

# ADVANCES IN PLASTID BIOLOGY AND ITS APPLICATIONS

EDITED BY : Niaz Ahmad, Steven J. Burgess and Brent L. Nielsen  
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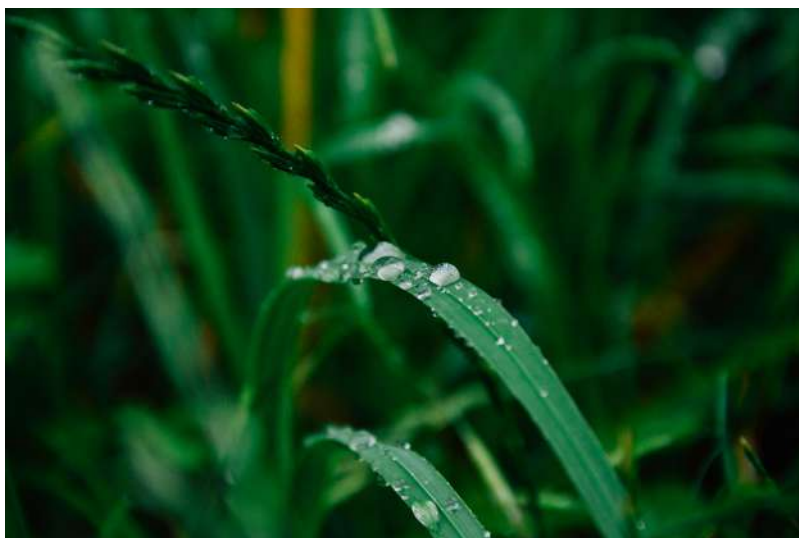
# ADVANCES IN PLASTID BIOLOGY AND ITS APPLICATIONS

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One of the distinguishing features of plants is the presence of membrane-bound organelles called plastids. Starting from proplastids (undifferentiated plastids) they readily develop into specialised types, which are involved in a range of cellular functions such as photosynthesis, nitrogen assimilation, biosynthesis of sucrose, starch, chlorophyll, carotenoids, fatty acids, amino acids, and secondary metabolites as well as a number of metabolic reactions. The central role of plastids in many aspects of plant cell biology means an in-depth understanding is key for a holistic view of plant physiology. Despite the vast amount of research, the molecular details of many aspects of plastid biology remains limited.

Plastids possess their own high-copy number genome known as the plastome. Manipulation of the plastid genome has been developed as an alternative way to developing transgenic plants for various biotechnological applications. High-copy number of the plastome, site-specific integration of transgenes through homologous recombination, and potential to express proteins at high levels (>70% of total soluble proteins has been reported in some cases) are some of the

technologies being developed. Additionally, plastids are inherited maternally, providing a natural gene containment system, and do not follow Mendelian laws of inheritance, allowing each individual member of the progeny of a transplastomic line to uniformly express transgene(s). Both algal and higher plant chloroplast transformation has been demonstrated, and with the ability to be propagated either in bioreactors or in the field, both systems are well suited for scale up of production.

The manipulation of chloroplast genes is also essential for many approaches that attempt to increase biomass accumulation or re-routing metabolic pathways for biofortification, food and fuel production. This includes metabolic engineering for lipid production, adapting the light harvesting apparatus to improve solar conversion efficiencies and engineering means of suppressing photorespiration in crop species, which range from the introduction of artificial carbon concentrating mechanisms, or those pre-existing elsewhere in nature, to bypassing ribulose biphosphate carboxylase/oxygenase entirely.

The purpose of this eBook is to provide a compilation of the latest research on various aspects of plastid biology including basic biology, biopharming, metabolic engineering, bio-fortification, stress physiology, and biofuel production.

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# Editorial: Advances in Plastid Biology and Its Applications

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**Keywords:** plastids, plastid transformation, retrograde signaling, biopharming, metabolic engineering

## The Editorial on the Research Topic

### Advances in Plastid Biology and Its Applications

Plastids originated from endosymbiosis around 1.5 billion years ago. They have been extensively studied to understand photosynthesis and other metabolic functions and to express foreign proteins, and knowledge about plastids has greatly increased. However, there are many aspects of plastid biology that remain unclear, and there have been difficulties in fully developing plastid transformation as an effective vehicle to express proteins. This research topic was launched to advance the knowledge of plastid biology, review recent progress, and address some of the challenges.

Tight coordination between plastid and nuclear genomes is essential for development and homeostasis in plant tissues. Bobik and Burch-Smith provide a detailed overview of this process including retrograde signaling between plastids and other organelles, plastid signaling in response to biotic and abiotic stress and the effect on the cell wall and intercellular symplasmic transport. By viewing chloroplast signaling in the context of the whole plant, they highlight the impact of chloroplast engineering on intracellular communication to avoid unintended consequences on growth and development.

An example of this concern is the alteration of carotenoid content of plants for the production of high value products. There is growing evidence that carotenoid cleavage products (apocarotenoids) can play an important role in modulating stress responses and impact upon plastid biogenesis; progress in identifying the signals and genes responsible is reviewed by Tian.

Chloroplast development from pro-plastids in angiosperms is dependent on light signaling pathways. Hills et al. demonstrated that classic plastid signaling also exists in gymnosperms but found that pine chloroplast biogenesis is light-independent. They investigated how light dependence might have evolved, and propose that suppression of photosynthetic gene responses to plastid signals in the dark occurred through recruitment of repressors of photomorphogenesis.

Organisms have evolved different mechanisms to cope with environmental stresses. The accumulation of osmoprotectants helps stabilize the active conformation of proteins and keeps cellular structures including membranes intact. D-arabitol accumulation in yeast provides protection against drought and salt stress. Khan et al. transferred the D-arabitol-mediated pathway into plants to test whether they could be made tolerant to drought and salinity. Overexpression of yeast arabitol dehydrogenase (ArDH)—an enzyme that reduces D-ribulose to D-arabitol—in tobacco chloroplasts conferred tolerance to NaCl up to 400 mM and 6% polyethylene glycol (PEG). This finding could have implications for developing stress-resilient crops to enhance yield.

Shimajima et al. utilized the inorganic phosphate (Pi) starvation response to stimulate triacylglycerol (TAG) accumulation in *Arabidopsis thaliana*. TAGs are useful as feedstocks for biofuel production or high value fatty acids, but are generally produced in seed tissues,

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which represent only a small proportion of overall plant biomass. TAG accumulation in vegetative tissues was increased in a starch deficient phosphoglucomutase mutant (*pgm-1*) background by overexpressing TAG synthesis enzymes under the low Pi inducible promoter of *monogalactosyldiacylglycerol synthase 3* (*MGD3*).

Infectious diseases continue to be a serious problem facing the growing human population. Significant research is focused on developing large-scale strategies for cost-effective production of therapeutics and vaccine antigens. Chloroplasts have the potential to express proteins at extraordinary levels, allowing plants to be used as green factories. Waheed et al. review the state-of the art of producing vaccines in plants via chloroplast transformation. They also discuss why chloroplast-made therapeutics have not reached the market despite a number of successful laboratory studies, and the major issues which should be addressed to fully exploit this technology.

Microalgal cells offer an alternative plastid expression system. Doron et al. provide a detailed overview of algal transformation systems, including techniques for transformation and genetic components, which encompasses selection markers, regulatory elements, promoters, inducible systems and plastid targeting signals.

Photosynthesis in  $C_3$  plants is relatively inefficient due to the partial loss of  $CO_2$  during recycling of 2-phosphoglycerate to 3-phosphoglycerate via photorespiration. Cyanobacteria and algae have  $CO_2$ /bicarbonate transporters that concentrate  $CO_2$  around Rubisco, the  $CO_2$  concentrating mechanism (CCM). Expression of cyanobacterial CCM proteins in chloroplasts may improve photosynthesis in  $C_3$  plants. However, CCM proteins function in the inner envelope membrane (IEM) and localizing foreign proteins there has been difficult. Uehara et al. expressed two cyanobacterial bicarbonate transporters, BicA and SbtA, each tethered with an IEM transit peptide, in Arabidopsis chloroplasts. The effects on photosynthesis have not yet been examined, but the successful localization of the protein in the IEM is an important step toward improving photosynthesis.

Most early studies on plastid division examined mesophyll cells. Fujiwara et al. studied the effects of a mutation in the Arabidopsis AtMinE1 chloroplast division site determinant gene using CFP, YFP, and GFP fusions. The mutant had dramatic effects on plastid size and shape in epidermal cells but no significant differences in mesophyll cells. Mutants have enlarged plastids, and stromules and bulges emerging from the large plastids led to smaller subcompartments after FtsZ-mediated constriction. The larger plastids were unable to divide due to the inability to form a productive interaction with FtsZ. The results

suggest that control of plastid division may differ among plant tissues.

Delfosse et al. reviewed the use of fluorescent proteins (FPs) to study chloroplast structure, extensions and stromules. The use of FPs has led to insights on interactions between chloroplasts, mitochondria and peroxisomes that suggest cooperation between organelles during photorespiration or in response to stress conditions or reactive oxygen species (ROS). Applications of FPs reviewed in this paper and a prior one (Hanson and Sattarzadeh, 2013) will lead to insights on other plastid types and interactions between intracellular organelles.

Plastid DNA levels change during plant development, with high amounts of DNA in young chloroplasts and significantly less in mature leaf cells. This should be considered in efforts to improve chloroplast transformation and plastid efficiency. Plants have two nuclear-encoded dual-localized DNA polymerases, and analysis of Arabidopsis mutants indicates that neither is totally essential for replication of either chloroplast or mitochondrial DNA. However, mutations in either result in decreased DNA levels in both organelles and slower growth depending on the mutant and age of the plant tissue tested (Morley and Nielsen).

In conclusion, this research topic summarizes current progress, challenges, and prospects for applications in plastid biology. Plastids vary in many significant characteristics depending on the tissue, age, and cell type. This includes changes in plastid division and structure, retrograde signaling, responses to stress, and DNA copy number. The complex signaling networks linking plastids to the nucleus and their evolution is only just starting to be understood. These reports underscore the importance of not over-interpreting findings on plastid properties or functions in specific plant tissues, and should be considered for full development of chloroplast genetic engineering for agricultural improvements and production of therapeutics.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Chloroplast signaling within, between and beyond cells

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The most conspicuous function of plastids is the oxygenic photosynthesis of chloroplasts, yet plastids are super-factories that produce a plethora of compounds that are indispensable for proper plant physiology and development. Given their origins as free-living prokaryotes, it is not surprising that plastids possess their own genomes whose expression is essential to plastid function. This semi-autonomous character of plastids requires the existence of sophisticated regulatory mechanisms that provide reliable communication between them and other cellular compartments. Such intracellular signaling is necessary for coordinating whole-cell responses to constantly varying environmental cues and cellular metabolic needs. This is achieved by plastids acting as receivers and transmitters of specific signals that coordinate expression of the nuclear and plastid genomes according to particular needs. In this review we will consider the so-called retrograde signaling occurring between plastids and nuclei, and between plastids and other organelles. Another important role of the plastid we will discuss is the involvement of plastid signaling in biotic and abiotic stress that, in addition to influencing retrograde signaling, has direct effects on several cellular compartments including the cell wall. We will also review recent evidence pointing to an intriguing function of chloroplasts in regulating intercellular symplasmic transport. Finally, we consider an intriguing yet less widely known aspect of plant biology, chloroplast signaling from the perspective of the entire plant. Thus, accumulating evidence highlights that chloroplasts, with their complex signaling pathways, provide a mechanism for exquisite regulation of plant development, metabolism and responses to the environment. As chloroplast processes are targeted for engineering for improved productivity the effect of such modifications on chloroplast signaling will have to be carefully considered in order to avoid unintended consequences on plant growth and development.

**Keywords:** retrograde signaling, plastid signaling, redox, phytohormones, plasmodesmata, cell wall, stromules, stress responses

## Introduction

According to the endosymbiotic theory plastids originated from free-living cyanobacteria that were engulfed by early eukaryotic cells. These cyanobacteria were retained by their hosts and have co-evolved with their host cells over 1.5 billion years to become an integral part of the modern plant cell (Yoon et al., 2004; Nakayama and Archibald, 2012). It is accepted that the successful stable integration occurred because of exceptional mutual benefits: the eukaryotic cell was able to establish an autotrophic lifestyle, while the engulfed cyanobacteria reached a pathogen-free asylum. Accumulating evidence suggests that this stable symbiosis between



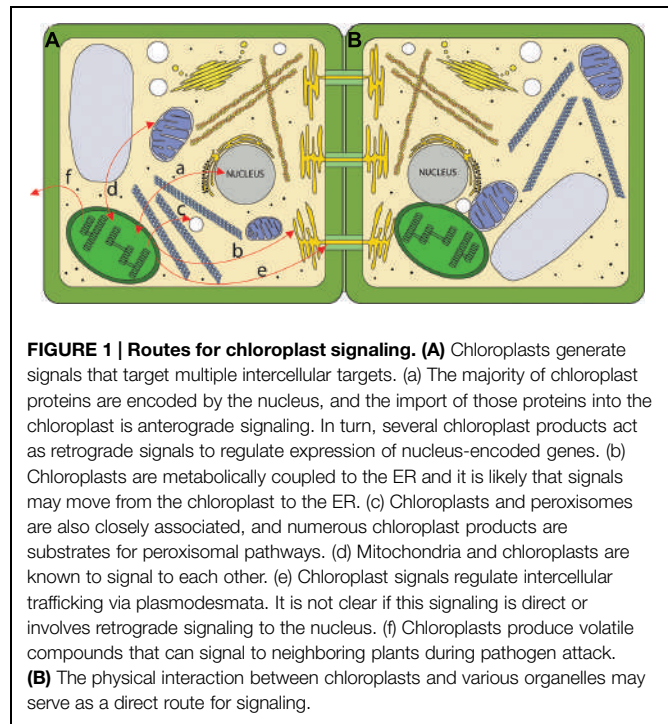
cyanobacteria and the eukaryotic cell was facilitated by infection of the latter with Chlamydiales pathogens (Ball et al., 2011, 2013).

The most conspicuous function of modern plastids is the sophisticated oxygenic photosynthesis performed by chloroplasts. However, plastids perform many other functions that are critical for proper plant development and physiology including the synthesis of amino acids, nucleotides and fatty acids, production of phytohormones, some vitamins and a multitude of secondary metabolites, as well nitrogen and sulfur assimilation. Many chloroplast secondary metabolites, besides being necessary for basic plant metabolic functions, are also important for interaction with the environment, as they function in plant defense against pathogen ingress and plant adaptation to stresses including heat, drought and high light. Thus, chloroplasts act as a hub in the cellular response to signals, generating a variety of signals that coordinate a fine-tuned and appropriate response to any given situation (Pfannschmidt and Yang, 2012).

The emerging view of the chloroplast is as a very dynamic signaling compartment. As a specific sensor of intra- and extracellular stimuli, chloroplasts constantly process and integrate a multitude of intracellular signals and pathways in order to sustain homeostasis at both the cellular and organismal levels (**Figure 1A**). An often over-looked aspect of cell biology is the physical interaction between organelles for coordination of signaling and metabolism (**Figure 1B**). This area is beginning to receive attention and we will examine these findings as they relate to chloroplasts and their roles in signaling. Given the dizzying array of signals that chloroplasts respond to and produce it is not surprising that there is considerable crosstalk between signaling pathways. This is particularly evident during responses to biotic and abiotic stress (Nakashima et al., 2014; Trotta et al., 2014; Zhou et al., 2015).

Chloroplast signaling is not limited to exerting its effects within the cell. It is clear that chloroplast-derived signals can travel far beyond their site of production to induce changes in distal parts of the plant (e.g., Petrillo et al., 2014). Further, chloroplasts apparently regulate intercellular trafficking via the channels known as plasmodesmata. Through this action, chloroplasts could regulate almost all aspects of plant growth and development as it is becoming clear that not only do metabolites but also hormones, transcription factors and small RNA molecules use these channels for intercellular communication. Beyond whole-plant signaling, chloroplast signaling can impact entire ecosystem through production of volatile compounds.

While we focus here on the chloroplasts in mature leaves, it can be expected that other types of plastids, e.g., proplastids, etioplasts, or leucoplasts, participate in signaling networks in response to their unique developmental states and environmental conditions. Further, it is also likely that at any given time different subpopulations of chloroplasts within a cell are in various metabolic or physiologic states and are therefore likely to be involved in distinct signaling processes. Thus chloroplast signaling is complex, and dissecting crosstalk and feedback mechanisms remains a daunting task. With attempts to engineer chloroplasts for specialized or improved metabolic outputs, attention must be paid to how these adjustments may impact



chloroplast behavior if unintended consequences are to be diminished. These consequences would not be limited to the chloroplasts, but could extend even to other plants cultivated in the vicinity of the engineered plants.

## Chloroplasts in Intracellular Signaling

### Chloroplast Signaling to the Nucleus

Over evolutionary time a significant number of the cyanobacterial genes were transferred to the host nucleus (Race et al., 1999; McFadden, 2001). These genes subsequently acquired sequences that function as transit peptides to enable import of their protein products back into plastids. The nucleus therefore exerts considerable control over chloroplast functions, and this nucleus-to-chloroplast signaling is termed anterograde signaling. Importantly, several genes encoding proteins that are integral components of photophosphorylation or photosynthesis were retained in the cyanobacterial genome (Allen, 1993; Race et al., 1999; Raven and Allen, 2003). Thus, in order to establish a stable eukaryotic plant cell it was necessary to synchronize the activities of both genomes. This has been achieved by creating a complex signaling system between the nucleus and plastids, able to transfer information and efficiently adjust gene expression in both compartments according to particular needs. Plastid derived-signals that regulate nuclear gene expression represent retrograde signaling. While anterograde signaling is well understood, it has been more challenging to unravel the molecular details of retrograde signaling.

Identification of molecules and signaling strategies underlying so-called retrograde signaling represents a long-standing quest in

plant biology. Historically, Bradbeer et al. (1979) provided the first report describing the existence of communication between those organelles. They observed that the barley (*Hordeum vulgare*) *albostrians* chloroplast ribosome-deficient mutant had severely decreased chloroplast protein synthesis, in conjunction with depressed expression of nucleus-encoded chloroplast genes. This revolutionary discovery was in opposition to Ellis' "cytoplasmic control principle" that posited control of organellar protein synthesis by cytoplasmic components (Ellis, 1977). Bradbeer's discovery was soon confirmed by other researchers who treated young seedlings of mustard, *Arabidopsis*, pea or barley with lincomycin, chloramphenicol or streptomycin – inhibitors of plastid protein synthesis (Oelmüller et al., 1986; Susek et al., 1993; Yoshida et al., 1998; Sullivan and Gray, 1999). Besides the strategy of relying on systems with compromised chloroplast ribosome function, other approaches to perturbing distinct aspects of chloroplast function have also successfully interrogated chloroplast-to-nucleus signaling. The induction of carotenoid deficiency in genetic mutants or in plants treated with norflurazon (a herbicide that inhibits phytoene desaturase and blocks carotenoid synthesis), as well as in plants with compromised tetrapyrrole synthesis (resulting in accumulation of intermediates) caused suppressed expression of nucleus-encoded chloroplast genes (Johanningmeier and Howell, 1984; Mayfield and Taylor, 1984; Susek et al., 1993; Kropat et al., 1997, 2000; Strand et al., 2003; Zhang et al., 2011). These and other studies led to the realization that the plastid functional status can regulate the expression of photosynthesis associated nuclear genes (PhANGs; Surpin et al., 2002). Moreover, analysis of the *Arabidopsis* chloroplast ribosomal protein mutant *rps1* revealed that chloroplast translational capacity is a critical factor in developing heat tolerance. This is mediated by inducing expression of the heat stress transcription factor HsfA2, a key regulator of heat tolerance (Yu et al., 2012). Therefore, the functional status of chloroplasts also regulates nuclear genes involved in heat-tolerance. The involvement of plastid translation in retrograde signaling and plant development was recently discussed in detail (Tiller and Bock, 2014).

The intense search for factors mediating this chloroplast-to-nucleus communication has identified a set of plastid metabolic intermediates. Importantly, proteins with functions in both chloroplasts and nuclei have been recently identified and have been proposed to participate in retrograde signaling. These retrograde signals are expected to work by modifying the expression of nuclear genes in order to adapt plant development and physiology to constantly changing environmental conditions. Currently two major modes of retrograde signaling are distinguished and they are involved in so-called biogenic and operational type of control. Whereas the former includes signals responsible for chloroplast and photosynthesis biogenesis, the latter act in response to changing environmental cues in fully developed chloroplasts (Pogson et al., 2008). The best-studied target of retrograde signaling is represented by PhANGs, but plastids are also involved in tuning the expression of nuclear genes involved in response to a plethora of biotic and abiotic conditions. A recent meta-analysis of microarray studies of systems where high levels of retrograde signaling were induced

has identified a core module of 39 nuclear genes that were subject to regulation in response to all signals examined (Glasser et al., 2014). The genes in this group, presumably representing the core retrograde-response module, are all known to be responsive to sugar, reactive oxygen species (ROS), abscisic acid (ABA) and/or auxin signaling pathways. Thus retrograde signaling may exploit a common component of these signaling pathways to mediate changes in gene expression.

## The GENOMES UNCOUPLED (GUN) Mutants – Aiming to the Nucleus with Guns

Very helpful in deciphering the retrograde signaling phenomenon were mutants isolated from genetic screens. The *gun* (*genome uncoupled*) mutants escaped the pattern of suppressed PhANG expression despite defective chloroplast physiology or inhibited biogenesis. There are numerous excellent reviews of the role of *guns* in retrograde signaling (Woodson and Chory, 2008, 2012; Barajas-Lopez Jde et al., 2013; Chi et al., 2013). So far, six *gun* mutants have been identified and they can be classified according to pathways they belong to. Whereas the *gun1* mutant results from mutation in a gene encoding a chloroplast-localized pentatricopeptide repeat-containing protein (PPR), the *gun2-6* mutants are associated with tetrapyrroles synthesis (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Strand et al., 2003; Koussevitzky et al., 2007). The exact role of GUN1 in PhANG regulation is far from understood, however, it is known to act upstream of ABSCISIC ACID INSENSITIVE 4 (ABI4), an APETALA 2-type transcription factor that binds to the ACGT motif of light- and ABA-responsive elements (Koussevitzky et al., 2007). Interestingly, the expression of ABI4 was regulated by PTM, a chloroplast PHD-type transcription factor (Sun et al., 2011). The involvement of the key enzymes of the tetrapyrrole synthesis pathway in the *gun* phenotype led to detailed investigations of tetrapyrroles, especially Mg-protoIX, as putative retrograde signals. However no correlation between Mg-protoIX levels and retrograde signaling could be established (Matsui et al., 2008; Moulin et al., 2008). Interestingly, the *gun6* mutant identified heme as a strong candidate for mediating plastid-to-nucleus signaling (Woodson et al., 2011). Moreover, it was proposed that the impact of tetrapyrrole biosynthesis on nuclear gene expression is mediated by singlet oxygen ( $^1\text{O}_2$ )-induced signaling and feedback regulated 5-aminolevulinic acid (ALA) synthesis (Schlicke et al., 2014).

## SAL1-PAP Chloroplast Retrograde Pathway

The detailed analysis of *sal1*, an *Arabidopsis* phosphonucleotidase mutant, has identified a known second messenger as acting in chloroplast-to-nucleus signaling. Estavillo et al. (2011) have demonstrated that the chloroplast and mitochondria-localized SAL1 phosphatase regulates the steady-state level of 3'-phosphoadenosine 5'-phosphate (PAP) by dephosphorylating it to an adenosine monophosphate (AMP). In the *sal1* mutant, or in response to drought stress or high light intensity, PAP levels increased, inducing expression of ASCORBATE PEROXIDASE 2 and EARLY LIGHT INDUCIBLE PROTEIN 2, two nuclear

genes whose expression is induced by high light stress (Harari-Steinberg et al., 2001; Caverzan et al., 2012). It has been proposed that PAP travels from chloroplasts to the nucleus where it regulates nuclear gene expression. Nucleus-localized exoribonucleases (XRN) are likely targets of PAP, and by repressing their activity PAP may stimulate expression of high light and drought-responsive genes, leading to increased tolerance (Estavillo et al., 2011; **Figure 2A**).

### Methylerythritol (MEcPP) Retrograde Pathway

Isoprenoid metabolism is a major biosynthetic pathway in plants (Cordoba et al., 2009). The *Arabidopsis* constitutively expressing *HPL* (*ceh1*) mutant displays enhanced expression of *hydroperoxide lyase* (*HPL*), a stress-inducible nuclear gene encoding a plastid-localized protein of the oxylipin pathway. The *ceh1* mutation disrupted a plastid-localized enzyme (HDS) that catalyzes conversion of methylerythritol cyclodiphosphate (MEcPP) to hydroxymethylbutenyl diphosphate (HMBPP; Xiao et al., 2012). The absence of CEH1 led to accumulation of MEcPP and induced the expression of a subset of stress-associated genes, including *ISOCHORISMATE SYNTHASE 1* (a key plastidial enzyme in the salicylic acid (SA)-biosynthetic pathway) and *HPL*, but not *ALLEN OXIDE SYNTHASE* [*AOS*, encoding a plastid-localized protein of the jasmonic acid (JA)-biosynthetic pathway]. HDS-depleted plants with increased levels of MEcPP accumulated SA and displayed increased resistance to infection by biotrophic pathogens. Importantly, elevated MEcPP levels and increased expression of *HPL* are observed upon both wounding and high light treatment, demonstrating involvement of MEcPP in a retrograde pathway involved in abiotic stresses (operational control) distinct from the *gun* signaling pathway. Therefore MEcPP is a retrograde signal inducing targeted stress responses. The proposed mechanism of MEcPP action involves direct modification of chromatin remodeling by disruption of DNA-histone interactions (Xiao et al., 2012; **Figure 2B**).

### Derivatives of Carotenoids as Signaling Molecules

Carotenoids are tetraterpenoid products of the isoprenoid biosynthetic pathway that also generates ABA and strigolactones (Ruyter-Spira et al., 2013; Giuliano, 2014). Carotenoids are constituents of the light harvesting complexes where they serve as accessory pigments to extend the absorption spectra of the chlorophylls, and they have critical protective roles as scavengers of singlet oxygen ( $^1\text{O}_2$ ) generated by the PSII reaction center (Telfer, 2014). Carotenoid derivatives have recently been proposed to act as chloroplast-generated signaling molecules that link chloroplast activity and nuclear gene expression (Ramel et al., 2012; Avendano-Vazquez et al., 2014; Van Norman et al., 2014).

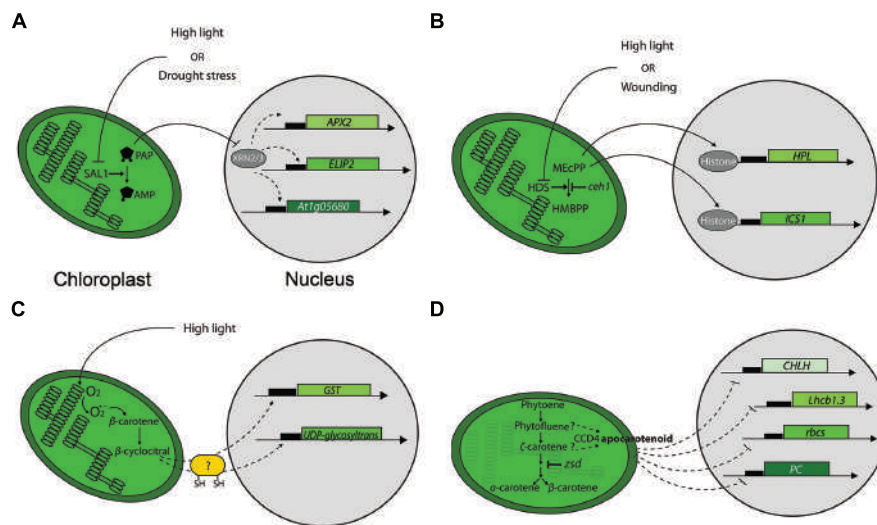
$\beta$ -cyclocitral is a product of singlet oxygen-induced  $\beta$ -carotene oxidation. This volatile molecule contains an  $\alpha,\beta$ -unsaturated carbonyl, designating it as a reactive electrophile species (RES).  $\beta$ -cyclocitral's accumulation in *Arabidopsis* leaves during high-light stress correlated with accumulation of singlet oxygen, supporting the notion of  $\beta$ -cyclocitral as an oxidation product of  $\beta$ -carotene (Ramel et al., 2012). Consistent with this,

transcriptome analysis by DNA microarrays revealed that about 80% of  $\beta$ -cyclocitral-induced or repressed genes are also responsive to singlet oxygen overproduction in the *Arabidopsis* fluorescent (*flu*) mutant (op den Camp et al., 2003). Using qRT-PCR Ramel and coworkers demonstrated that all singlet oxygen marker genes tested are also induced by  $\beta$ -cyclocitral. Importantly, this effect seemed to be specific to  $\beta$ -cyclocitral as the overlap between gene expression changes induced by  $\beta$ -cyclocitral and other RES like methyl vinyl ketone or malondialdehyde (MDA) was smaller. Among the genes most strongly induced by  $\beta$ -cyclocitral were 10 glutathione-S-transferase (GST) genes and 12 UDP-glycosyltransferases (**Figure 2C**). Both groups of genes are involved in detoxification processes conferring tolerance to singlet oxygen in *Chlamydomonas reinhardtii* (Ledford et al., 2007). The protective effect of  $\beta$ -cyclocitral resulted in better PSII quantum efficiency and lower lipid peroxidation under high light stress (Ramel et al., 2012). Finally, the current model proposes  $\beta$ -cyclocitral as a stress molecule generated in chloroplasts under photooxidative stress that reprograms gene expression leading to stress acclimation. Thus, in addition to their roles in light harvesting and as antioxidants, carotenoids can also act as signaling molecules. The exact mechanism of  $\beta$ -cyclocitral action or its receptor in the nucleus are unknown, however, it is proposed that as an electrophile with a  $\alpha,\beta$ -unsaturated carbonyl group it could react with electron donors such as proteins containing sulphhydryl groups (Ramel et al., 2012, 2013). Very recently, elevated levels of  $\beta$ -cyclocitral were reported in *Arabidopsis* plastoglobule kinase mutants that have defective plastoglobule metabolism (Lundquist et al., 2013). The *abc1k1 abc1k3* double mutant shows rapid chlorosis under high light stress, confirming  $\beta$ -cyclocitral's role in mediating stress responses.

In addition to  $\beta$ -cyclocitral, apocarotenoids are also potential chloroplast retrograde signaling components. Analysis of the *Arabidopsis*  $\zeta$ -carotene desaturase mutant (*zds/clb5/spc1/pde181*) displaying arrested chloroplast biogenesis at a very early stage of development led to the conclusion that the accumulation of an uncharacterized apocarotenoid can act as a retrograde signal (Avendano-Vazquez et al., 2014). This apocarotenoid is likely generated by the activity of the carotenoid cleavage deoxygenase 4 (CCD4) enzyme on  $\zeta$ -carotene (**Figure 2D**). Accumulation of this putative cleavage product was shown to modulate expression of many nuclear genes required for leaf development leading to a severe phenotype that included arrested chloroplast development and leaves with defective adaxial-abaxial patterning. Since neither ROS nor ABA nor strigolactone signaling pathways were responsible for the observed phenotypes, it was concluded that the putative phytofluene or  $\zeta$ -carotene-derived apocarotenoid is part of a novel retrograde signaling pathway. Interestingly, the observed defects were restricted to primary leaves, underscoring the differences in developmental regulation between plastids in different organs (Avendano-Vazquez et al., 2014).

A third carotenoid derivative is implicated in plastid retrograde signaling, this time with respect to lateral root (LR) development (Van Norman et al., 2014). Reduced LR formation in *Arabidopsis* seedlings treated with norflurazon was observed and further investigation indicated that a  $\beta$ -carotene





**FIGURE 2 | Mechanisms of chloroplast-to-nucleus signaling. (A)** Retrograde signaling by PAP. High light or drought stress inhibits SAL1 phosphatase and leads to the accumulation of PAP. PAP likely inhibits specific exonucleases (XRN2) to modify nuclear gene expression. *APX2* and *ELIP2* stand for *ASCORBATE PEROXIDASE 2* and *EARLY LIGHT INDUCIBLE PROTEIN 2* genes, respectively. **(B)** Retrograde signaling by MEcPP. High light or wounding inhibits 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS), leading to the subsequent accumulation of MEcPP. MEcPP affects nuclear gene expression via a mechanism proposed to involve chromatin remodeling by destabilizing DNA-histone interactions. *HPL* and *ICS1* stand for *HYDROPEROXIDE LYASE* and *ISOCHORISMATE SYNTHASE 1* genes, respectively. **(C)** Carotenoid-derivative  $\beta$ -cyclocitral mediates retrograde signaling. The ROS singlet oxygen induces formation of  $\beta$ -cyclocitral during high light treatment.  $\beta$ -cyclocitral's action on selected nuclear genes is proposed to involve proteins containing sulphhydryl groups. The genes depicted are *GLUTATHIONE-S-TRANSFERASE (GST)* and *UDP-glycosyltransferase*. **(D)** An unidentified apocarotenoid affects expression of nuclear genes. It is proposed that the putative signaling apocarotenoid accumulates in chloroplasts due to compromised  $\zeta$ -carotene desaturase activity that results in accumulation of phytofluene and  $\zeta$ -carotene, putative substrates for the carotenoid cleavage deoxygenase 4 (CCD4) enzyme that is prerequisite for the putative apocarotenoid synthesis. *CHLH*, *Lhcb1.3*, *rbcS* and *PC* stand for genes encoding the subunit H of the Mg-chelatase complex, light-harvesting complex 1.3 isoform, the Rubisco small subunit and plastocyanin, respectively.

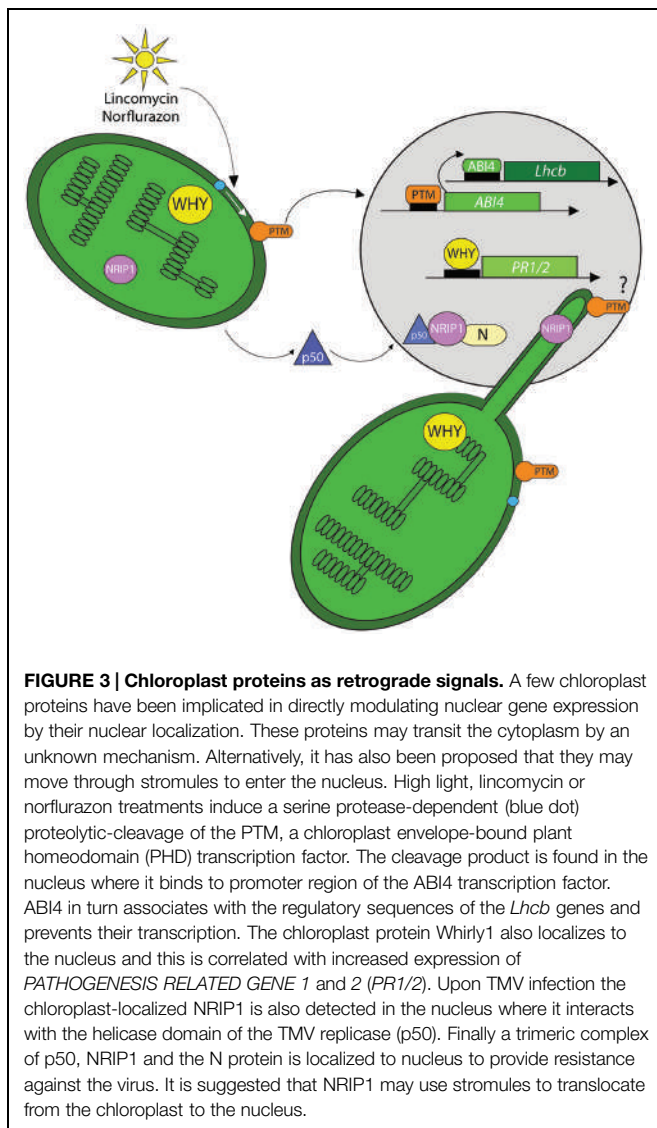
derivative is required for prebranch site formation (Van Norman et al., 2014). Extensive genetic analyses ruled out ABA and strigolactone as the carotene-derived signaling molecule involved in LR formation. Additionally, treatment of *Arabidopsis* seedlings with D15, a candidate inhibitor of carotenoid cleavage at the 9,10 position, resulted in a highly significant decrease in LR capacity, suggesting that this unknown apocarotenoid is likely cleaved at this position. Interestingly, even though reduced LR capacity and small albino shoots were observed in carotenoid biosynthesis mutants and in plants treated with norflurazon, plants treated with D15 had green shoots of comparable size to wild type. This demonstrates that the involvement of carotenoids in LR formation is separate from their photoprotective function (Van Norman et al., 2014). Intriguingly, carotenoid biosynthesis was found to occur in differentiated parts of the root at some distance from the oscillating zone where prebranch sites and eventually LRs formed. Thus the non-cell autonomous function of (apo)carotenoids seems to be required for development of LRs (Van Norman et al., 2014).

### Chloroplast Proteins as Retrograde Signals

It is a broadly accepted paradigm that most nucleus-encoded chloroplast proteins reach this compartment due to the transit peptides located at their N-termini. Cleavage of the signal peptides after entry in to the chloroplast generates the functional chloroplast proteins. However, accumulating data show that

some chloroplast proteins also act in the nucleus. While it is tempting to speculate that this is attributable to two-way protein movement between chloroplasts and cytoplasm, this has not been unambiguously demonstrated. However, a physiological function for this chloroplast-nucleus dual localization is apparent and it seems to be indispensable for proper plant response to pathogen attack and abiotic stress, and emphasizes the function of chloroplasts as signaling compartments.

One such protein, PTM (PHD type transcription factor with transmembrane domains) was shown to provide a physical link in signaling between chloroplasts and nucleus to regulate gene expression (Sun et al., 2011). This membrane-bound transcription factor (MTF) is localized to chloroplast outer envelope by four transmembrane domains at its C-terminus (**Figure 3**). The N-terminus of PTM contains a DNA-binding homeodomain box, a different transcription factors (DDT) domain and a plant homeodomain (PHD). Interestingly, a shorter variant of this protein, lacking the transmembrane domains, was detected in nuclear fractions. Notably, increased amounts of the shorter PTM variant were detected upon treatment with either norflurazon or lincomycin, and on exposure to high light. Through the application of protease inhibitors, it was demonstrated that the shorter form of PTM was the result of serine protease activity (Sun et al., 2011; Adam, 2015). According to the proposed model, chloroplast signals induce the intramembrane proteolytic cleavage of full



length PTM, producing a soluble shorter variant (~58 kDa) that is released to the cytoplasm and finally translocates to the nucleus where it binds, through its PHD domain, to the *ABI4* promoter to induce *ABI4* expression. *ABI4*, in turn binds to the *Lhcb* promoter, close to the CUF1 element and precludes binding of G-box-binding factors required for the expression of *Lhcb* and other PhANGs. This model explains the *gun* phenotype observed in *ptm* and *abi4* mutants. Moreover, the amount of processed PTM declined in the *gun1* mutant, suggesting a complex regulatory network.

WHIRLY1 provides another example of a chloroplast protein with a role in the nucleus. A WHIRLY1 fusion protein expressed in the plastid genome of tobacco, localized to both plastids and nuclei, and the two subpopulations were the same molecular size (Isemer et al., 2012). As a consequence WHIRLY1-regulated *PR* (*PATHOGENESIS RELATED*) genes were upregulated under normal growth conditions (Figure 3). WHIRLY1 is part

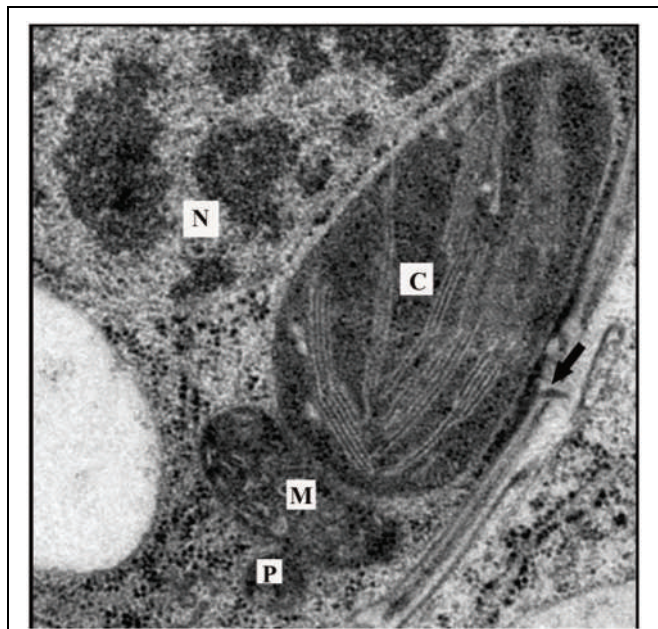
of the transcriptionally active chromosome in plastids, and interestingly, another component of this complex, pTAC12 (HEMERA), also showed dual localization to chloroplasts and nuclei with unchanged molecular mass, excluding any proteolytic modification (Chen et al., 2010). However, unlike in the case of WHIRLY1, the dual localization of HEMERA in a single cell has not yet been demonstrated. Such a demonstration is important for ruling out the possibility that dual localization could be caused by fluctuating distribution resulting from specific cell types or developmental stages. It has been proposed that WHIRLY1 conveys information about the chloroplast redox state to the nucleus, and SA regulates this communication (Foyer et al., 2014). The proposed mechanism of WHIRLY1 action would be similar to that of NPR1 (See below). The mechanism and consequences of the proposed translocation of NRIP1 (another chloroplast-localized protein) to the nucleus is discussed later.

There are a few hypotheses that aim to explain the nuclear localization of chloroplast-targeted proteins and they invoke mechanisms enabling translocation of chloroplast-localized proteins to nuclei. One of them proposes permeabilization of the chloroplast outer membrane by an unknown mechanism. Another posits that stress-induced modification of chloroplasts results in the formation of stromules that contact nuclei could facilitate direct trafficking of chloroplast proteins (Caplan et al., 2008). In agreement with the former, a recent communication described GFP-fusion protein leakage from functional chloroplasts upon pathogen attack (Kwon et al., 2013). Interestingly, this occurrence was shown to be dependent on ROS. Evidence for the latter hypothesis of stromule involvement in chloroplast protein translocation is now being reported (see Stromules below). Regardless of the mechanism governing the distribution of chloroplast proteins to the cytoplasm and/or nucleus, this phenomenon may represent an important pathway for direct communication between chloroplasts and nuclei, and provides important insights in understanding the molecular basis of retrograde signaling.

## Chloroplasts and Inter-Organellar Signaling

It has long been appreciated that there is metabolic crosstalk between organelles. Metabolic pathways often involve multiple organelles and metabolic intermediates may be transported across membranes by diffusion or by specialized transporters in an energy-dependent manner after traversing the cytosol. However, there is emerging evidence that direct physical contact between organelles may provide a major route for metabolic exchange (Figure 4). Such direct contact would also provide routes for inter-organelle signaling, although evidence for this is still limited. Thus, chloroplasts, besides producing signals that may travel long distances from plastids to their targets, may also communicate directly with other organelles through physical contacts. Here we consider evidence for chloroplast-organelle contacts and possible roles for these contacts in signaling.





**FIGURE 4 | Arrangement of organelles in a leaf cell.** Transmission electron microscopy images often reveal chloroplasts in close proximity with peroxisomes and mitochondria. Chloroplasts can also be observed near the nucleus and cell wall. Note the presence of a plasmodesma in the cell wall (arrow). Such arrangements of organelles would minimize distances that signals must traverse to arrive at their target. C, chloroplast; M, mitochondria; P, peroxisome; N, nucleus.

## Stromules for Plastid-to-Plastid and Plastid-to-Nucleus Signaling

Rediscovered by Kohler et al. (1997), the observation of tubular protrusions from plastids changed our thinking about chloroplasts and possible pathways for signaling (Kohler et al., 1997). These so-called stromules are stroma-filled tubules enclosed by the inner and outer plastid envelope membranes, and are 0.4–0.8  $\mu\text{m}$  in diameter and of variable length typically up to 65  $\mu\text{m}$  (Gray et al., 2001). They are more abundant in non-green plastids than in chloroplasts. Stromules are distinct from the chloroplast protrusions (CPs) that form during stress (Holzinger et al., 2007a,b), and that are involved in the sequestration of Rubisco from the rest of the chloroplast body (Yamane et al., 2012).

Stromule formation is dependent on both intrinsic and extrinsic factors. The size of plastid, plastid identity, state of differentiation and density of plastids all determine stromule formation (Waters et al., 2004). The actin cytoskeleton has also been reported to be important for stromule formation and movement (Kwok and Hanson, 2003; Kwok and Hanson, 2004b; Gunning, 2005; Holzinger et al., 2007b), and the myosin XI motor is required for stromule formation in *Nicotiana benthamiana* chloroplasts (Natesan et al., 2009; Sattarzadeh et al., 2009). In contrast, it has recently been reported that isolated chloroplasts can form stromules (Brunkard et al., 2015b). Stromule formation is also temperature sensitive, and temperatures around 20°C appear optimal for stromule formation (Holzinger et al., 2007a),

while lower temperatures inhibit their formation (Gray et al., 2012) and higher temperatures induce CP formation (Holzinger et al., 2007a). The light-dependence of stromule formation is somewhat controversial. There have been reports that light is not required for stromule formation (Kwok and Hanson, 2003; Gray et al., 2012), but recent findings demonstrate increased formation of stromules from *Arabidopsis* mesophyll chloroplasts during the day as compared to the night (Brunkard et al., 2015b). It is possible the differing results can be explained by differences in plant growth conditions and the types of plastids examined in each experiment. Besides light and temperature the hormone ABA, likely generated in response to environmental stresses, also induces stromule formation (Gray et al., 2012). Consistent with this, salt and osmotic stress also increased the fraction of stromule-bearing chloroplasts.

The most obvious consequences of stromules are significant enlargement of both plastid envelope surface and plastid volume. These modified plastid properties could affect the rate of plastid import and export, and also cause changes in plastid compartmentalization, respectively. Consistent with these presumed functions, it was shown that chlorophyll and thylakoid membranes are absent from stromules (Kohler et al., 1997; Holzinger et al., 2007a; Newell et al., 2012). The trafficking of plastid genomes or genetic material via stromules has also been ruled out (Newell et al., 2012). On the other hand proteins like GFP, aspartate aminotransferase and Rubisco complexes of a molecular weight around 550 kDa have been localized within stromules (Kwok and Hanson, 2004a). Moreover, interconnections between individual plastids via stromules have been reported, and photobleaching experiments and the use of photoconvertible proteins have demonstrated the transfer of proteins between them (Kohler et al., 2000; Hanson and Sattarzadeh, 2011, 2013, but see Schattat et al., 2012, 2015). Given that connections between plastids are rare, the biological significance of possible plastid-to-plastid trafficking remains unclear (Hanson and Sattarzadeh, 2013).

Stromules are involved in metabolic responses to stress, namely chloroplast autophagy in response to nutrient starvation (Ishida et al., 2008). Through observations of fluorescently tagged proteins and the *Arabidopsis atg5* autophagy mutant, it was demonstrated that plastid stromal proteins can be remobilized to the vacuole via the ATG-dependent autophagic pathway, without destroying the chloroplast. Similar observations have been reported from rice (Izumi et al., 2015). According to the proposed model stressed-induced autophagy sequesters stromules by forming an isolation membrane that eventually clips off a given stromule and its stromal contents, and then transports the cargo to the vacuole for degradation (Ishida et al., 2008). Interestingly, both stromules and protrusions were identified in potato tuber amyloplasts (Borucki et al., 2015). These protrusions, unlike stromules, are likely involved in starch accumulation in the parenchyma storage cells. The involvement of protrusions in accumulation of starch had been previously demonstrated (Langeveld et al., 2000). Thus stromules are involved in metabolism.

Plastids and stromules have been repeatedly observed in close proximity to other organelles including mitochondria, other

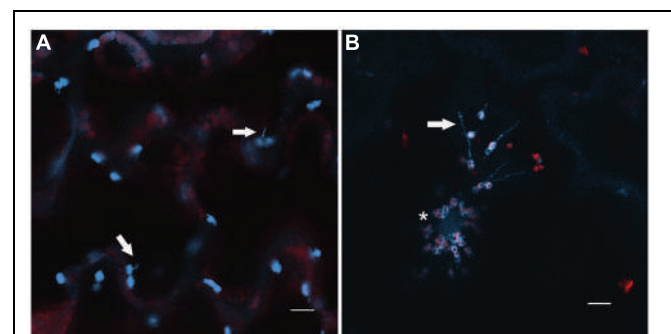
plastids, ER, plasma membrane and nuclei. From the perspective of signaling, however, the most interesting seems to be the distinct distribution of plastids around nuclei, including concentration of stromules around and, most intriguingly, inside the nucleus (Collings et al., 2000; Kwok and Hanson, 2004c). Clusters of plastids with long stromules of 20–30  $\mu\text{m}$  localized around the nucleus were observed in *N. tabacum* petioles of cotyledons (Kwok and Hanson, 2004c), as well as in the lower part of the hypocotyl where the plastids were preferentially arranged around the nucleus with long stromules of up to 100  $\mu\text{m}$  extending to the cell periphery (Natesan et al., 2005). Such concentration of plastids around nucleus was also observed in petal cells and in shoot meristems (Kohler and Hanson, 2000; Kwok and Hanson, 2004c). Stromules have also been observed lying in grooves and invaginations of the nuclear membranes in tobacco epidermal cells (Kwok and Hanson, 2004c). Such direct connections were proposed to increase efficiency in plastid-nucleus communication. Stromules have also been implicated in chloroplast-to-ER signaling (Schattat et al., 2011a,b), and in intercellular signaling (Kwok and Hanson, 2004c) but this has not been explicitly tested or proven. These suggestions are based on interaction between stromules and ER or the plasma membrane, respectively, and further characterization of these interactions is warranted.

Stromules and stromule-nucleus contacts may have important roles in host–pathogen interactions. The induction of stromules and remobilization of chloroplasts to surround nuclei was observed in *N. benthamiana* leaves in response to infiltration with GV3101, common lab strain of *Agrobacterium tumefaciens* (Erickson et al., 2014). In addition, starch accumulated in GV3101-treated leaves and the levels of soluble sugars also increased. However, another lab strain LBA4404 did not produce these effects. The introduction of the *trans zeatin synthase* (*tsz*) gene from the GV3101 plasmid into LBA4404 led to the induction of stromules and other cellular changes typically observed on GV3101 infiltration. Indeed, direct application of cytokinin to leaves produced phenotypes similar to those obtained with GV3101. Thus, cytokinin may mediate the production of stromules and chloroplast movements during some plant–pathogen interactions.

The question of why stromule induction and chloroplast-nucleus associations occur during plant–pathogen interactions has recently been addressed. *Nicotiana* N RECEPTOR INTERACTING PROTEIN (NRIP)1 is a chloroplast rhodanese sulphurtransferase that is required for effector triggered immunity (ETI) against TMV mediated by the N innate immune receptor (Caplan et al., 2008). In the presence of the viral p50 helicase effector NRIP1 was recruited to the cytoplasm and nucleus where it formed a protein complex with the TIR domain of the N immune receptor (Figure 3). The interaction with N in the nucleus and cytoplasm is necessary for ETI. These results raised the intriguing questions of whether NRIP1 was remobilized from the chloroplast and how this could be accomplished. Recent data suggest that stromules may provide a route for NRIP1 to traffic from the chloroplast to the nucleus. Co-expression of N and p50 induced formation of stromules during HR-PCD associated with this interaction (Caplan et al., 2015; Figure 5).

Similarly, stromule induction was observed during ETI initiated in response to bacterial pathogens, and on treatment with the defense-related signaling molecules SA and the ROS  $\text{H}_2\text{O}_2$ . Through correlative EM-fluorescence microscopy, the authors provide convincing evidence that stromules contact the nucleus during N-mediated defense. In elegant experiments that fuse a nuclear exclusion sequence (NES) to NRIP1, the authors provide quantitative evidence that NRIP1 is indeed trafficked from the chloroplast to the nucleus.  $\text{H}_2\text{O}_2$  also traffics to the nucleus via stromules. In plants overexpressing chloroplast outer membrane protein CHLOROPLAST UNUSUAL POSITIONING 1 (CHUP1) stromule formation is abolished, suggesting that this membrane plays a role in stromule formation. This was confirmed in *chup* mutants and knockdown plants, where constitutive stromule formation was observed. The importance of stromules to HR-PCD and the defense response was underscored by the accelerated HR-PCD observed in those plants. These results are suggestive of a role for stromules in intracellular trafficking and possibly signaling during plant–pathogen interactions, and these possibilities warrant further examination. It will be exciting to test whether plastids and/or stromules are able to create hemifusion membranes with the nuclear membrane and whether other signaling molecules or metabolites use this route for chloroplast signaling.

A separate study raises the possibility that stromules may also function in intercellular and intracellular trafficking of pathogens. The chloroplast-localized chaperone heat shock cognate 70 kDa protein (cpHsc70-1) was identified as interacting with the AbMV movement protein (MP; Krenz et al., 2010). In uninfected tissues cpHsc70-1-YFP homo-oligomerization was demonstrated by bimolecular fluorescence complementation (BiFC). cpHsc70-1-YFP oligomers localized to chloroplasts and near the cell periphery. Intriguingly, cpHsc70-1-YFP localized to punctate structures in chloroplasts and filaments that stretched like a



**FIGURE 5 | Chloroplast behavior during defense. (A)** Stromules (arrows) are observed intermittently from chloroplasts in the epidermis of *Nicotiana benthamiana* leaves. **(B)** Upon infection with Tobacco mosaic virus, chloroplasts cluster around the nucleus (asterisk), and stromule formation is induced (arrow). The chloroplasts shown are expressing NRIP1-CFP (Caplan et al., 2008, 2015). Note the NRIP1-CFP signal detected in the nucleus in TMV-infected chloroplasts, indicative of the translocation of NRIP1 from the chloroplasts to the nucleus. Images were collected on a Zeiss LSM 710 confocal laser scanning microscope and single focal plane images are shown. Scale bar is 10  $\mu\text{m}$ .

“string of pearls” between chloroplasts or to the cell periphery in AbMV-infected leaves. Reduced levels of the Hsc70 ortholog in *N. benthamiana* by virus induced gene silencing (VIGS) led to decreased intercellular trafficking of AbMV while viral replication was unaffected, suggesting that the chloroplast chaperone and stromules may have a role in viral trafficking that is independent of viral replication and accumulation (Krenz et al., 2010). Indeed, it has been proposed that the Hsc-70 chaperone in stromules may facilitate viral transport from chloroplasts to the cell periphery and plasmodesmata, from where intercellular spread could occur (Krenz et al., 2012). AbMV is a geminivirus that replicates in the plant nucleus, but AbMV is also found in plastids (Groning et al., 1987); therefore stromule-nucleus contacts could also provide a route for viral trafficking to nuclei in newly infected cells.

Another interesting question regarding the involvement of stromules in chloroplast signaling is when during evolution would such an innovation have arisen. The R gene *RESISTANCE TO POWDERY MILDEW (RPW)8.2* confers broad resistance to fungi that cause powdery mildew. RPW8.2 localizes to the extrahaustorial membrane (EHM) during fungal penetration of the host cell (Wang et al., 2009). Interestingly, RPW8.2 mutants have recently been reported to localize to an unknown membrane that appears to surround stromules (Wang et al., 2013a). This membrane has been termed the peristromule membrane (PSM) and it is proposed that they may share some as-yet-unknown characteristics with the EHM. Some RPW8.2 mutants localize to both the EHM and PSM, and while others localize to the nucleus. Notably, invasion by fungal haustoria induced the formation of stromules that purportedly connect plastids with haustoria. The authors speculate that stromules may represent an ancient interface for host–pathogen interactions. However, wild-type RPW8.2 was not observed labeling the PSM, and the authors speculate that this may be due to rapid cycling of RPW8.2 on and of the PSM (Wang et al., 2013a).

Together, these studies suggest that stromules could function in signaling in mediating responses to both biotic and abiotic stress, however, this remains to be demonstrated. Understanding the cellular functions of stromules will no doubt reveal intriguing aspects of plant cell biology and possibly identify new targets for engineering plants with modified responses to biotic and abiotic stresses.

### Chloroplast-Endoplasmic Reticulum Contacts: Lipid Signaling

A conundrum in plant biology is that the enzymes for a given metabolic pathway are often found in different subcellular compartments. One striking example of this is the biogenesis of complex lipids in some plants including *Arabidopsis*. In these plants fatty acids synthesized *de novo* in the chloroplast by the prokaryotic pathway may be exported to the ER where they are assembled into lipids by the eukaryotic pathway before import back into chloroplasts (Benning, 2009). Despite concerted efforts to identify candidate transporters that would allow the substrates to translocate from the chloroplast to the ER, these transporters have not yet been discovered (Wang

and Benning, 2012). This raises the intriguing possibility that metabolites may traffic directly from the chloroplast to the ER through membrane interactions. Moreover, direct physical contact sites between chloroplasts and ER, named plastid-associated membranes (PLAM), have long been reported by transmission electron microscopy in many plant and algal species (Cran and Dyer, 1973; Crotty and Ledbetter, 1973; Renaudin and Capdepon, 1977; McLean et al., 1988). More recently, confocal microscopy has also suggested chloroplast-ER membrane contact sites (MCS; Andersson et al., 2007; Tan et al., 2011). Indeed, these MCS are likely held together through strong protein–protein interactions since the ER remained associated with chloroplasts even after application of forces of 400 pN (Andersson et al., 2007). Once synthesized in the ER glycerolipids have to be imported into the chloroplasts. Mathematical modeling supports the import of diacylglycerol (DAG) from the ER to chloroplasts (Marechal and Bastien, 2014). Genetic analyses have identified the TGD complex as essential for import of ‘eukaryotic’ precursors into chloroplasts (Boudiere et al., 2014). TGD1-3 constitute a bacterial ABC transporter, while TGD4 apparently forms a  $\beta$ -barrel that localizes to both chloroplasts and ER (Wang and Benning, 2012). Thus transporters are indeed important mediators of lipid trafficking.

The DellaPena group has proposed an elegant hypothesis to explain chloroplast-ER membrane continuity (Mehrshahi et al., 2014). This group has developed a transorganellar assay to test whether non-polar metabolites, exemplified by tocopherols (vitamin E), located in the plastid envelope could directly access the lumen of the ER and modulate enzymes located there (Mehrshahi et al., 2013). To do this, tocopherol cyclase (TC) that is normally a chloroplast resident protein was retargeted to the ER in the background of the *vte1* mutant that does not make TC and tocopherols (Porfirova et al., 2002). Excitingly, this ER-localized TC complemented the *vte1* mutation and tocopherol levels were restored to almost wild-type levels in the rescued lines (Mehrshahi et al., 2013). This study revealed similar access to chloroplast-localized substrates for ER-localized  $\gamma$ -tocopherol methyltransferase ( $\gamma$ TMT) and  $\alpha$ -carotene  $\epsilon$ -ring hydroxylase LUTEIN DEFICIENT1 (LUT1). Based on their successful transorganellar complementation assay, the authors propose that an exchange of non-polar metabolites between plastids and endoplasmic reticulum occurs, most likely due to direct contacts between those organelles at PLAM sites according to the membrane hemifusion model (Mehrshahi et al., 2014). The hemifusion model postulates that the fused membrane of the ER and chloroplast outer envelope would consist of the inner leaflet of the ER and the plastid outer envelope membranes, two envelopes with similar compositions of non-polar metabolites (Mehrshahi et al., 2014). A membrane of this nature would create an easy path for chloroplast-to-ER signaling by non-polar metabolites.

### Chloroplast-Peroxisome Contacts

Peroxisomes are dynamic membrane-bound organelles of remarkable metabolic plasticity. They are found in all eukaryotic cells, and in plant cells are usually found in close association with mitochondria and chloroplasts (Figure 3). Peroxisomes are able



to adjust their complement of enzymes in response to changes in environmental and developmental signals (reviewed in Goto-Yamada et al., 2015; Sandalio and Romero-Puertas, 2015). Peroxisomes were first recognized for their action in scavenging  $H_2O_2$ , however, it is clear that peroxisomes have much more extensive roles in cellular metabolism (Sandalio and Romero-Puertas, 2015). A subset of these reactions highlights the intimate metabolic coupling between chloroplasts and peroxisomes.

The photorespiratory pathway is the conversion of phosphoglycolate to  $CO_2$  and 3PGA, via a complex series of reactions that takes place across three separate subcellular compartments: chloroplasts, peroxisomes, and mitochondria (Foyer et al., 2009). Severe photorespiratory conditions initiate ROS-dependent lipid peroxidation in the chloroplast that leads to activation of the lipoxygenase (LOX)-mediated reaction, which is one of the starting points in the oxylipin metabolic pathway. LOX also initiates the synthesis of many cell constituents and signaling molecules, including jasmonates via oxo-phytodienoic acid (OPDA; Weber et al., 1997; Montillet et al., 2004). OPDA is then transported into the peroxisomes where two rounds of beta-oxidation serve to modify the fatty-acid side-chain of the ring. It is believed that the transport of OPDA is carried out by both active and passive transport (Leon, 2013).

Recent work has revealed that in addition to close metabolic coupling, peroxisomes and chloroplasts physically interact with each other in a light-dependent manner. Using the newly developed femtosecond laser technology and confocal laser scanning microscopy, Oikawa and colleagues demonstrated that peroxisomes adopted an elliptical shape to increase their surface area and more tightly adhered to chloroplasts in light (Oikawa et al., 2015). A force of 61 fN  $nm^{-2}$  was needed to disrupt the chloroplast-peroxisome interaction in the light compared to 23 fN  $nm^{-2}$  required to so do in the dark. Interestingly, these changes in peroxisome shape and location depended on photosynthesis, but were independent of photorespiration or the activity of photoreceptors, and the actin cytoskeleton negatively regulated the interaction between the chloroplasts and peroxisomes (Oikawa et al., 2015). The chloroplast-peroxisome physical interaction is consistent with reports from other systems that suggest direct interaction between organelles is necessary for metabolite exchange (Binns et al., 2006; de Brito and Scorrano, 2008). Thus the trafficking of compounds like OPDA during JA synthesis could potentially contribute to signaling between plastids and peroxisomes.

The *Arabidopsis* *snowy cotyledon3* (*sco3-1*) mutant also provides support for signaling between chloroplasts and peroxisomes. In seedlings, the *sco3-1* mutation interrupted chloroplast biogenesis, decreased chlorophyll accumulation, and disrupted thylakoids and, as a consequence, photosynthesis (Albrecht et al., 2010). This mutation also resulted in photoinhibition in mature leaves under high  $CO_2$  concentrations. It is quite interesting that the SCO3 protein is initially targeted to peroxisomes. Also interesting is that loss of SCO3 function led to cytoskeletal defects, specifically affecting microtubules. Thus, both the cytoskeleton and peroxisomes are necessary for normal chloroplast development. Further investigation of SCO3

function will illuminate the process of communication between chloroplasts and peroxisomes.

## Chloroplast-Mitochondrion Contacts

Like chloroplasts, mitochondria are the end products of an endosymbiotic event, and they have also retained a portion of their ancestral genome (Woodson and Chory, 2008). Mitochondria-to-nucleus retrograde signaling is critical for coordinating expression of nuclear genes encoding mitochondrial proteins with expression of the mitochondrial genome (Woodson and Chory, 2008; Rhoads, 2011). Given their central roles in energy capture and utilization it is perhaps not surprising that chloroplasts and mitochondria exchange metabolites. Chloroplasts and mitochondria are also coupled by cellular redox status.

Mutant analyses have shed light on chloroplast-mitochondrion signaling. The expression of *alternative oxidase* (AOX), a nucleus-encoded mitochondrial gene, seems to be regulated by chloroplasts as increased expression of *Arabidopsis* and soybean AOX has been observed upon high light treatment (Finnegan et al., 1997; Blanco et al., 2014). Intriguingly, the white leaves of the chloroplast ribosome-deficient barley mutant *albostrians* as well as photo-bleached leaves of wild type obtained upon treatment with norflurazon, displayed elevated levels of mitochondrial DNA and transcripts (Hedtke et al., 1999). Consistent with this observation, recent analyses of leaves with green/white variegation in 12 ornamental plants confirmed that chloroplast dysfunction leads to increased levels of mitochondrial DNA in white sectors (Toshioji et al., 2012). Communication between chloroplasts and mitochondria seems to be bidirectional as mutation of genes encoding mitochondrial proteins can have profound effects on chloroplasts (e.g., Xu et al., 2008; Burch-Smith et al., 2011).

Several possible routes for communication between chloroplasts and mitochondria are proposed. There is coordinated expression of nuclear genes encoding chloroplast and mitochondria proteins, and it is therefore likely that some of the chloroplast signaling to mitochondria is accomplished by modulating nuclear gene expression. Indeed, expression of the *MITOCHONDRIAL DYSFUNCTION STIMULON* (MDS) suite of mitochondria-associated genes was regulated in response to chloroplast perturbations that included increased ROS and NO production (Ng et al., 2014). These MDS genes carry a common regulatory motif in their promoters that mediates their induction in response to mitochondrial retrograde signals (De Clercq et al., 2013; Ng et al., 2013). Moreover, it was demonstrated that *Arabidopsis* ABI4 regulates both *Lhcb* and *AOX1A* genes, providing a molecular link for nucleus-coordinated chloroplast-mitochondria communication (Koussevitzky et al., 2007; Giraud et al., 2009). Analysis of the *Arabidopsis* *regulator of alternative oxidase 1* (*rao1*) mutant deficient in a nucleus-localized cyclin-dependent kinase E1 (CDKE1) that is a prerequisite for AOX induction, indicates it is another nucleus-localized sensor integrating mitochondrial and chloroplast retrograde signals (Blanco et al., 2014). Unlike wild type plants the *rao1*(*cdke1*) mutant was unable to induce *AOX1A* expression upon application of antimycin A

or DCMU (that specifically targets chloroplasts). Further, the mutant displayed the *gun* phenotype upon induction of redox stress originating specifically from chloroplast photoelectron transport.

Another possible mechanism for chloroplast-to-mitochondrion signaling could involve dual targeting of proteins. The localization of proteins to multiple subcellular compartments is an ancient feature of land plants that can be observed in *Physcomitrella patens* (Xu et al., 2013b) and diatoms (Gile et al., 2015). In *Arabidopsis*, over 100 proteins are targeted to both chloroplasts and mitochondria (Carrie and Whelan, 2013). It is tempting to propose that, analogous to the situation where proteins have been shown to localize to both chloroplasts and nuclei, proteins may move from the chloroplast to the mitochondria. The regulated translocation of proteins from chloroplasts to mitochondria to modulate mitochondrial gene expression would mediate chloroplast signaling. Such translocation would be made much easier by direct contact between chloroplasts and mitochondria.

Third, there may be direct communication by physical interaction. In leaves, chloroplasts, mitochondria and peroxisomes have often been observed in close association, consistent with metabolic exchange among these organelles (Figure 4). It seems that the formation of this tri-organelle unit is regulated, with the chloroplast-peroxisome association being established first and then recruiting mitochondria (Oikawa et al., 2015). Application of the femtosecond laser pulses to chloroplast-mitochondria complexes in a variety of tissues should illuminate the biophysical characteristics of their association.

In all these instances of organelle interactions, it is apparent that more detailed analysis at the level of resolution provided by electron microscopy is needed. Such studies should incorporate state-of-the-art fixation techniques like tandem high-pressure freezing and freeze substitution in order to minimize artifact formation and to maintain intact the presumably delicate membrane extensions and contact sites. These techniques are becoming easier and less time-consuming (McDonald, 1999, 2014) and can easily be adopted for plant cell biology (Bobik et al., 2014). When coupled with fluorescence microscopy, this will be a powerful approach for ultrastructural interrogation, as exemplified by recent work from Caplan et al. (2015).

## Hormones and Reactive Molecules as Chloroplast Signals

As sessile organisms plants have evolved to cope with extreme environmental conditions and fluctuations. Some of the most common environmental challenges to plant survival include drought, flooding resulting in reduced oxygen availability, and temperature extremes. In addition, the photosynthetic machinery is sensitive to excessive light and such exposure results in oxidative stress at the cellular level. In addition to these abiotic stresses, pathogens pose a constant threat of disease. Plants have therefore evolved a complex suite of responses that are exquisitely fine-tuned to allow them to cope with these stresses. It is widely recognized that chloroplasts both sense and respond

to environmental conditions. Indeed, the chloroplast-generated hormones SA, JA and ABA and other secondary messengers including ROS and reactive nitrogen species (RNS) as well as redox signals are critical components of the plant stress response. Therefore chloroplast signaling is indispensable for plant survival of abiotic and biotic stress. The roles of all these chloroplast-associated molecules in coping with biotic and abiotic stress have been extensively examined and several excellent reviews are available (Miller et al., 2008; Padmanabhan and Dinesh-Kumar, 2010; Kangasjarvi et al., 2012; Pfannschmidt and Yang, 2012; Trotta et al., 2014).

### Salicylic Acid

Salicylic acid is best known for its role plant-pathogen interactions and particularly in plant defense. However, SA also has roles in plant developmental processes including germination, root and shoot growth and senescence, and also functions in abiotic stress responses (Rivas-San Vicente and Plasencia, 2011). A phenolic compound, SA is largely the product of the isochorismate pathway in the chloroplasts. Pathogen infection induces the production of SA by chloroplasts mainly through the action of the chloroplast-localized ISOCHORISMATE SYNTHASE (ICS)1 enzyme although there may be some contribution by ICS2 (Wildermuth et al., 2001; Garcion et al., 2008). Analysis of the *ics1 ics2* double mutant has revealed that there are other cellular sources of SA (Garcion et al., 2008), most likely via phenylpropanoid metabolism in the cytoplasm (Vlot et al., 2009). SA conjugates with glucose or a methyl side-group are the commonly active forms of SA. Indeed, methyl salicylate is a critical mediator of systemic acquired resistance (SAR; Park et al., 2007), and it may also function in ecological defense signaling when it becomes airborne (Shulaev et al., 1997).

Many SA-mediated defense responses rely on the action of the transcriptional activator NON-EXPRESSOR OF PR1 (NPR1; Fu and Dong, 2013). NPR1 interacts with TGA transcription factors, and it is believed to act with them as co-activators of defense gene expression (Zhang et al., 1999; Zhou et al., 2000; Yan and Dong, 2014). The action of NPR1 is dependent on the cellular redox state. In the absence of SA, NPR1 oligomerizes in the cytoplasm, but on perception of SA, NPR1 is reduced and the oligomer disassembles into monomers that then relocate to the nucleus to modulate gene expression (Kinkema et al., 2000; Mou et al., 2003). The redox state of NPR1 is mediated by the glutathione and thioredoxin redox systems (Mhamdi et al., 2010; Han et al., 2013). In the nucleus, NPR3 and 4, two other SA-binding proteins (SABPs) that are closely related to NPR1, modulate NPR1's activity (Fu et al., 2012). It should be noted that there are also NPR1-independent pathways that mediate SA signaling (Rairdan et al., 2001; Shah, 2003; Uquillas et al., 2004; Blanco et al., 2005). Downstream of NPR1 the SA signaling pathway is well understood (Spoel et al., 2003; Fu and Dong, 2013).

Salicylic acid is important for basal defense as well as for ETI (Alazem and Lin, 2015), and application of SA or overexpression of its biosynthesis genes leads to increased pathogen resistance (Malamy et al., 1990; Ryals et al., 1996;



Mur et al., 2008; Shah, 2009; Coll et al., 2011). Chloroplast  $\text{Ca}^{2+}$  signals are induced in a stress-specific manner, and this response is mediated by the calcium-sensing receptor (CAS; Nomura et al., 2012). CAS mediates the  $\text{Ca}^{2+}$  signals in ETI and in response to the presence of highly conserved pathogen associate molecular patterns (PAMPS). CAS, and thus  $\text{Ca}^{2+}$ , regulates chloroplast SA biosynthesis and plants depleted of CAS failed to induce SA production in response to pathogen infection. In addition, expression of several nuclear defense-related genes was shown to be dependent on CAS, and the pattern of gene regulation was most similar to that observed in response to  $^1\text{O}_2$ .

Interestingly, exogenous application of SA induces closure of plasmodesmata (Wang et al., 2013b). This is mediated by PLASMODESMATA LOCALIZED PROTEIN (PDL)5, and likely involves the action of a callose synthase (Lee et al., 2011; Wang et al., 2013b). Consistent with these findings, *Arabidopsis pdlp5* mutants have decreased resistance to bacterial pathogens. These results clearly demonstrate crosstalk between SA signaling and plasmodesmata, and illustrate how chloroplast signals can act to regulate intercellular trafficking via plasmodesmata (discussed below). It will be interesting to see if similar mechanisms are deployed for viral resistance since viruses use plasmodesmata for intercellular trafficking.

There has been some controversy over the SA-binding properties of NPR1. Binding assays using recombinant GST-NPR1 and tritiated-SA ( $[^3\text{H}]\text{-SA}$ ) suggested that NPR1 did not bind SA (Fu et al., 2012). In contrast, equilibrium dialysis experiments revealed that NPR1 bound SA with a  $K_D$  similar to that observed for other receptor-ligand interactions for plant hormones (Wu et al., 2012). Recent results confirm that SA can indeed bind NPR1 (Manohar et al., 2014). However, it is clear that NPR1 is not the only protein that binds SA. The metabolic enzymes catalase (Chen et al., 1993), ascorbate peroxidase (Durner and Klessig, 1995) and methyl salicylate esterase (SABP2; Forouhar et al., 2005) have been shown to bind SA. Recent work reveals that SA potentially has numerous targets in the cell including, not unexpectedly, numerous chloroplast proteins.

Many of these SABPs have been identified by high-throughput approaches (Moreau et al., 2013; Manohar et al., 2014). By probing *Arabidopsis* protein microarrays with 4-azido SA (azSA), an SA analog, the Popescu lab identified numerous chloroplast-localized proteins with roles in photosynthesis and oxidative phosphorylation as proteins interacting with AzSA (Moreau et al., 2013). Two other interesting candidate SABPs were also identified: thimet metalloendopeptidase At5g65620 (TOP1) and its homolog encoded by At5g10540 (TOP2), both of unknown cellular function. TOP1-GFP fusions localized to chloroplasts and TOP2 is likely cytosolic. Interestingly, *top1 top2* double mutants had compromised ETI and PCD to bacterial pathogens. Further, TOP1 and TOP2 dimerize in an SA- and redox-dependent manner (Westlake et al., 2015). However, TOP1 and TOP2 have distinct responses to the reductant DTT, suggesting they have different activities *in planta*. A role for TOP1 and TOP2 in the oxidative stress response was demonstrated by treating

various *top* mutants with methyl viologen, a potent inducer of oxidative stress, but this function is likely restricted to early in plant development. Two additional high throughput screens have recently identified another 100 candidate SABPs (Manohar et al., 2014). Of these, nine were already known SABPs and the SA binding of nine of new candidate SABPs was verified. Notably, four of the new SABPs have roles in redox regulation, reiterating the interaction between these two pathways.

## Jasmonates

Jasmonic acid is a lipid-derived hormone that is perhaps best known for its roles in insect herbivory and wounding, but also has roles in plant growth and development (Leon, 2013). The term jasmonates refers to a group of compounds that are derived from linoleic acid. JA is synthesized via the octadecanoid pathway and JA synthesis is initiated in the chloroplasts but is completed in the peroxisome. JA is then derivatized to yield a diverse array of metabolites that have different functions, ranging from storage to inactivation (reviewed in Leon, 2013; Wasternack and Hause, 2013). The most active of these compounds is a JA conjugate with isoleucine, (+)-7-*iso*-JA-Ile (Wasternack, 2014). JA biosynthesis and signaling pathways have been elucidated (Turner et al., 2002; Antico et al., 2012; Wasternack and Hause, 2013; Zhu, 2014). JA signaling in stress is closely linked with that of another hormone, ethylene (Kunkel and Brooks, 2002). Interestingly SA and JA/ET signaling are often antagonistic to each other (Robert-Seilanian et al., 2011). This highlights the crosstalk between chloroplast signals.

The final chloroplastic intermediate in JA-biosynthesis is *cis*-(+)-12-oxophytodienoic acid (OPDA). OPDA is then translocated to the peroxisome, where its hydrocarbon chain is shortened by  $\beta$ -oxidation. It is not clear how OPDA is transported to the peroxisomes, but the process must be tightly regulated, given that OPDA itself is able to act as a signaling molecule. Indeed, OPDA has been implicated in tendril coiling (Blechert, 1999); in *Arabidopsis* seed germination (Dave et al., 2011); in tomato embryo development (Goetz et al., 2012), and fertility in *P. patens* (Stumpe et al., 2010). OPDA is also known to be an important signal for defense (Stintzi et al., 2001; Scalschi et al., 2015). Interestingly, *P. patens* does not make JA but instead uses OPDA for defense (Stumpe et al., 2010; Ponce De Leon et al., 2012). However, SA is used as a defense signal in this moss (Ponce De Leon et al., 2012). In the liverwort *Marchantia polymorpha* wounding induces OPDA production, and exogenous application limited *M. polymorpha* growth (Yamamoto et al., 2015). As in *P. patens*, JA was not detected in *M. polymorpha*. This finding was supported by OPDA regulating growth of *M. polymorpha* while JA could not. Thus, the production of lipid-derived signals by the chloroplast is an ancient feature of plants, and the production of JA may have developed more recently.

## Crosstalk of RNA Silencing and Chloroplast Hormones

There is also crosstalk between the chloroplast-derived phytohormones and the RNAi machinery of plant cells. Several studies have reported increased expression of RNA-DEPENDENT RNA POLYMERASE1 (*RDR1*) on exogenous

application of SA (Xie et al., 2001; Yu et al., 2003; Hunter et al., 2013), JA (Pandey and Baldwin, 2007) and ABA (Hunter et al., 2013). RDR1 is known to have a role in antiviral RNA silencing, in the production and amplification of virus-derived siRNAs (Donaire et al., 2008; Wang et al., 2010). JA interaction with SA is mostly antagonistic, and there are few genes whose expression is induced by both hormones (Pieterse et al., 2009). The finding RDR1 can be induced by multiple hormones suggests that there is crosstalk between hormones and the RNA silencing machinery to mediate stress responses. However, RDR1 does not seem to have a role in drought resistance (Hunter et al., 2013), although other components of the RNAi components have been demonstrated to function in stress tolerance (Earley et al., 2010; Li et al., 2012; Westwood et al., 2013). Together, these findings extend the role of chloroplast signaling in the stress response.

### Abscisic Acid

Abscisic acid is one of the most important hormones mediating plant biotic and abiotic stress responses. ABA also has major roles in various plant physiological processes including stomatal movement and seed dormancy (Wensuo and Zhang, 2008; Rodriguez-Gacio Mdel et al., 2009; Kim et al., 2010). ABA is a sesquiterpenoid that is produced by the methylerythritol phosphate (MEP) pathway in plastids that produces carotenoids (Finkelstein, 2013). ABA levels in a cell are the result of both its synthesis and catabolism. Interestingly, several subcellular compartments are involved in ABA metabolism (Xu et al., 2013a). All the steps of the *de novo* ABA biosynthetic pathway occur in plastids except for the last two, which occur in the cytosol. The first committed step in ABA synthesis is the cleavage of the carotenoid xanthophyll by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to produce the C15 compound xanthoxin, that is transported from the plastids into the cytosol by an unknown mechanism, where it will be converted into ABA (Finkelstein, 2013). Once synthesized, ABA is transported from the sites of synthesis to sites action via the xylem and phloem and is thus made available to both roots and shoots. ABA catabolism occurs via one of two pathways: oxidation or conjugation to glucose to produce a glucosyl ester ABA-GE. ABA-GE is stored in the vacuole or ER until it mobilized under stress conditions. Because of its central roles in modulating responses to various stresses, ABA signaling is an attractive target for engineering plants with increased tolerance to those stresses.

The mechanisms governing perception of, and signaling by, ABA are being discovered (Yoshida et al., 2015). Members of the PYR/PYL/RCAR family of soluble proteins are ABA receptors (Ma et al., 2009; Park et al., 2009). This protein family has 14 members, almost all of which appear capable of forming an ABA-receptor complex that is able to activate the transcription of ABA-responsive genes (Kline et al., 2010). Downstream of the receptors, several kinases, including calcium dependent kinases (CDPKs), and phosphatases mediate ABA signaling, culminating in changes in nuclear gene expression (Finkelstein, 2013; Yoshida et al., 2015). The 26S proteasome is also important in mediating ABA signaling (Ludwikow, 2015).

Abscisic acid signaling is particularly important during drought, salinity and cold stress. During pathogen infection, ABA signaling is antagonistic to JA/Et signaling (Soosaar et al., 2005); and it can also antagonize SA signaling (Alazem and Lin, 2015). Indeed, crosstalk between SA, JA, and ABA signaling pathways during pathogen defense is well documented (Tuteja, 2007; Flors et al., 2008; Cao et al., 2011) and underscores the role of chloroplasts in integrating inputs for plant survival. Importantly, the H subunit of Mg chelatase was shown to be an ABA receptor, and moreover, the observations that ABA can repress *Lhcb* expression and the gun phenotype of the *abi4* mutant link this hormone to retrograde signaling (Shen et al., 2006; Koussevitzky et al., 2007).

### Reactive Oxygen Species

Reactive oxygen species are formed by the reduction of molecular oxygen and the term ROS includes superoxide ( $O_2^{\cdot-}$ ), hydroxyl, alkoxyl ( $\cdot RO$ ), and peroxy radicals as well as non-radical molecules like hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ). It is well established that while large amounts of ROS are damaging, small amounts act as signaling molecules (Miller et al., 2008; Gill and Tuteja, 2010). In an effort to avoid the toxicity of ROS, plants have evolved multiple antioxidant systems. It is clear then that ROS signaling is complex and is often the outcome of the balance between production and scavenging.

In a typical plant cell ROS may be generated by a variety of subcellular compartments including chloroplasts, peroxisomes, mitochondria and the apoplast. In a photosynthesizing leaf most ROS is the product of the chloroplasts and peroxisomes with smaller contributions from mitochondria (Foyer and Noctor, 2005). During photosynthesis, in the reaction center of photosystem II (PSII), excited triplet center chlorophyll P680 interacts with oxygen to generate  $^1O_2$ . The acceptor side of PSI produces superoxide and hydrogen peroxide as electrons are transferred from reduced ferredoxin to molecular oxygen. Photorespiration in the peroxisomes to recycle Rubisco that has reacted with oxygen is a major source of  $H_2O_2$  (Yoshida and Noguchi, 2011).

Plastid-generated ROS molecules have been shown to act as signals that modulate expression of nuclear genes. Different ROS molecules have been shown to induce expression of distinct suites of genes (Desikan et al., 2001; Vandenabeele et al., 2004; Vanderauwera et al., 2005; Laloi et al., 2007; Li et al., 2011; Balazadeh et al., 2012; Mor et al., 2014). This has made it possible to draw distinctions between the various ROS initiating responses to different stresses, (e.g., Gadjev et al., 2006). By virtue of their nature, ROS are known to interact with a variety of biological molecules. For example, as a strong electrophile,  $^1O_2$  can react spontaneously with many classes of biological molecules including proteins, lipids and nucleic acids. However, the question remains whether they act directly or indirectly through modification of other biomolecules.

Hydrogen peroxide is the least reactive ROS, and at high light intensities up to 5% of chloroplast-generated  $H_2O_2$  has been detected outside the organelle (Mubarakshina et al., 2010).  $H_2O_2$  may exit the chloroplasts via aquaporins (Bienert et al.,

2007). Recently,  $H_2O_2$  has also been shown to relocate from chloroplasts to the nucleus via stromules (Caplan et al., 2015). Indeed, chloroplast ROS production induces stromule formation (Brunkard et al., 2015b). These findings suggest that  $H_2O_2$  may act directly on its targets to regulate their expression or behavior.

In contrast,  $^1O_2$  is highly reactive, and,  $^1O_2$  is involved in signaling pathways leading to cell death or to acclimation (Wagner et al., 2004; Ledford et al., 2007). Yet, because of its very high reactivity and therefore short lifetime *in vivo*,  $^1O_2$  is not considered to be a molecule directly involved in chloroplast-to-nucleus signaling. Studies in the *Arabidopsis fluorescent (flu)* mutant have shed light on  $^1O_2$  signaling (Kim and Apel, 2013). The *flu* mutant accumulates protochlorophyllide and when plants are exposed to light they generate large amounts of  $^1O_2$  (Meskauskiene et al., 2001). Increased  $^1O_2$  production in *flu* chloroplasts was associated with induction of stress responses including dramatic changes in nuclear gene expression and enhanced accumulation of the stress hormones, SA, Et, and the oxylipins OPDA and JA (op den Camp et al., 2003). Interestingly, these  $^1O_2$ -induced changes were dependent on the chloroplastic EXECUTER1 (EX1) and EX2 proteins (Kim et al., 2012). To date, two direct protein targets of  $^1O_2$  have been identified: one is  $\beta$ -carotene and the other is the D1 protein of PSII (Kim and Apel, 2013). Reaction of  $^1O_2$  with  $\beta$ -carotene produces  $\beta$ -cyclocitral, shown to be involved in retrograde signaling (Ramel et al., 2012; see above). D1 is potent scavenger of  $^1O_2$ , and this activity leads to the destruction of D1 by protease activity. However, to date no biological activity for these D1 fragments in plants has been reported.

Plants possess several enzymes including catalase and ascorbate peroxidase that detoxify ROS (Miller et al., 2008). In addition plants have evolved antioxidant systems that allow them to not only cope with ROS production during stress but also transduce ROS signals. Such systems include tocopherols (vitamin E), ascorbate (vitamin C), and glutathione (a tripeptide thiol) and they all associate with chloroplasts. Glutathione is the most important antioxidant in plants (Zechmann, 2014) and it can act on ROS directly or through the ascorbate-glutathione system. Glutathione synthesis is initiated in chloroplasts (Wachter et al., 2005). The importance of glutathione highlights the importance of thiol-disulfide reactive proteins as antioxidants (Foyer and Noctor, 2003). Proteins including thioredoxins, glutaredoxins, glutathione peroxidases are found in chloroplasts as well as other subcellular compartments, and these proteins convey the redox state of chloroplasts to the rest of the plant cell. The involvement of redox signaling in mediating plant stress responses are extensively documented (Foyer et al., 2009).

### Nitric Oxide and Reactive Nitrogen Species

Nitric oxide (NO) is a small gaseous molecule whose role in signaling in plant and non-plant systems is well established. While NO is an important second messenger in plants, major aspects of NO synthesis and action remain undiscovered. NO has critical roles in normal plant physiology including seed dormancy, germination, the floral transition, and during stress responses (Wendehenne et al., 2004; Moreau et al., 2008; Sanz et al., 2015). In plants NO is synthesized in chloroplasts (Mandal

et al., 2012; Tewari et al., 2013), and synthesis is closely linked to lipid metabolism, specifically that of oleic acid (Mandal et al., 2012).

In animals NO is synthesized by NITRIC OXIDE SYNTHASEs (NOSs) that oxidize L-arginine to NO (Stuehr, 2004). Curiously, NOS-like activity has long been described in plants but the identity of this enzyme remains elusive (Besson-Bard et al., 2008). In plants, nitrate reductase can reduce nitrate to NO (Desikan et al., 2002). Another enzyme implicated in NO synthesis is AtNOA1 (formerly AtNOS1). However, AtNOA1 is a GTPase that binds ribosomes, and it lacks NOS activity (Moreau et al., 2008). Nonetheless, *atnoa1* mutants displayed reduced levels of NO (Guo et al., 2003).

One of the primary mechanisms through which NO exerts its effects is by direct regulation of protein activity through nitrosylation of cysteine residues in a redox-dependent manner (Mengel et al., 2013). Nitration of tyrosine residues also occurs, and it is mediated by the peroxynitrite ( $ONOO^-$ ) RNS formed on reaction of NO with  $O_2$  (Corpas et al., 2013). Tyrosine nitration is viewed as a hallmark of plants under a variety of biotic and abiotic stresses (Corpas et al., 2013). While proteomic analyses have identified only relatively few nitrosylated proteins in *Arabidopsis* (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Ortega-Galisteo et al., 2012; Camejo et al., 2013; Puyaubert et al., 2014), a recent computational approach identified more than 16,000 potential protein targets for nitrosylation (Chaki et al., 2014). Refined versions of this program should be very useful in streamlining investigation of NO function and effects in signaling.

There is substantial crosstalk between ROS and RNS signaling pathways (Lounifi et al., 2013). Indeed, even small amount of NO had profound effects on chloroplast PET and redox state (Vladkova et al., 2011; Misra et al., 2014). It is therefore not surprising that NO is important for plant defense (Zaninotto et al., 2006; Bellin et al., 2013). Further, NO signaling interacts with SA and JA signaling pathways (Wendehenne et al., 2004; Zhou et al., 2015). NO production has been proposed as a potential target for engineering plant fitness (Foresi et al., 2015). However, this approach should be carefully considered given the potentially deleterious effects of runaway NO production and the extensive crosstalk between NO and other stress signaling pathways.

### Chloroplasts as Targets of Pathogen Effectors

The previous sections have highlighted the role of several chloroplast products in stress tolerance and plant defense. That chloroplasts have critical roles in pathogen defense is supported by the identification of numerous chloroplast proteins as direct targets of pathogen effectors (Table 1). Several of these targets are components of PET, suggesting that photosynthesis and chloroplast function are important for defense against pathogens. A series of experiments aimed at addressing the role of chloroplasts beyond phytohormone production in plant defense has been reported. VIGS of two genes encoding components of the PSII oxygen-evolving complex, *PsbQ* and *PsbO*, resulted in a three to four-fold increase in the number of Turnip mosaic virus (TuMV) infection foci (Manfre et al.,

**TABLE 1 | Chloroplast proteins targeted by pathogen effectors.**

Pathogen	Pathogen protein	Target	Reference
<b>Viruses</b>			
Alfalfa mosaic virus	Coat protein	PsbP	Balasubramaniam et al., 2014
Tobacco mosaic virus	Replicase (helicase) Replicase	NRIP1	Caplan et al., 2008
		PsbO	Abbink et al., 2002
		AtpC	Bhat et al., 2013
		Rca	Bhat et al., 2013
Tomato mosaic virus	Movement protein	RbcS	Zhao et al., 2013
Plum pox virus	CI	PSI-K	Jimenez et al., 2006
Potato virus Y	HC-Pro	MinD	Jin et al., 2007
		XDS	Li et al., 2015
		Rieske Fe/S	Shi et al., 2007
Soybean mosaic virus	P1	Ferredoxin	Cheng et al., 2008
Sugarcane mosaic virus	HC-Pro	Rubisco	Lin et al., 2011
Turnip mosaic virus	P3	PsbO	Jang et al., 2013
Alternanthera mosaic virus	TGB3		
<b>Bacteria</b>			
<i>Pseudomonas syringae</i> pv.	HopI1	Cytosolic	Jelenska et al., 2007
Tomato DC3000	HopN1 HopU1 HopK1	Hsp70*	Jelenska et al., 2010
		PsbQ	Rodriguez-Herva et al., 2012
		RNA binding proteins (CP-RBPs)	Fu et al., 2007
		Unknown	Li et al., 2014
<i>P. syringae</i> pv pisi	AvrRps4	Unknown	Li et al., 2014
<b>Fungi</b>			
Melampsora larici-populina	MLP10772	Unknown	Petre et al., 2015
(rust fungus)	MLP124111		

2011). A similar increase was observed when the chloroplast protease FtsH or Rubisco were depleted by VIGS (Manfre et al., 2011). These findings were further supported by the observation that treatment with lincomycin, and low-light conditions led to increased rates of TuMV infection. Importantly, it was demonstrated that these effects were independent of SA, as lincomycin-treated plants induced SA production to the same levels on TuMV infection as observed in non-treated controls. Given that TuMV replicates in association with the chloroplast outer envelope (Wei et al., 2010), it is perhaps not surprising that perturbing chloroplast function results in increased viral infection. However, chloroplast proteins are targets for other viruses, e.g., Tobacco mosaic virus (TMV), which are not known to replicate in association with chloroplast membranes. Interestingly, PsbO has been shown to interact the helicase domain of the TMV replicase, and VIGS of *PsbO* lead to a 10-fold increase in TMV infection in *N. benthamiana* (Abbink et al., 2002).

## Chloroplasts and Intercellular Signaling

The engulfment of cyanobacteria and subsequent evolution of the cellulosic cell wall surrounding the new plant cell likely created a physical barrier that hampered cell-to-cell communication and further development of multicellular, three-dimensional plants. It is important to highlight that modern

chloroplasts are a sister group to the filament and heterocyst-forming cyanobacteria *Nostoc* and *Anabaena* (Falcon et al., 2010). Intercellular movement of molecules between the cells of *Anabaena cylindrica* filaments has been demonstrated (Mullineaux et al., 2008). Heterocysts are specialized cells that fix N<sub>2</sub> and lack O<sub>2</sub>-evolving PSII and typically they have thick cell walls with limited permeability to gases. Interestingly, heterocysts are connected to vegetative cells by microplasmodesmata (Falcon et al., 2010; Padmanabhan and Dinesh-Kumar, 2010). Thus, we propose that chloroplasts, as the successors of these ancient cyanobacteria, participate in regulation of the cell-to-cell connectivity of modern plant cells by regulating plasmodesmata biogenesis in contemporary plants. This hypothesis is supported by a number of reports showing that mutants with defective chloroplasts display aberrant trafficking and altered expression of cell wall-related genes (see below). The relationship between chloroplasts and plasmodesmata and thus cell wall becomes more palpable when one considers sugar transport from source to sink tissues through plasmodesmata.

## Chloroplast Signaling and Symplasmic Transport

Communication between plant cells is enhanced by the presence of plasmodesmata, specialized channels traversing the cell wall between adjacent cells. Even though discovered more than 100 years ago, the structure and regulation of plasmodesmata are



poorly understood. In contrast, recent advances in plant biology and genetics have helped reveal much about their function. Plasmodesmata are indispensable for proper plant development as they provide a route for exchange of metabolites including water, ions and photoassimilates, as well as information encoded by hormones, nucleic acids and proteins including transcription factors (reviewed in Gallagher et al., 2014; Brunkard et al., 2015a; Heinlein, 2015; Jackson, 2015). Plant viruses have evolved to take advantage of the intercellular connectivity afforded by plasmodesmata, and viruses use these channels for cell-to-cell spread during infections of their plant hosts. Recent studies on grafted plants resulted in a tantalizing hypothesis that plasmodesmata facilitate the movement of the entire chloroplast genomes between neighboring cells (Stegemann and Bock, 2009; Stegemann et al., 2012; Thyssen et al., 2012). However, the dimensions of plasmodesmata would constrain movement of intact organelles, and whether the translocated genomes use intact chloroplasts as vehicles or are transported through plasmodesmata as “naked” but specifically folded DNA remains to be elucidated.

Interestingly, genetic screens aimed at isolating genes regulating plasmodesmata have identified genes involved in the biogenesis and/or functioning of chloroplasts. The first such reported mutant was maize *sucrose export defective1* (*sxd1*; Russin et al., 1996). The *sxd1* mutants failed to export photosynthate from sites of photosynthesis and have reduced intercellular transport due to accumulation of callose at plasmodesmata located at the bundle-sheath and vascular parenchyma interfaces (Botha et al., 2000). Callose is a polymer composed of  $\beta$ -1,3-linked glucose residues and callose accumulation at the necks of plasmodesmata is a major strategy for limiting plasmodesmal trafficking (De Storme and Geelen, 2014). Notably, despite accumulating sugars and starch in source tissues, *sxd1* does not repress photosynthesis (Provencher et al., 2001). *SXD1* is the maize ortholog of *Arabidopsis* *VTE1*, encoding a chloroplast TC required for the production of the antioxidant vitamin E (Provencher et al., 2001; Porfirova et al., 2002). The *sxd1* mutant provided the first clue that chloroplast redox state may influence plasmodesmata. However, under optimal growth conditions *vte1* mutants were indistinguishable from wild-type plants, although they were more sensitive to photooxidative stress (Porfirova et al., 2002). *Arabidopsis* mutants with defects in tocopherol metabolism should be carefully examined for plasmodesmata-related changes in intercellular trafficking.

A genetic screen for *Arabidopsis thaliana* mutants with altered plasmodesmal function identified the *gfp arrested trafficking* (*gat*) mutants (Benitez-Alfonso et al., 2009). In these mutants GFP synthesized in the companion cells of the phloem failed to move via the plasmodesmata into the surrounding tissues as occurred in wild-type tissues; that is, *gat* mutants have decreased intercellular trafficking. The *gat1*, 2, 4, and 5 mutations are all seedling lethal, with development ceasing about 10 days after germination. *GAT1* encodes a plastid-localized thioredoxin-m3 (TRX-m3), and *gat1* roots accumulate higher levels of ROS than do wild-type roots. In addition, callose was found to accumulate at plasmodesmata in *gat1* roots. Similar observations have been made for the other *gat* mutants (Benitez-Alfonso and Jackson,

2009). Notably, approximately five percent of plasmodesmata in *gat1* seedling roots are occluded by electron-dense material (Benitez-Alfonso et al., 2009). Overexpression of *GAT1* results in the reciprocal phenotype of increased intercellular transport. Thus, like *SXD1/VTE1*, *GAT1* likely functions in redox homeostasis involving the chloroplasts and perturbation of plastid redox state results in altered plasmodesmata. In addition, it has been proposed that altered metabolic flux in the *gat1* mutant may alter the redox state of TRX-m3 and ultimately influence plasmodesmata function (Benitez-Alfonso et al., 2009).

The Zambryski lab conducted a separate screen for *Arabidopsis* mutants with altered plasmodesmata-mediated intercellular trafficking (Kim et al., 2002; Burch-Smith and Zambryski, 2012). Several *increased size exclusion limit* (*ise*) mutants were identified by screening embryonically lethal mutants and monitoring their ability to traffic fluorescent dyes between cells. *ISE1* and *ISE2* have been mapped and cloned (Kobayashi et al., 2007; Stonebloom et al., 2009). In addition to increased plasmodesmal trafficking *ise1* and *ise2* embryos also contain increased numbers of plasmodesmata with multiple branches (Burch-Smith and Zambryski, 2010). Interestingly, nuclear *ISE2* encodes a chloroplast-localized DEVH-type RNA helicase, (Burch-Smith et al., 2011) while *ISE1* encodes a mitochondrial DEAD-box RNA helicase (Stonebloom et al., 2009). Further analyses in *Arabidopsis* and *N. benthamiana* revealed that loss of *ISE1* and/or *ISE2* leads to defective chloroplasts and ultimately leaves become yellow (Burch-Smith and Zambryski, 2010; Burch-Smith et al., 2011; Stonebloom et al., 2012). Gene expression analyses on *ise1* and *ise2* embryos by tiling microarrays revealed that the largest class of nuclear genes affected in both mutants, representing ~20% of the total, encode chloroplast-localized proteins (Burch-Smith et al., 2011). Importantly, genes encoding products for chlorophyll biosynthesis, photosynthetic light-harvesting reactions, and the Calvin cycle for carbon fixation were all affected in both mutants. Thus, the overlapping plasmodesmal phenotypes of the *ise1* and *ise2* mutants are likely due to defective chloroplasts.

The changes in plasmodesmata and intercellular trafficking are likely mediated by altered redox signaling from chloroplasts and mitochondria. Using redox-sensitive fluorescent reporters the redox state of chloroplasts depleted of *ISE1* or *ISE2* was measured (Stonebloom et al., 2012). Significantly, chloroplasts in *ISE1*- or *ISE2*-silenced *N. benthamiana* leaves were more reduced than in non-silenced control leaves. These results are consistent those from the *gat1* mutant (Benitez-Alfonso et al., 2009). Thus, oxidized chloroplasts are associated with decreased intercellular trafficking while reduced chloroplasts increase plasmodesmata-mediated trafficking. Further experiments focused on identifying the redox pathways and signal transduction involved in regulating plasmodesmata will yield important insight into how the physiological state of the chloroplasts is communicated to the rest of the plant.

## Redox Signals Travel between Cells

Recently the involvement of chloroplasts in remote control of nucleus-localized alternative splicing was reported. Specifically, the light-regulated redox state of plastoquinone is the source of



a chloroplast-generated signal able to control alternative splicing (Petrillo et al., 2014). In the presence of a reduced or oxidized plastoquinone pool, the alternative splicing of selected nuclear genes is promoted or inhibited, respectively. Importantly, even though photosynthetic tissues generated it, the putative signal is able to travel to roots to control alternative splicing. Thus chloroplast redox signals can act non-cell-autonomously. The redox pathway leading to defective alternative splicing has not been identified; however, the redox-regulated protein kinases of chloroplasts are proposed to contribute to the signaling machinery through a process of phosphorylation of some other proteins (Petrillo et al., 2014). This result is consistent with the observation that an *Arabidopsis* plasma-membrane-localized thioredoxin, Trx h9 moved intercellularly (Meng et al., 2010), presumably via plasmodesmata. The potential for intercellular trafficking by redox signals greatly extends the reach of chloroplasts in signaling.

The question remains: how could chloroplasts regulate plasmodesmata and intercellular trafficking? It is possible that as-yet-unidentified chloroplast signals could directly target plasmodesmata. Indeed, chloroplasts are often observed in the vicinity of plasmodesmata, and regardless of the reasons for this close proximity, this means signals would not have to travel across long distances to act (Figure 4). Studies in the algae *Chara australis* also suggest a relationship between chloroplast and cell wall structure (Foissner et al., 2015). It is also possible that chloroplast-to-nucleus retrograde signaling may be involved in regulating events at the cell wall. Indeed, disruption of chloroplast function often results in significant changes of expression of genes involved in cell wall synthesis and modification (Burch-Smith et al., 2011). Similarly, expression of genes with functions related to the cell wall was down regulated in plants accumulating  $\beta$ -cyclocitral upon high light treatment (Ramel et al., 2012). It is also perhaps significant that three of the 39 genes identified as the core response module of chloroplast retrograde signaling encode proteins involved in synthesizing or modifying cell walls (Glasser et al., 2014). Future experiments should determine the pathways used for chloroplast-to-plasmodesmata signaling.

## Chloroplast Signaling in Ecosystem Functioning – the Power of Scent

In addition to being the center for production of oxygen and sugars, chloroplasts are also the source of plethora of secondary metabolites. Chloroplast-produced biogenic volatile organic compounds (BVOCs) seem to represent a fascinating signaling system that enables a plant to communicate with not only itself (e.g., propagation of a systemic response), and with the entire ecosystem that includes plant–plant, plant–animal/insect interaction, but could potentially also impact atmospheric chemistry (Atkinson, 2000; Seybold, 2006; Heil and Silva Bueno, 2007; Heil, 2010).

Volatiles directly produced in chloroplasts include isoprene, monoterpenes, diterpenes, hemiterpenes and volatile carotenoid derivatives, all synthesized by the MEP pathway. Also generated by the chloroplasts are green leaf volatiles (GLVs), synthesized

in the LOX-pathway that consists of C6 and C9 aldehydes, alcohols and their acetate esters, that are responsible for the ‘mowed grass odor’ (Dudareva et al., 2013). These volatiles have important physiological roles, for example, stabilization of thylakoid membranes, reduction of ROS, as well as an ecological function, emphasizing an often underestimated contribution of chloroplasts/plastids to fine regulation of the ecosystem (Velikova et al., 2012). It is estimated that each year about 500 Tg C of isoprene is emitted to the atmosphere. This significant amount has some consequences as the highly reactive isoprene molecule can oxidize OH groups to yield peroxy radicals that in turn can convert NO into NO<sub>2</sub>, producing O<sub>3</sub> (Thompson, 1992; Carter, 1994; Atkinson, 2000). Therefore isoprene as a reactive molecule plays a crucial role in establishing the content of atmospheric greenhouse gasses and pollutants (ozone, methane, secondary organic aerosols, etc.; Harrison et al., 2013).

Chloroplast-produced BVOCs have other ecological functions that are a consequence of their signaling propensity. One of these is the so-called priming effect that leads to enhanced and more effective defense response to pathogen or insect attack in plants pretreated with volatiles (Engelberth, 2004). Although they are released continuously, there is enhanced emission of volatiles upon biotic or abiotic stresses including drought, high light, parasite or herbivore activity (wounding), resulting in so-called HIPVs (herbivore induced plant volatiles) emissions that include monoterpenes and GLVs (Brilli et al., 2009; Bonaventure and Baldwin, 2010; Dicke and Baldwin, 2010; Niinemets, 2010, 2013; War et al., 2011; Heil, 2014). Leaves of attacked plants emit volatiles that can prime defense responses in intact leaves of the same plant as well as on neighboring plants. Moreover, as demonstrated in maize, the intact plants exposed to HIPVs (termed receiver plants) are able to store the information conveyed by volatiles and recall it upon herbivore attack to activate defense genes encoding proteinase inhibitors (Ali et al., 2013).

The molecular mechanism of volatile signaling is far from understood, however, an attractive scenario of volatile-driven epigenetic modification through DNA methylation has been demonstrated as a very reliable strategy for priming defense induction. Moreover, since the JA-signaling pathway is triggered upon herbivore attack (wounding), it is expected that induced JA formation as a downstream effect would be relevant to the HIPV-primed defense responses (Ali et al., 2013). However, some of the most important questions concern the perception and the very first steps in volatiles signaling in receiver plants. To this end it is proposed that volatiles, as hydrophobic compounds, could diffuse through the outer, lipophilic leaf layer (cuticle) or enter through stomata (Baldwin et al., 2006). Indeed, strong membrane depolarization as well as calcium influx were observed upon treatment of tomato plants with GLVs, two phenomena often associated with a signal transduction cascade in other systems (Zebelo et al., 2012). Rearrangements of cytoskeleton and expression of selected genes encoding, among others, protein kinases and transcription factors as well as changes in stomatal aperture were observed upon treatment with the monoterpenes camphor and menthol (Kriegs et al., 2010). However, the concentrations used in the assay are

inconsistent with physiological levels, thus the observed effects are questionable. Elucidating the impact of BVOCs on gene expression and impacts on general cell biology are a likely future direction of this field.

Another intriguing aspect of chloroplast originating volatiles is that their release from attacked plants can serve as attractants of the offending herbivore's natural biological enemies, a phenomenon known as tritrophic chemical communication or tritrophic interaction (Heil, 2008; Mooney et al., 2012; Scala, 2013). Importantly, some volatiles also have allelopathic and insecticidal properties that could be deterrents for oviposition or can attract pollinators and/or seeds dispersers (Schulz et al., 2007; McCallum et al., 2011; Rodriguez et al., 2011; Reis et al., 2014). Together, these reports shed new light on chloroplasts as factories that generate volatile compounds that are important not only from the perspective of a single plant, but also the entire ecosystem. As new strategies for chloroplast biotechnology are deployed, it will be important to examine their effects on these volatiles so that susceptibility to herbivore predation and pathogen ingress are not inadvertently introduced through unintended modification of volatile production and perception.

## Conclusion and Future Directions

The era of transcriptomics initiated by the microarray platform and continued by Next-generation sequencing technology,

together with metabolomics and proteomic approach provided by mass-spectrometry, established new possibilities and new directions in addressing questions in biology. Inevitably, due to these two strategies contemporary plant biology is progressing toward a more holistic view, which translates into studying not a single protein *per se*, but a single protein in the context of an entire cell, tissue, organ, individual plant and ultimately the entire ecosystem biology. Current progress in studying chloroplast biology shows their function goes far beyond photosynthesis and includes all aspects of plant biology, making these organelles an integral part and a full-fledged player in the cell. Understanding the signaling pathways chloroplasts and other plastids use to communicate with the rest of the plant cell under normal and stress conditions will provide new ideas for developing powerful strategies for engineering improved plants. Moreover, a thorough knowledge of chloroplast signaling will also allow the design of rational approaches that could minimize if not avoid unintended crosstalk and undesirable outcomes.

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# Recent advances in understanding carotenoid-derived signaling molecules in regulating plant growth and development

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Carotenoids (C<sub>40</sub>) are synthesized in plastids and perform numerous important functions in these organelles. In addition, carotenoids can be processed into smaller signaling molecules that regulate various phases of the plant's life cycle. Besides the relatively well-studied phytohormones abscisic acid (ABA) and strigolactones (SLs), additional carotenoid-derived signaling molecules have been discovered and shown to regulate plant growth and development. As a few excellent reviews summarized recent research on ABA and SLs, this mini review will focus on progress made on identification and characterization of the emerging carotenoid-derived signals. Overall, a better understanding of carotenoid-derived signaling molecules has immediate applications in improving plant biomass production which in turn will have far reaching impacts on providing food, feed, and fuel for the growing world population.

**Keywords:** apocarotenoid, carotenoid, chloroplast, development, retrograde signaling, root, shoot, signaling

Plants harness solar energy through photosynthesis to support their growth, development, reproduction, and amassment of carbon and nutrients in storage tissues. Carotenoid pigments play indispensable roles in light harvesting complexes and reaction centers of the photosystems. In addition to their direct involvement in photosynthesis, carotenoids and their cleavage products (i.e., apocarotenoids) also participate in controlling the plant's life cycle via coordinating activities among different organelles, cells, tissues, and organs. Research progress on two carotenoid-derived phytohormones, abscisic acid (ABA) and strigolactones (SLs), has been reviewed recently (Cutler et al., 2010; Chater et al., 2014; Seto and Yamaguchi, 2014; Waldie et al., 2014; Mittler and Blumwald, 2015). Here, we focus on several additional carotenoid derivatives that have been investigated for their functions in regulating chloroplast biogenesis, shoot and/or root development, as well as stress response.

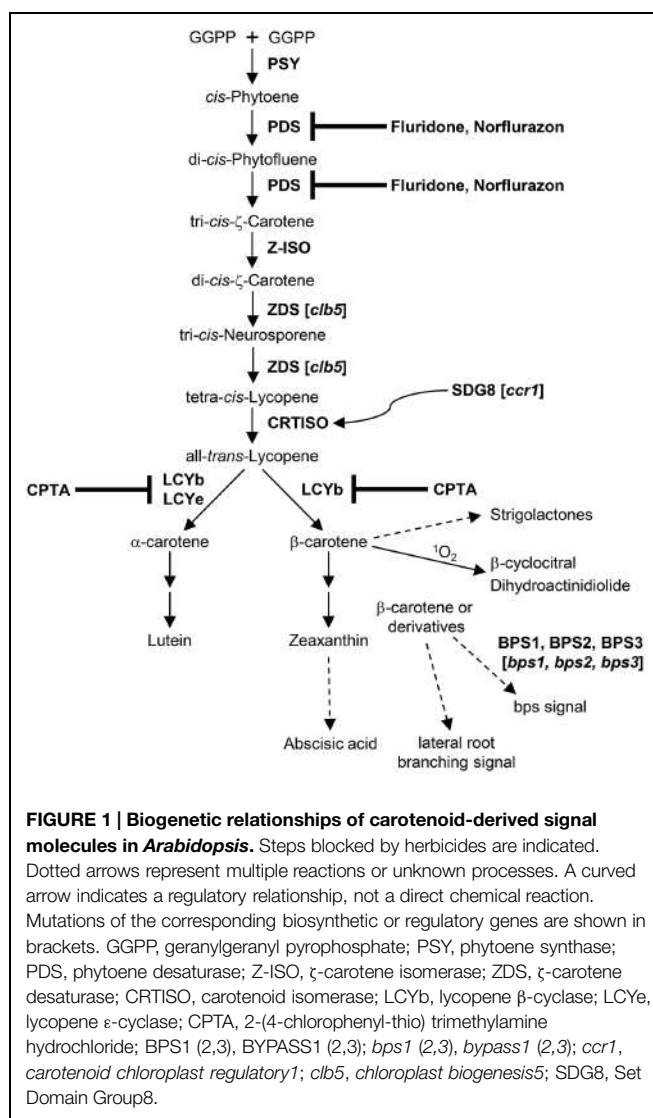
## Carotenoid-derived Signals in Shoot and Root Development

About a decade ago, allelic *bypass1* (*bps1*) mutants that lack leaf vasculature and trichomes, produce short roots and exhibit arrested shoot apical meristem (SAM) activities were isolated from ethyl methanesulfonate (EMS) mutagenized *Arabidopsis* grown at 16°C (Van Norman et al., 2004). The shoot and root phenotypes of *bps1* were alleviated when the mutant plants were grown at higher temperatures (Van Norman et al., 2004). Through elegant genetic and biochemical analyses of the *bps1* mutant and wild type plants, the Sieburth group showed that a mobile

bypass (bps) signal is generated in roots of wild type *Arabidopsis* plants, which regulates root and shoot development through root-to-shoot signal transmission (Van Norman et al., 2004). Since application of carotenoid biosynthesis inhibitors or introduction of the *pds1* mutation (containing an impaired carotenoid biosynthetic enzyme phytoene desaturase/PDS) could partially rescue the abnormal *bps1* growth phenotype, it was hypothesized that bps could be a signal originated from carotenoids (Van Norman et al., 2004; Van Norman and Sieburth, 2007). Further molecular, biochemical and genetic examinations indicated that the bps signal is distinct from ABA and SLs; its production entails synthesis of  $\beta$ -carotene branch carotenoids but does not require the activity of a single carotenoid cleavage enzyme (9-*cis*-epoxycarotenoid dioxygenase/NCED or carotenoid cleavage dioxygenase/CCD) (Van Norman and Sieburth, 2007; **Figure 1**). Besides *BYPASS1* (*BPS1*), two additional *BPS* genes (*BPS2* and *BPS3*) were identified in *Arabidopsis* and the *bps* triple mutant (*bps1bps2bps3*) has aberrant cell divisions during early embryogenesis that result in defective SAM, root apical meristem (RAM), and vascular meristem (VM) (Lee et al., 2012). Such embryonic defects are not evident in *bps1* and less pronounced in the *bps1bps2* double mutant, suggesting that the three *Arabidopsis* *BPS* genes possess overlapping yet distinct functions in generating the bps signal (Lee et al., 2012). Analyses of the auxin response markers as well as localization and trafficking of the PIN1 auxin efflux transporter in *bps* mutants revealed that the auxin signaling pathway is not a primary target of the bps signal for regulation of plant development (Lee and Sieburth, 2012; Lee et al., 2012). Though the chemical structure of bps is currently unknown, progress has been made recently toward elucidation of the bps signal using a bioassay-based purification and identification scheme (Adhikari et al., 2013).

Another shoot development mutant, *carotenoid chloroplast regulatory1* (*ccr1*), was identified by the Pogson group in a screen for *Arabidopsis* mutants defective in carotenoid and chloroplast regulation (Cazzonelli et al., 2009). Unlike *bps1* that contains a lesion in a gene of unknown function, *ccr1* encodes a malfunctioning histone methyltransferase Set Domain Group8 (SDG8) (Cazzonelli et al., 2009; **Figure 1**). As a result of the *ccr1* mutation, histone methylation of chromatin around carotenoid isomerase (CRTISO, an enzyme that converts tetra-*cis*-lycopene to all-*trans*-lycopene) and *CRTISO* expression were reduced, which led to significantly decreased accumulation of lutein and increased levels of  $\beta$ -carotene branch xanthophylls (Park et al., 2002). The *ccr1* mutant exhibited more abundant rosette and cauline branching than wild type plants, yet, unlike the *Arabidopsis* shoot branching mutants deficient in SLs, the *ccr1* mutant phenotype could not be suppressed by a root generated, transmissible signal when grafted to the wild type *Arabidopsis* root stock (Cazzonelli et al., 2009). Though changes in carotenoid content were observed in *ccr1*, it remains to be elucidated whether increased shoot branching in this mutant is indeed associated with a carotenoid-derived signal.

In addition to mutant analysis (e.g., *bps1*, *ccr1*) that allows efficient identification of altered plant growth and the corresponding causal mutations, chemical treatment of



wild type *Arabidopsis* seedlings has also been valuable in uncovering a carotenoid-derived signal that controls lateral root (LR) branching. Van Norman et al. (2014) measured the root's capacity for LR formation (i.e., LR capacity) based on the number of prebranch sites and observed a decrease in LR capacity in *Arabidopsis* seedlings treated with carotenoid biosynthesis inhibitors, norflurazon (NF) and 2-(4-chlorophenyl-thio) trimethylamine hydrochloride (CPTA). Further support for association of reduced LR capacity with carotenoid deficiency was provided by application of NF and CPTA to *Arabidopsis* expressing a transcriptional reporter of auxin response, *pDR5:LUC*; it was shown that inhibition of carotenoid production disturbed the rhythmic oscillation of the LR clock necessary for establishment of prebranch sites (Van Norman et al., 2014). Since ABA or SL deficiency does not impose a similar impact on LR capacity as carotenoid biosynthesis inhibitor treatment, a novel carotenoid-derived signal in LR determination and development has been proposed.

Generation of this signal appears to require a CCD activity as treatment of *Arabidopsis* seedlings with a CCD inhibitor D15 led to significantly decreased LR capacity (Van Norman et al., 2014). Since D15 treatment, which blocks CCD activities and prevents carotenoid turnover, caused substantially more increases in root carotenoid content than in shoots, the authors hypothesized that D15 could inhibit a root-specific CCD activity. However, it is also possible that D15 could be inhibiting the same CCD activity in both roots and shoots. This is because if D15 is a competitive inhibitor for carotenoids binding to CCD enzymes, it is conceivably more effective in roots that contain low levels of carotenoids than in shoots where abundant carotenoid accumulation is found. Understanding the mechanistic basis of D15 inhibition of CCD activities will help discern these possibilities. On another note, it will be interesting to evaluate the impact of overexpressing a root-specific *CCD1* or *CCD4* on LR capacity in the future.

## Carotenoid-derived Signals in Chloroplast Biogenesis, Leaf Development, and Stress Response

While enzymatic cleavage by NCEDs or CCDs is necessary for production of several apocarotenoid signals (e.g., ABA and SLs), an exciting new line of research by the Havaux group indicated that chemical oxidation of  $\beta$ -carotene by singlet oxygen ( $^1\text{O}_2$ ) can result in a series of short chain, cleavage derivatives of  $\beta$ -carotene, which are collectively designated carotenoid reactive electrophile species (RES) [e.g.,  $\beta$ -cyclocitral ( $\beta$ -CC,  $\text{C}_7$ ) and dihydroactinidiolide (dhA,  $\text{C}_{11}$ )] (Ramel et al., 2012a,b, 2013a,b,c; Havaux, 2014; Shumbe et al., 2014). In *Arabidopsis* plants exposed to high light, oxidative cleavage of  $\beta$ -carotene by  $^1\text{O}_2$  produces  $\beta$ -CC and dhA, which elicit  $^1\text{O}_2$  responsive gene expression in the nucleus via a retrograde signaling mechanism and subsequently lead to high light acclimation of the plants (Ramel et al., 2012b; Shumbe et al., 2014). Based on the highly specific impact of carotenoid RES on the  $^1\text{O}_2$ , but not the  $\text{H}_2\text{O}_2$  marker gene expression, it was suggested that they facilitate plant acclimation to high light stress via the  $^1\text{O}_2$  signaling pathway and is independent of  $\text{H}_2\text{O}_2$ . Carotenoid RES regulation also differs from the EXECUTER1 protein mediated programmed cell death response to  $^1\text{O}_2$  as the *executer1* mutant plants could still acclimate to high light stress and respond to  $\beta$ -CC treatment in a similar way as wild type *Arabidopsis* (Ramel et al., 2012b). Interestingly, high light stress acclimation in the *Arabidopsis* chlorophyll *b* deficient mutant *chl1* and the double mutant between *chl1* and *dde2* [a mutant deficient in jasmonate (JA)] is accompanied by a suppression of JA biosynthesis and decreased JA accumulation in leaves, suggesting an interaction between the carotenoid RES induced  $^1\text{O}_2$  response and JA signaling pathways (Ramel et al., 2012b, 2013a,b). It further suggests that high light acclimated plants may be somewhat compromised for JA-mediated responses to pathogens and herbivores. Since  $^1\text{O}_2$  could also be generated in plant defense against microbial pathogens (Triantaphylidès and Havaux, 2009), it will be informative to explore whether carotenoid RES signals

also directly participate in biotic stress responses by plant hosts.

Besides the carotenoid RES control of nuclear gene expression, involvement of  $\zeta$ -carotene desaturase (ZDS) precursors in retrograde signaling was also proposed upon characterization of an *Arabidopsis* mutant of ZDS (Dong et al., 2007). After a close examination of null *Arabidopsis zds/chloroplast biogenesis5 (clb5)* mutant alleles, abnormal leaf development and cell differentiation as well as compromised auxin responses were also apparent in *clb5*, in addition to the albino phenotype common to mutants that lack a functional ZDS enzyme (Avendaño-Vázquez et al., 2014). Consistent with the report by Dong et al. (2007), the *clb5* mutation modified expression of plastid- and nuclear-encoded genes involved in carotenoid biosynthesis and chloroplast development (Avendaño-Vázquez et al., 2014). Application of an inhibitor of the PDS activity fluridone or introduction of the *pds3* mutation rescued the *clb5* mutant gene expression and leaf development phenotypes, suggesting that products of the PDS reaction and precursors of the ZDS reaction, including di-*cis*-phytofluene and  $\zeta$ -carotene isomers ( $\zeta$ -carotene isomerase/Z-ISO catalyzes the isomerization of tri-*cis*- $\zeta$ -carotene to di-*cis*- $\zeta$ -carotene), may be substrates for the underlying signals regulating chloroplast biogenesis and leaf development (Avendaño-Vázquez et al., 2014; **Figure 1**). Moreover, cleaved signals could be generated from di-*cis*-phytofluene and/or  $\zeta$ -carotene isomers by CCD4 as *ccd4* mutation in the *clb5* mutant background was able to rescue the *clb5* mutant leaf phenotypes. Exogenous application of ABA or SLs did not affect the appearance of *clb5* leaves, providing further support for novel carotenoid-derived signals controlling chloroplast and leaf development (Avendaño-Vázquez et al., 2014). It remains to be investigated whether these signals are generated locally in leaves or they could be mobile signals transported from roots.

## Perspectives

Overall, substantial progress has been made on identification and characterization of carotenoid-derived signaling molecules in plants. With the exception of ABA, SLs,  $\beta$ -CC, and dhA, a primary unaddressed question regarding these carotenoid-derived signals is their chemical identities. Aside from structure elucidation, there are several additional questions that remain to be answered: (1) How are the structurally uncharacterized apocarotenoid signals produced? Biochemical and genetic analyses suggested that carotenoid precursors could be subjected to catalysis by carotenoid cleavage enzymes. However, direct evidence for involvement of a single or multiple CCD enzymes is still lacking. (2) How are the carotenoid-derived signals transported between different cell types, tissues, and organs? (3) Thus far, work on carotenoids-derived signals, other than ABA and SLs, has been mainly conducted in *Arabidopsis*. Do these signals perform similar functions in other plants? (4) Do overlapping activities or antagonistic effects exist among different carotenoid-derived signaling molecules? (5) Are there additional carotenoid-derived signals remaining to be discovered?



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# Chloroplast Biogenesis-Associated Nuclear Genes: Control by Plastid Signals Evolved Prior to Their Regulation as Part of Photomorphogenesis

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The assembly of photosynthetically competent chloroplasts occurs in angiosperm seedlings when first exposed to light, and is due to the control by light of photosynthesis-associated nuclear genes (PhANGs), also dependent upon plastid-to-nucleus “biogenic” communication signals. The relationship between light- and plastid signal-regulation of PhANGs is close but poorly understood. In contrast, many conifers green in the dark and the promoter of a pine PhANG, *Lhcb*, is active in the dark in tobacco. Here, we show that the activity of this promoter in tobacco is sensitive to plastid photobleaching, or to the inhibition of plastid translation in the light or the dark, and the same interventions reduce expression of the native gene in pine seedlings, demonstrating classic plastid biogenic signaling in gymnosperms. Furthermore, *Arabidopsis* mutations causing defective plastid biogenesis suppress the effect in darkness of mutations in COP1 and DET1, repressors of photomorphogenesis, for the expression of several PhANGs but not a photosynthesis-unrelated, light-regulated gene. GLK transcriptional regulators mediate the response of *LHCB* but not of other tested PhANGs. We propose the ability to suppress PhANG response to positive plastid biogenic signals in the dark may have contributed to the evolution of light-controlled chloroplast biogenesis.

**Keywords:** chloroplast development, photomorphogenesis, plastid signals, LHCB, DET1, COP1 *Arabidopsis*, GLK, gymnosperm photomorphogenesis

## INTRODUCTION

The development of flowering plant chloroplasts occurs in the light, and involves the expression of 1000s of genes encoded in the nucleus, the import of their products into developing plastids, as well as the expression of ca. 120 protein and RNA-encoding genes by the genome of the chloroplast itself (Waters and Langdale, 2009; Jarvis and López-Juez, 2013). Light is a key inductive signal for the expression of genes involved in the assembly of a photosynthetically competent chloroplast, the so-called photosynthesis associated nuclear genes (PhANGs). In seedlings germinated in the absence of light leaf development is repressed and plastids in the cotyledons develop as etioplasts, containing partially developed internal membranes and a chlorophyll precursor, protochlorophyllide, associated to a light-requiring protochlorophyllide oxido-reductase (Reinbothe et al., 1996). This renders seedlings photosynthetically incompetent.

At the same time the expression of PhANGs in cells developing chloroplasts is closely coordinated with the functional state of the plastid. If ongoing plastid biogenesis is impaired, by failure to safely complete chlorophyll biosynthesis (because of photooxidative damage to membrane complexes, caused by carotenoid synthesis mutations or chemical inhibitors like norflurazon), or to express the chloroplast genome (because of organelle translation mutations or inhibitors), or to import nuclear-encoded proteins, PhANG expression is also down-regulated (Inaba et al., 2011; Chi et al., 2013). This reveals the existence of plastid-to-nucleus communication, also called plastid retrograde signaling, more specifically plastid biogenic signaling. The term “biogenic” is used to distinguish it from operational or environmental signaling, which refers to the later influence of functional but stressed chloroplasts on nuclear genes when subjected to environmental challenges (Woodson and Chory, 2012; Pogson et al., 2015). Biogenic signals resulting at least from defects in tetrapyrrole (primarily chlorophyll) biosynthesis and from deficiencies of organellar gene expression can themselves also be, to a degree, genetically separated (Vinti et al., 2000; Mochizuki et al., 2001; Gray et al., 2003; Chi et al., 2013).

Light control of PhANG expression is part of a broader program of control by light of development overall, the so-called photomorphogenesis program, initiated by the activation of phytochrome and cryptochrome photoreceptors. This program contrasts with that of development in the dark, skotomorphogenesis, which instead ensures investment into elongating organs and prevents the development of photosynthesising leaves (Arsoovski et al., 2012). The result is a vast reprogramming of the transcriptome of seedlings when first exposed to light (Jiao et al., 2007). Plastid biogenic signaling, on the other hand, probably involves the production or regulated export of one or more tetrapyrrole signals (Terry and Smith, 2013), and the monitoring of the activity of the chloroplast transcription/translation machinery, involving the plastidic GUN1 protein (Koussevitzky et al., 2007; Woodson and Chory, 2008). In spite of their apparent dissimilarity, multiple observations have highlighted a close relationship between light and chloroplast biogenic control of PhANGs. For example promoter truncations could not remove one response without the other, and plastid development defects led to reduced light responsiveness (Simpson et al., 1985; Bolle et al., 1996; McCormac et al., 2001; Vinti et al., 2005; Ruckle et al., 2012). Even gain of function experiments using light-regulated pairs of promoter elements (Puente et al., 1996; Martínez-Hernández et al., 2002) recreate the light response and the plastid dependence simultaneously.

Such evidence raises the intriguing possibility of light and plastid-to-nucleus signaling to PhANGs sharing mechanisms, even of one potentially being based on the other. Plastid-to-nucleus biogenic signals have been revealed in flowering plants, with their nature and very existence in the green alga *Chlamydomonas reinhardtii* being the subject of mixed evidence (Johanningmeier and Howell, 1984; Johanningmeier, 1988; Ramundo et al., 2013). Meanwhile the presence of phytochrome and cryptochrome photoreceptors has been shown

in all major groups of land plants (Sharrock and Mathews, 2006) but the nature of photomorphogenic responses varies. Many gymnosperm seedlings grow partially skotomorphogenically but green in the dark (Alosi et al., 1990; Yamamoto et al., 1991; Burgin et al., 1999). This stems from the presence of a light-independent protochlorophyllide oxido-reductase (Forreiter and Apel, 1993), and from the expression of PhANGs in the dark (Yamamoto et al., 1991; Peer et al., 1996). In fact the promoter of the *Lhcb6* (*cab-6*) gene from black pine, encoding a form of the apoprotein of the major light harvesting complex of photosystem II, is able to drive the expression of a beta-glucuronidase (GUS) reporter gene constitutively in the dark in an angiosperm, tobacco (Kojima et al., 1994). This observation provided a tool which could now be used to analyze the relationship between light and plastid-to-nucleus biogenic signals in the expression of PhANGs.

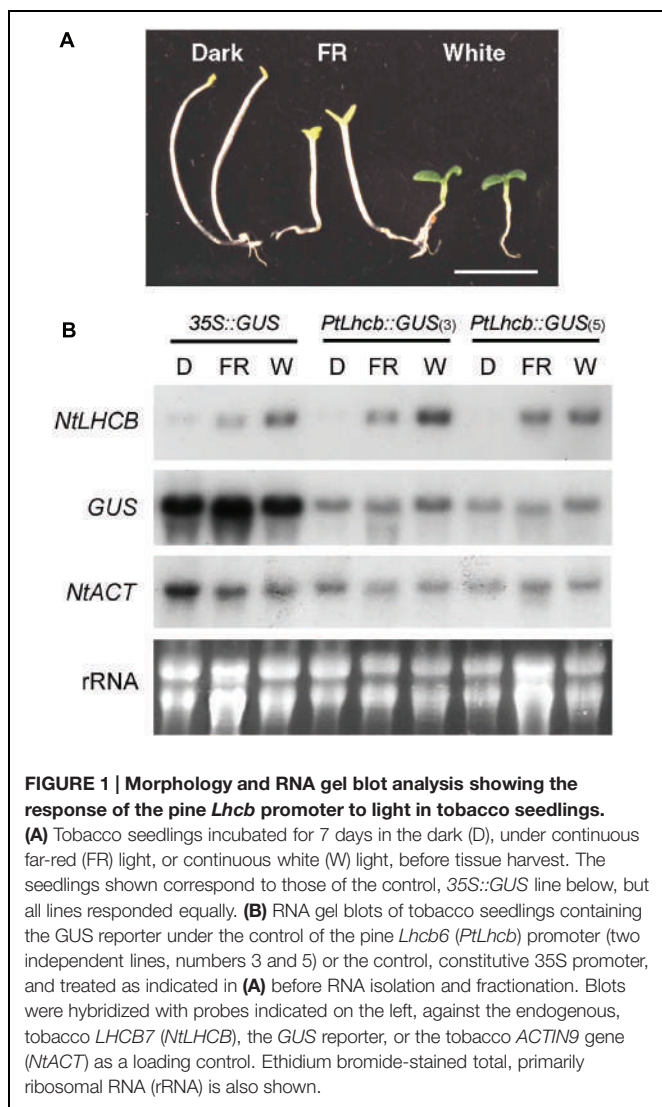
A central theme in photomorphogenesis is the activation of light-induced genes by transcription factors (notably HY5) whose accumulation is prevented in the dark by proteasomal degradation, triggered by the activity of ubiquitin ligase and associated proteins of the DET/COP class; those proteins therefore act as repressors of photomorphogenesis. The activity of these repressors is abolished in the light by photoreceptor activation (Jiao et al., 2007; Lau and Deng, 2012). Nuclear events of plastid-to-nucleus biogenic signaling are less clear, with a negative role for the ABI4 transcription factor, acting downstream of chloroplast-localized GUN1, having been shown for a subset of genes (Koussevitzky et al., 2007); furthermore the GOLDEN2 LIKE (GLK) family of transcription factors has been demonstrated to play a conserved, positive role in the expression of selected PhANGs across land plants (Fitter et al., 2002; Yasumura et al., 2005; Waters et al., 2009), and to also be involved in the response to defective plastid protein import on such genes (Kakizaki et al., 2009).

In this study we have asked the following questions: is the pine *Lhcb* promoter truly phytochrome-independent in tobacco? Is it dependent on plastid-to-nucleus biogenic signals and, if it is, does this reflect the situation in the original cellular environment in pine? Is there, in *Arabidopsis*, a genetic interaction in the dark between plastid biogenesis and repressors of photomorphogenesis? And what role do GLK transcription factors play in the response to light and plastid-to-nucleus communication? Our results lead us to propose a model in which the photoreceptor control of chloroplast development evolved, in part at least, through the recruitment of DET/COP repressors to suppress to ability of PhANGs to respond to plastid-to-nucleus signals of a positive nature in the dark.

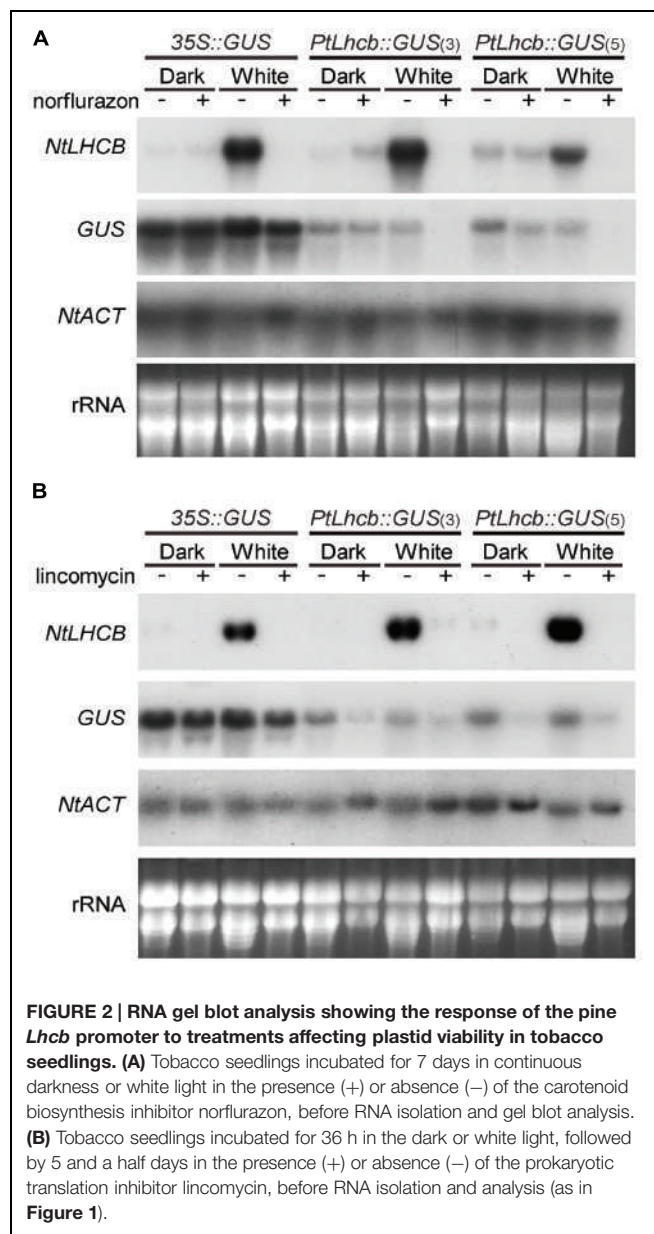
## MATERIALS AND METHODS

### Plant Material, Light, and Growth Conditions

Tobacco (*Nicotiana tabacum* cv. Petit Havana) lines (numbers 3 and 5) carrying 1900 bp upstream of the start codon of the pine *Lhcb6* (*cab-6*) gene driving the GUS-coding sequence, or the 35S promoter GUS fusion gene (line 18 for experiments in **Figures 1** and **2**, or lines 1 and 18 for **Figure 3**), were described



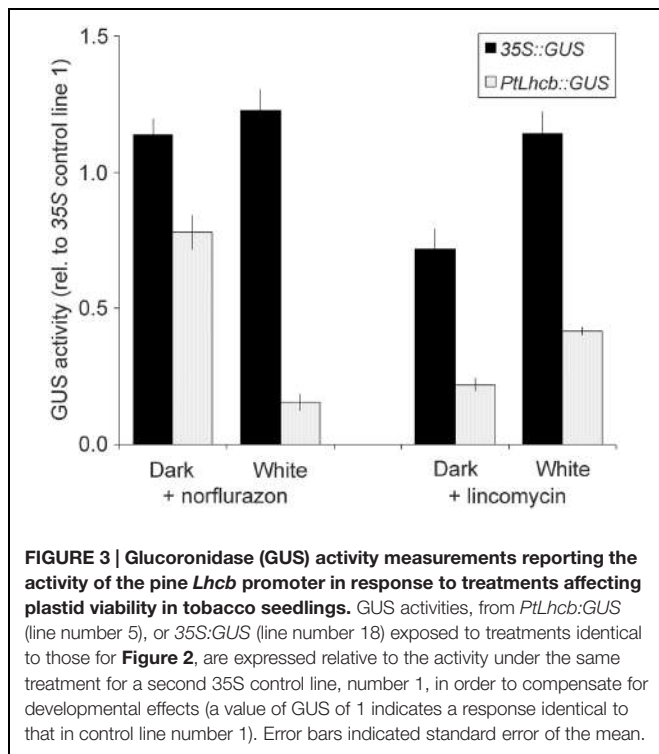
previously (Kojima et al., 1994). Unless otherwise stated, seeds were surface-sterilized, plated on agar-solidified MS medium containing 1% sucrose, and incubated as previously described for *Arabidopsis* seeds (Vinti et al., 2000). Pine (*Pinus thunbergii*) seeds were obtained from Chiltern seeds, Ulverston, UK. Seeds were stored at 4°C for at least 2 months before use. Seeds were surface-sterilized with a 22% solution of hydrogen peroxide for 30 min, before placing on MS medium in magenta boxes (Sigma Aldrich, Poole, UK). Due to erratic germination and slightly uneven growth rate under the different conditions, seeds were monitored daily for less than 30 s under a green safe light (Vinti et al., 2000) until radicle emergence, and grown subsequently for a period between 14 and 21 days (as determined in preliminary experiments), until they reached a stage comparable to that reached by seedlings in the light at 14 days. *Arabidopsis* seeds of the *det1-1* mutant (Chory et al., 1989), *cop1-4* (Deng et al., 1991), *ppi1-1* (Jarvis et al., 1998), and *glk1 glk2* double mutants (Fitter et al., 2002) were kind gifts from J. Chory (Salk Inst.), J. Sullivan (Queen Mary University of London), P. Jarvis and



J. Langdale (both University of Oxford), respectively. Seeds of the *cue8* mutant, and its wild type, the reporter gene-containing pOCA108 line (Bensheim ecotype) were lab stocks (López-Juez et al., 1998). Double mutants were isolated from the respective crosses, selecting in the second generation for a deetiolated phenotype in the dark, followed by a visibly pale (*ppi1*) or slow greening (*cue8*) phenotype upon transfer to soil, or by genotyping assays of *glk1* and *glk2* as described (Fitter et al., 2002). For *det1 ppi1* deetiolated mutants were selected in the F2, and those segregating with a paler phenotype identified in the F3. The segregation ratios of the phenotypes as seedlings were consistent with single or double mutant (triple for *det1 glk1 glk2*) genotypes, although the survival rate of combined mutants was reduced.

The growth temperatures for every experiment were 25°C (for tobacco and pine) and 21°C (for *Arabidopsis*).





## Light and Inhibitor Treatments

FR and white light sources were as previously described (Vinti et al., 2000). The fluence rate of white light was  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ , and that of FR  $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Norflurazon, a kind gift of P. Bramley (Royal Holloway, University of London), was incorporated into agar-solidified growth media at a final concentration of  $10 \mu\text{M}$  (tobacco) or  $100 \mu\text{M}$  (pine). Lincomycin (Sigma Aldrich, Poole, UK) was added to growth media at a final concentration of  $0.5 \text{ mM}$  (tobacco) or  $10 \text{ mM}$  (pine). For lincomycin treatment, seeds were placed on sterile filter paper laid onto lincomycin-free, agar-solidified medium, and transferred to lincomycin-containing plates after 36 h (tobacco) or on the day of radicle emergence (pine).

## GUS Activity Assays

Glucuronidase activity was measured using a fluorogenic substrate as described (López-Juez et al., 1998). Each experiment used three samples of 5–10 seedlings and all experiments were repeated 2–4 times, with results given being the average of all combined samples.

## RNA Gel Blot Analysis and Quantitation

RNA was extracted as previously described after 7 days (tobacco) or 5 days (*Arabidopsis*) of growth of seedlings in continuous light or continuous darkness (Vinti et al., 2000). Tissue harvest nevertheless took place at the same time of day as the start of growth, to avoid any remaining circadian effects. RNA was extracted from cotyledons of pine seedlings as above, followed by purification through the RNA-binding column of a commercial

kit (RNeasy Plant Mini, Qiagen, Manchester, UK). RNA gel blot analysis, hybridisation with  $^{32}\text{P}$ -labeled probes and quantitation using a phosphorimager and ImageQuant software were carried out as described (Vinti et al., 2000). Probe templates used for hybridisation were produced by PCR-amplification from genomic DNA or plasmids, and are given as (Supplementary Table S1).

## Quantitative Real-time RT-PCR

*Arabidopsis* RNA was extracted using the RNeasy Plant Mini kit (see above), quality-checked by agarose gel electrophoresis, and  $1 \mu\text{g}$  aliquots reverse-transcribed using the Quantitec kit (Qiagen). cDNA was used for real-time amplification in a Rotorgene Q (Qiagen) as previously described (López-Juez et al., 2008), except that a JumpStart SYBR® Green Quantitative PCR master mix (Sigma Aldrich) was utilized. Relative quantitation for each target gene used the  $\Delta\text{Ct}$  method against the geometric mean of the expression of two constitutive genes, *ACT2* and *UBQ10*, and is presented relative to wild type in the dark. Data for *cop1 cue8* are presented twice, relative to Col-0 (wild type of *cop1*) and to pOCA108 (wild type of *cue8*). Gene identifiers and corresponding primers were as listed (Supplementary Table S2).

## Sequence Analysis

Bioinformatic searches of homologs of the *Arabidopsis* DET1 (At4g10180) and COP1 (At2g32950) proteins were carried out in GenBank<sup>1</sup>, among sequenced full or partial genomes of embryophytes and among translated versions of mRNAs and expressed sequenced tags of conifers. Alignment of sequences used Clustal Omega<sup>2</sup> and the result was displayed using BoxShade<sup>3</sup>.

## RESULTS

### Expression of the Pine *Lhcb* Promoter is Largely Phytochrome-independent in Tobacco

The study by Kojima et al. (1994) described a small degree of light responsiveness of the pine *Lhcb* (*cab-6*) promoter in tobacco seedlings, which could be attributed to developmental effects as it could also be observed in control lines with a constitutive reporter driven by the 35S promoter. We reanalyzed this issue by exposing the transgenic tobacco seedlings to treatments specifically activating the main deetiolation photoreceptor, phytochrome A, and comparing the expression of the pine *Lhcb*-driven *GUS* reporter with that of the endogenous tobacco *LHCB* gene. We used two independent lines containing the pine *Lhcb::GUS* construct -lines 3 and 5 (Kojima et al., 1994)- and a control line containing 35S::GUS -line 18 (Kojima et al., 1994)-, and *GUS* and *NtLHCB7* probes. The results show a clear de-etiolation response of the tobacco seedlings under continuous far-red light

<sup>1</sup><http://www.ncbi.nlm.nih.gov/genbank/>

<sup>2</sup><http://www.ebi.ac.uk/Tools/msa/>

<sup>3</sup><http://www.ch.embnet.org/>

(FR) treatment (**Figure 1A**), and a very large increase under FR or white light of steady-state mRNA levels of the native *LHCB* genes, but only small (35S, *PtLhcb* line 3) or none at all (*PtLhcb* line 5) of those of the *GUS* reporter (**Figure 1B**; for blot quantitation see Supplementary Figure S1).

### Expression of the Pine *Lhcb* Promoter is Dependent on Plastid-to-nucleus Biogenic Communication Signals in Tobacco

The lack of phytochrome response of the *PtLhcb6* gene could correlate with a lack of plastid signal response or, alternatively, the responses to plastid and light signals could be different from those of the endogenous tobacco gene. We therefore examined the response of the pine promoter to plastid signals, both those revealed by photooxidative damage that follows carotenoid synthesis inhibition by norflurazon, and those dependent on plastid translation which is blocked by the antibiotic lincomycin (**Figure 2**, with blot quantitation in Supplementary Figure S1). Activity of the pine *Lhcb* promoter (represented by steady-state *GUS* mRNA levels) was clearly sensitive to norflurazon treatment in the light (**Figure 2A**). The treatment had negligible effects on pine *Lhcb* promoter activity or tobacco *LHCB* mRNA level in the dark as expected (when carotenoid absence causes no phototoxic damage). Plastid translation-dependent signals were capable of regulating the pine *Lhcb* promoter activity in the light and in the dark (**Figure 2B**). The native *NtLHCB* mRNA decreased in response to lincomycin to a much greater extent than the *PtLhcb*-driven *GUS* mRNA did (**Figure 2B**). This could be evidence that the gymnosperm promoter is less tightly regulated by plastid translation-dependent signals than the angiosperm one is, or it could be explained by additional, post-transcriptional regulatory mechanisms of the angiosperm mRNA.

We confirmed the plastid signal-dependence of the pine *Lhcb* promoter in tobacco by carrying out further seedling *GUS* activity assays (**Figure 3**). To avoid developmental effects of the treatments on overall protein synthesis capacity (Kojima et al., 1994), activities were expressed relative to those in a separate control 35S line. Again these data showed a very clear decrease in *PtLhcb*-dependent *GUS* (of around 85%) upon plastid photooxidative damage, and a smaller (60–80%) decrease upon plastid translation inhibition.

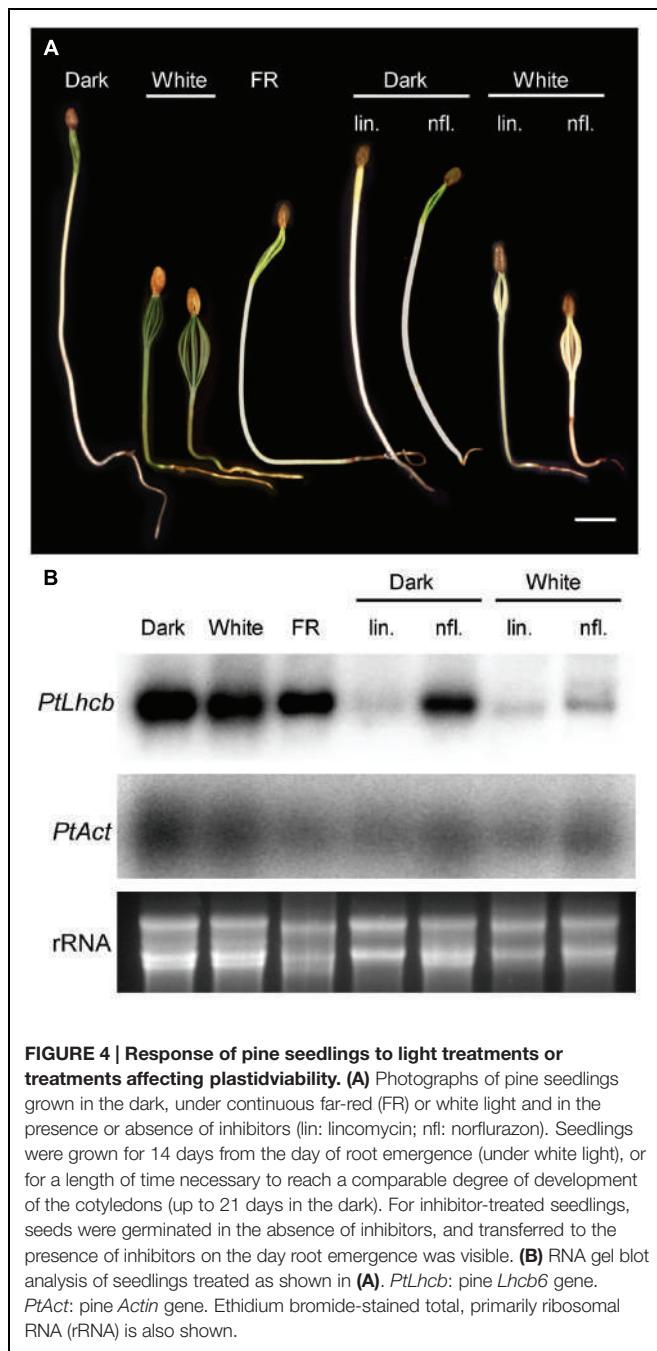
### The Expression of the *Lhcb* Gene in Pine is Dependent on Plastid-to-nucleus Communication Signals

The results above prompted us to investigate the existence of plastid-to-nucleus signals in seedlings of pine itself. This was necessary to assess whether the *GUS* reporter activity in tobacco was an accurate representation of the behavior of the native gymnosperm promoter, as well as to establish whether the signaling machinery is indeed present in pine cells. We examined phytochrome-dependent responses at the same time. These were challenging experiments because of the non-synchronous germination of seeds, and because the inhibitors were only effective at high doses possibly due to inefficient take-up. We

monitored the germination of seeds individually through a very brief, safe light exposure, and examined the expression of the PhANG *Lhcb6* relative to that of a constitutive *Act* gene, encoding actin. The results in **Figure 4** confirm the ability of seedlings to green in the absence of light. However, it is clear that other known photomorphogenic responses do take place: seedlings were substantially shorter and cotyledons larger under white light. FR was able to trigger a partial photomorphogenic response. Correspondingly with the green phenotype, no change in the expression of *Lhcb* under light control was apparent. However, the *PtLhcb* expression responded to inhibition of plastid function. Growth of seedlings on norflurazon had a small effect in the dark and caused a very substantial reduction of *Lhcb* mRNA levels in the light, demonstrating that most of the response is due to photooxidative damage. Plastid translation inhibition caused decreases in *Lhcb*, relative to *Act*, in the dark and in the light (**Figure 4**, blot quantitation provided in Supplementary Figure S2). This confirms that this gymnosperm *Lhcb* gene is dependent on both of these sources of plastid-to-nucleus biogenic communication signals in its native cellular context.

### Plastid Biogenesis Mutations Suppress the Photosynthesis-associated Nuclear Gene Expression in the Dark Caused by *cop1* and *det1* Mutations

The data above show that plastid control of *Lhcb* expression occurs in pine and, therefore, is likely to have been functional in the last common ancestor of conifers and angiosperms, before the skotomorphogenesis program suppressed the expression of PhANGs in the dark. Data in **Figure 4**, meanwhile, show that the difference between the skotomorphogenic and the photomorphogenic programs can also be observed in pine, albeit it does not affect *Lhcb* expression. One attractive hypothesis is that the repression of PhANGs like *Lhcb* in the dark evolved through the recruitment of the repressors of photomorphogenesis to repress the ability of PhANG promoters to respond to plastid signals (of a positive nature) in the dark, with this repression being relieved in the light. A prediction of this hypothesis would be that removal of the photomorphogenesis repressors would allow the response to plastid signals to be manifest in full, in the dark as well as in the light; in other words, that removal of the photomorphogenesis repressors would result in angiosperm seedlings showing responses of PhANGs to plastid signals more comparable to those of the conifer, determined by the state of the plastids. We set out to test this hypothesis using genetic tools, both mutations in the *DET1* (Chory et al., 1989) and *COP1* (Deng et al., 1991) genes for repressors of photomorphogenesis, and mutations in plastid biogenesis genes necessary in the dark and the light. As plastid biogenesis mutants we used *ppi1*, defective in *TOC33*, a component of the plastid protein translocon of the outer chloroplast membrane (Jarvis et al., 1998) and *cue8*, with a mutation in a housekeeping plastid protein causing reduced *LHCB* expression in both dark and light, and affecting both chloroplast and etioplast development (Vinti et al., 2005). The prediction of this scenario would be that mutations in plastid



biogenesis genes would reduce or suppress the PhANG gene expression phenotype in the dark caused by those in *DET1* or *COP1*. Seedling phenotypes of the combined mutants generated are shown (Figure 5), compared to those of the wild type grown in the absence or presence of norflurazon. A *det1 cue8* double mutant was generated but was severely growth-impaired, had poor seedling survival, and could not be examined for gene expression. Single mutants were grown in the dark and the light. Gene expression results (Figure 6) confirmed those previously reported: higher transcript levels were observed in the dark for photo-regulated genes in *det1* and *cop1* relative to

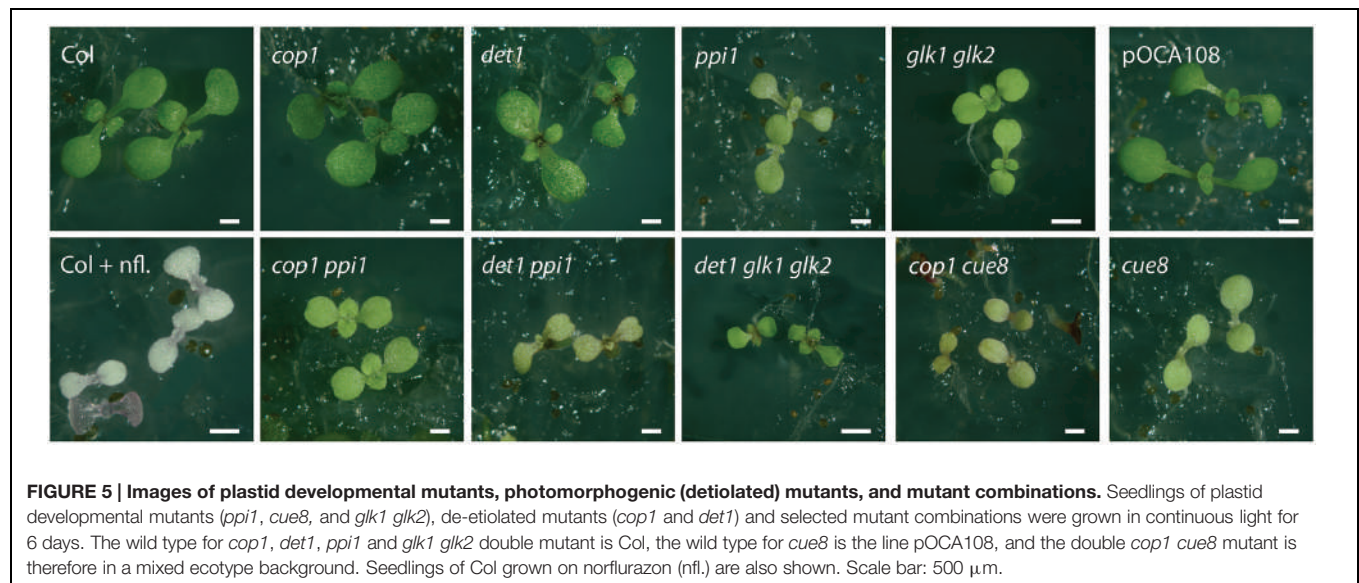
those in their wild types; reduced expression of *LHCB* and of a second light and plastid-dependent PhANG, *RBCS* (the gene for the nuclear-encoded small subunit of Rubisco), was seen in *ppi1* and *cue8*, in the dark (mildly for *ppi1*) and the light. The gene expression phenotype of double mutants, compared with that of the single *cop1* or *det1* mutants, is shown in Figures 7A,B. Both plastid mutations, particularly *cue8*, caused reductions in the double mutants of the expression of both PhANGs in the dark and the light, when compared with the mRNA levels in the corresponding single *cop1* or *det1* mutant. In general *cop1* appeared to cause a greater derepression of PhANGs in the dark (even though a response to light remained present), and *cue8* a greater suppression in dark and light. Meanwhile the expression of *CHALCONE SYNTHASE* (*CHS*), encoding an enzyme involved in flavonoid biosynthesis, induced by light as a photoprotectant but unrelated to photosynthesis, was strongly elevated in the *det1* mutant, and the presence of the second, plastid-biogenesis mutation caused not a reduction, but a further increase in the light (Figure 7B). In summary, the prediction that mutations causing defects in plastid biogenesis would reduce or suppress the effect in the dark of mutations in repressors of photomorphogenesis, specifically for PhANGs, appeared upheld.

### Mutations in Greening-related GOLDEN2-LIKE Transcription Factors Suppress the Action of *det1* Mutations on Photosynthetic Antenna Genes

GOLDEN2-LIKE transcription factors (GLKs) have been shown to drive the expression of a number of greening-related genes, and to mediate the nuclear response to defects in chloroplast protein import. We sought to establish whether the response to plastid-to-nucleus signals, which the data above are consistent with photomorphogenesis repressors suppressing in the dark, is mediated by these transcriptional regulators. To do this we generated a *det1 glk1 glk2* mutant (see Figure 5). The prediction was that, as with defects in essential plastid proteins, the expression of greening-related genes would be impaired, in spite of the *det1* mutation. We included in the analysis the combined mutants above, for comparison purposes, and examined two members of two subfamilies of *LHCB*, as well as the *CARBONIC ANHYDRASE 1* (*CA1*) gene, one of the top responders to repression by norflurazon (Koussevitzky et al., 2007), and whose product is involved in the photosynthetic carbon reactions.

The results (Figure 8) demonstrated the anticipated regulation of *LHCB1*, its expression requiring functional plastids (as shown by the reduction in *ppi* in the light or in *cue8* in dark and light). As expected, repression of *LHCB1* in the dark required the *DET1* and *COP1* products (note the elevated expression in the mutants), and the impairment in plastid function suppressed the positive effect in the dark of the loss of *DET1* or *COP1*. Loss of GLKs reduced expression of *LHCB1* in the light but also, fully, suppressed the action of the *det1* mutation on this gene, demonstrating that such expression, occurring in the dark if *DET1* is absent, necessitates functional GLKs. Nearly identical conclusions could be drawn monitoring the expression

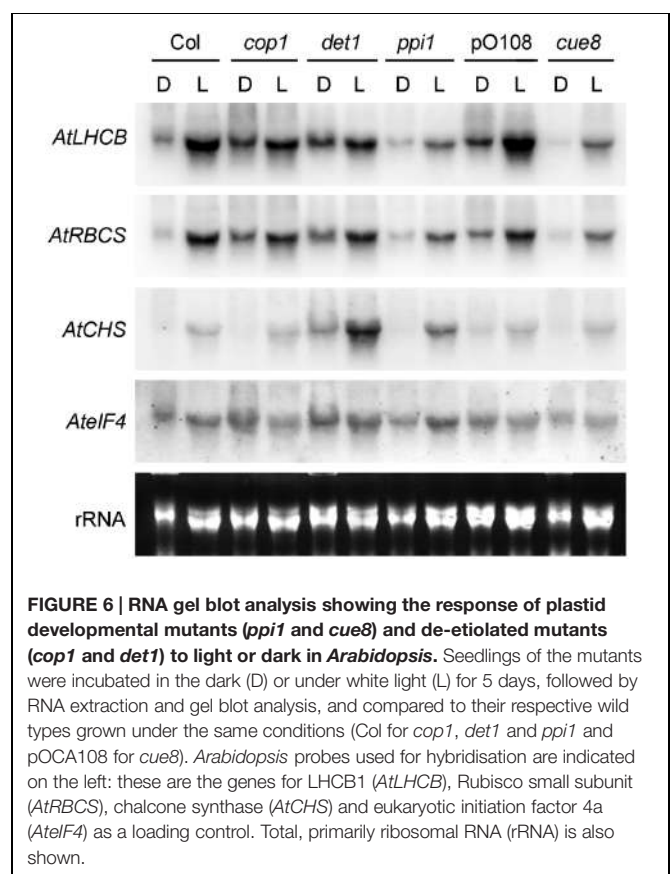




of *LHCB2*, albeit the extent of regulation, by both plastid-to-nucleus and light signals, was much attenuated compared to that of *LHCB1* (note also the reduced impact of norflurazon treatment on the wild type). This, however, was not the case for the *CA1* gene, whose expression was light-induced and norflurazon and *CUE8*-dependent, but proved almost completely independent of both the defect in *ppi1* and loss of GLKs. Accordingly, the *glk1 glk2* mutations had no impact on *CA1* expression when combined with loss of *det1*, demonstrating that expression of *CA1*, while suppressed by *DET1* in the dark, does not necessitate GLKs. Lastly, expression of *CHS* was, as expected, light-induced, suppressed by *DET1* and *COP1* in the dark, not reduced by mutations causing plastid defects (if any, elevated), and indeed elevated in the wild type in response to norflurazon. Unsurprisingly, GLK defects had no impact on the expression of this gene.

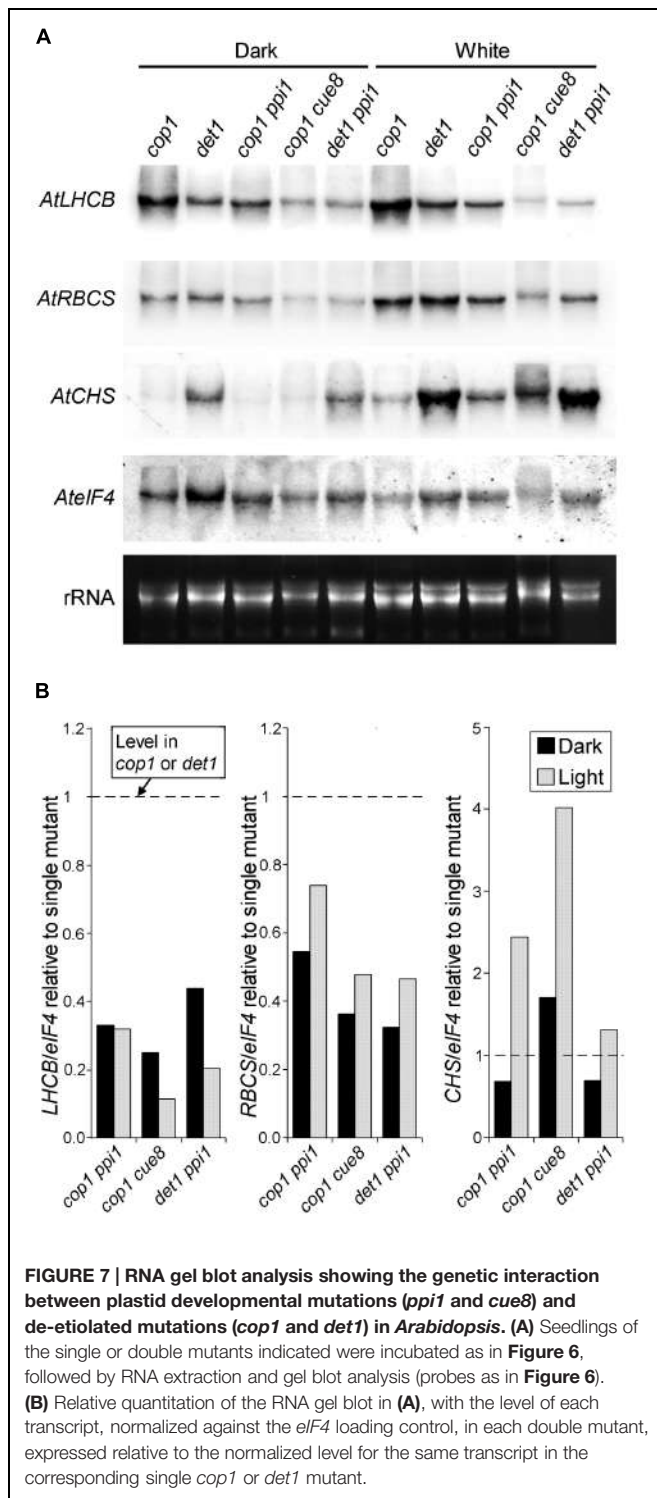
## DISCUSSION

Our results demonstrate that plastid-to-nucleus communication pathways regulate the expression of the PhANG *Lhcb7* in pine, as well as the activity of its promoter heterologously, in tobacco. Clearly, plastid biogenic signals reporting chloroplast viability either predate the divergence of angiosperms from other seed plants, including conifers, or have evolved repeatedly, the first of those explanations being the more parsimonious. Such divergence occurred at least 140 million years ago, and possibly much earlier (Willis and McElwain, 2014). Evidence exists for plastid-to-nucleus “operational” or environment-dependent signals and even the control of global cellular processes (like cell division) occurring in primitive photosynthetic eukaryotes (Escoubas et al., 1995; von Gromoff et al., 2008; Kobayashi et al., 2009). In *Chlamydomonas*, chlorophyll precursors and their chloroplast export have been shown to mediate the light response of an HSP70 heat-shock protein (Kropat et al., 2000),



but the product of this gene is not directly photosynthesis-associated. Also in *Chlamydomonas*, norflurazon was found to cause a loss of chlorophyll but no specific gene expression changes (Johanningmeier, 1988), and changes brought about by tetrapyrrole molecules included very few photosynthesis-associated genes (Voss et al., 2011). Suppression of transcription

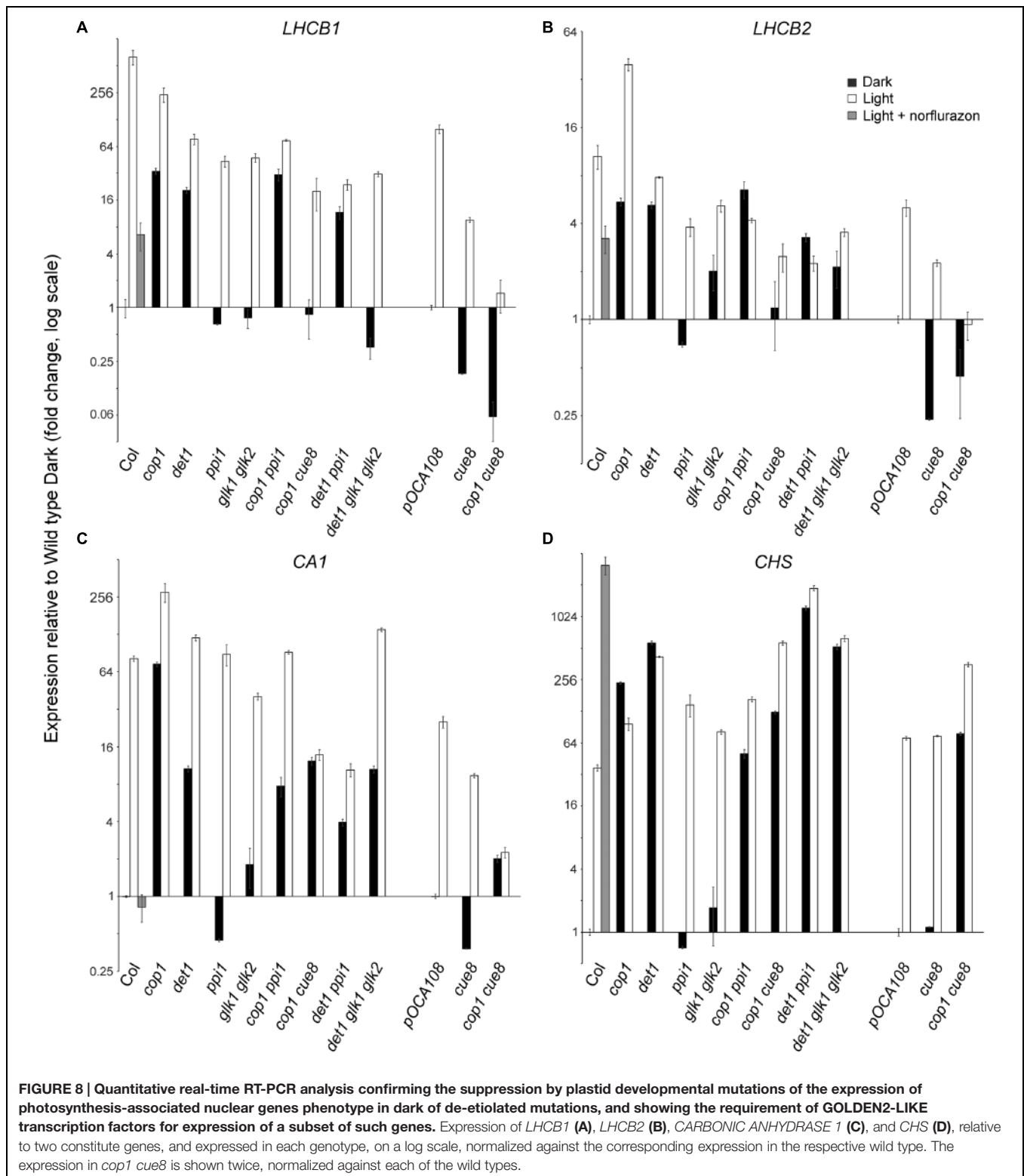




or translation by very elegant means in this alga does cause reduced expression of some nuclear photosynthesis associated genes, notably *Lhca1* (Ramundo et al., 2013). Hence, the range of plastid function monitored in *Chlamydomonas* is distinct, narrower than that monitored by plastid biogenic signals in flowering plants. This raises the question of whether

such regulatory pathways are orthologous. Arguably prior to multicellularity there was little need for the existence of non-photosynthetic proplastids, required to be inherited via germ cells or meristematic plant stem cells, and consequently for the assembly of photosynthetically competent chloroplasts from them as photosynthetic cells differentiated (Andriankaja et al., 2012; Charuvi et al., 2012; Jarvis and López-Juez, 2013). Our evidence, on the other hand, indicates the existence of the classic plastid-to-nucleus 'biogenic' signaling pathways controlling chloroplast development outside the angiosperms, and is consistent with such signals evolving or at least expanding in association with the need for differentiation of chloroplast-containing cells from proplastid-containing ones in embryophytes.

Close links between light and plastid responsiveness of PhANG expression have been uncovered at multiple levels. In cereal leaves, which show a basal/apical gradient of chloroplast development, no response of PhANGs to photoreceptors occurs in regions below those in which plastids are transcriptionally active (Rapp and Mullet, 1991). This could be a developmental, rather than a pathway-sharing connection, since a global gene expression analysis of the developing maize leaf has revealed a transition region, before chloroplasts become photosynthetically competent and the tissue converts from sink to source, in which the genes for transcription factors like HY5 and Golden2 become expressed (Li et al., 2010). Nevertheless evidence that light and plastid signals act on common promoter regulatory elements abounds (Bolle et al., 1996; Kusnetsov et al., 1996; Puente et al., 1996; Martínez-Hernández et al., 2002; Gray et al., 2003). One known exception, involving the response element of an *Arabidopsis* *LHCB* promoter to high light and reactive oxygen (Staneloni et al., 2008), probably reflects the distinction between plastid-to-nucleus biogenic and operational signals. Such overall evidence led to the original proposal (Arguello-Astorga and Herrera-Estrella, 1998) that light-responsive elements may have had an ancestral function as response elements to plastid retrograde (biogenic) signals. We observed a nearly complete lack of response (similar to that in photoreceptor mutants) of the *LHCB* promoter to phytochrome-activating light pulses in plastid-defective *cue8* and *cue3* mutant seedlings, even when some chlorophyll accumulation, and therefore basal *LHCB* expression, was readily detectable (Vinti et al., 2005). Others have also shown the degree of light responsiveness of PhANGs to be greatly attenuated by impairments of chloroplast biogenesis (Ruckle et al., 2007). Our evidence is consistent with the proposal of an ancestral role of light-responsive elements in the response to plastid-to-nucleus signals, and extends it by showing that the action of plastid signals occurs downstream that of the *det1* and *cop1* mutations in the dark, and therefore that the DET1 and COP1 proteins, dark repressors of photomorphogenesis, may repress the action of plastid signals (Figure 9). It has been previously shown (Sullivan and Gray, 1999) that lincomycin treatments suppress the elevated *LHCB* expression in the dark of *Arabidopsis cop1* and pea *lip1*. *LIP1* is orthologous to *COP1*. These authors interpreted their data as evidence for plastids producing retrograde signals that repress PhANG expression



also in the absence of light. An alternative explanation is that in flowering plants COP1 and DET1 act in the dark to suppress the response to *positive* signals of plastid origin. These plastid-derived signals would not be produced when plastids

are inactive (Figure 9). Haem, specifically that produced in plastids by the *FERROCHELATASE 1* gene product (Woodson et al., 2011) has been shown to act as precisely such a kind of signal. We should note that placed in the context of



dependence on plastid signals but independence on light (Figure 9).

While the present study was not intended to reveal the sources or nature of plastid-to-nucleus signals themselves, our genetic analysis of the interaction between photomorphogenic repressors and plastid functional state led to some interesting observations. While DET1 acts as a repressor of PhANG expression in the dark, its loss also causes a reduction of expression in the light (Figures 7 and particularly 8). This apparent action of DET1 as a repressor in the dark and an activator in the light seems paradoxical, but it has been observed in the past (Chory et al., 1989; Ruckle et al., 2012), and could be related to oxidative damage, which might also explain the large increase in *CHS* (photoprotectant) expression. It was useful to note differences in the responses of the two mutants with dysfunctional plastids, *ppi1* and *cue8*. *ppi1* is defective in TOC33, a component of the receptor complex at the outer envelope for chloroplast protein import specializing in the assembly of photosynthetically competent chloroplasts (Bauer et al., 2000; Kubis et al., 2003). Accordingly, defects in PhANG expression extended to most PhANGs tested (see Figures 6–8) but occurred clearly in the light, being mild (Figures 6 and 7) or barely, if at all, detectable (Figure 8) in the dark. On the other hand, *CUE8* encodes a chloroplast housekeeping protein, defects in which cause altered etioplast and chloroplast development (Vinti et al., 2005). This correlates with defective PhANG expression in both the dark and the light (see, in particular, Figure 8). This confirms the capacity of all plastids, not just chloroplasts, to communicate their physiological state to the nucleus, and the need for both housekeeping and photosynthesis-associated protein function to maintain PhANG expression. Our classic inhibitor treatments (norflurazon and lincomycin) also lead to similar conclusions. While lincomycin is a translation inhibitor specifically affecting chloroplast ribosomes (see Gray et al., 2003), regardless of light, norflurazon applied from germination has been shown to prevent functional photosynthetic membrane complex assembly in the light (Voigt et al., 2010; Kim and Apel, 2013). It is worth remembering that tetrapyrrole, including chlorophyll, biosynthesis occurs in association with the inner chloroplast envelope as well as the thylakoids (Tanaka et al., 2011). These inhibitors reveal the broad “base” of plastid physiology (protein synthesis machinery, tetrapyrrole synthesis, membrane state/function) being reported by biogenic plastid signals.

Also of interest is the observation that defects in GLKs did not affect all PhANGs tested. This is consistent with the original, extensive analysis (Waters et al., 2009), which identified as GLK targets primarily the genes for light harvesting, reaction center, and electron transport proteins and for chlorophyll biosynthesis. The authors did not observe defects in, for example, *RBCS*, the nuclear gene for the core protein of the photosynthetic carbon reactions, nor did we observe an effect for *CA1*. GLKs are themselves targets of plastid and light signals (Waters et al., 2009) and they can mediate the action of plastid signals on other genes (Kakizaki et al., 2009, and this

study), but they cannot be solely responsible for this action. Interestingly, the response of LHCs in *glk1 glk2* mutants to light was mildly affected, but expression clearly did not occur in such mutants in the dark, even in the absence of DET1, revealing GLKs as possible targets for the dark repression of LHCs by DET1. An alternative explanation, that *glk1 glk2* mutants simply displayed globally impaired chloroplast development, and therefore reduced plastid signaling, is not compatible with the fact that the impact of the mutations was gene-specific. Instead the evidence supports their direct role in the expression in the light of a subset of genes. The normal light response of *glk* mutants and the apparent additive effect (implying independence) of reduced greening between the phenotypes of *glk* and *phytochrome B* mutations observed in a previous study (Waters et al., 2009) could have arisen from a combination of photoreceptor redundancy and the fact that the pale phenotype of *phytochrome B* is due in part to its altered, shade-type, reduced leaf cellular development (Tsukaya, 2005).

Perhaps one unexpected consequence of the present work is that current progress in the understanding of terminal, mechanistic steps in both light and plastid-to-nucleus signal action might lead to mutually revealing findings, and assist an eventual goal of rational engineering of chloroplast biogenesis.

## AUTHOR CONTRIBUTIONS

AH and EL-J designed research. AH, SK, and EL-J carried out research. AH and SK contributed equally to this work. EL-J wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01078>



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# Metabolic engineering of the chloroplast genome reveals that the yeast *ArDH* gene confers enhanced tolerance to salinity and drought in plants

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Osmoprotectants stabilize proteins and membranes against the denaturing effect of high concentrations of salts and other harmful solutes. In yeast, arabinol dehydrogenase (*ArDH*) reduces D-ribulose to D-arabinol where D-ribulose is derived by dephosphorylating D-ribulose-5-PO<sub>4</sub> in the oxidized pentose pathway. Osmotolerance in plants could be developed through metabolic engineering of chloroplast genome by introducing genes encoding polyols since chloroplasts offer high level transgene expression and containment. Here, we report that *ArDH* expression in tobacco chloroplasts confers tolerance to NaCl (up to 400 mM). Transgenic plants compared to wild type (WT) survived for only 4–5 weeks on 400 mM NaCl whereas plants remained green and grew normal on concentrations up to 350 mM NaCl. Further, a-week-old seedlings were also challenged with poly ethylene glycol (PEG, up to 6%) in the liquid medium, considering that membranes and proteins are protected under stress conditions due to accumulation of arabinol in chloroplasts. Seedlings were tolerant to 6% PEG, suggesting that *ARDH* enzyme maintains integrity of membranes in chloroplasts under drought conditions via metabolic engineering. Hence, the gene could be expressed in agronomic plants to withstand abiotic stresses.

**Keywords:** chloroplast transformation, arabinol dehydrogenase, salt and drought tolerance, osmoprotectants

## Introduction

Crop production is severely affected by the accumulated salts in the soil. For example, about 20% of the world's agricultural soils are affected by salinity (Zhu, 2001) and about 25% of US soils are subjected to drought (Boyer, 1982; Tanji, 1990; Zhu, 2001). The damaging effects of salts on plants are mainly because of two reasons; one, water deficit resulting in osmotic stress and two, ions stress to biochemical processes (Ward et al., 2003). To withstand such osmotic stresses, plants synthesize, and accumulate compatible solutes, commonly known as osmoprotectants. These molecules stabilize proteins, membranes, and even transcriptional and translational machineries in the cells against the denaturing effect of accumulated salts and other damaging solutes

**Abbreviations:** *ArDH*, arabinol dehydrogenase; NaCl, sodium chloride; PEG, poly ethylene glycol.

(Yancey, 1994; Wani et al., 2013). The physicochemical basis of the osmoprotective effect of osmolytes requires the exclusion of osmoprotectants from the hydration sphere of proteins (Timasheff, 1992). Under this situation, structures of the native proteins are thermodynamically favored as they offer minimal surface area to the water (McNeil et al., 1999; Rontein et al., 2002). Whereas most salts interact directly with the protein surfaces, causing denaturation of proteins. It means under stressful conditions these osmoprotectants can advance cellular osmotic pressure and protect constituents of cells.

Osmoprotectants, based on their chemical properties, are of three types: betaines; amino acids like proline and non-reducing sugars such as trehalose and arabinol. All of these types do not occur in crop plants, however, their beneficial effects are generally not species-specific. Increasing crop tolerance by engineering plant metabolic pathways is one of the candidate approaches (LeRudulier et al., 1984; Sakamoto and Murata, 2001). Majority of plants cannot metabolize most sugar alcohols including D-arabinol (Stein et al., 1997) hence, a gene that encodes an enzyme for developing sugar alcohol can be expressed via plant chloroplast genome to provide osmoprotection under stress regimes.

Chloroplast transformation compared with conventional transgenic technologies has the unique advantages of very high level gene expression and accumulation of expressed proteins and increased transgene containment. In most cultivated plant species the chloroplasts are inherited uniparentally in a strictly maternal fashion thereby greatly reduce the risk of genetic outcrossing. Therefore, incorporating a gene conferring salt and/or drought tolerance will not call upon the question of potential drought resistance development in wild-type species. For example, chloroplast transgenic plants expressed very high level of transprotein (McBride et al., 1995; Khan and Maliga, 1999; Kota et al., 1999; Viitanen et al., 2004) due to polyploid nature of plastids and engineered translation control elements (Eibl et al., 1999; Kuroda and Maliga, 2001). Similarly, chloroplast derived herbicide resistance and salt tolerance overcome outcross problems of nuclear transformation because of strict maternal inheritance of plastid genomes (Daniell et al., 1998, 2002; Scott and Wilkinson, 1999; Lee et al., 2003; Khan et al., 2005). Currently, plastid transformation technology is regarded as one of the best approaches to express pharmaceuticals and vaccines with biological activity (Daniell and Khan, 2003; Fernandez-San Millan et al., 2003; Tregoning et al., 2003; Magee et al., 2004). This study has been undertaken to engineer plastid genome for stress tolerance through accumulating a non-reducing plant sugar, arabinol in tobacco plants. The spectinomycin-resistant plants were developed in the laboratory of the corresponding author and were analyzed for transgene integration into the plastome (Plastid genome). Later on, an M Phil student (Sarwar, 2007) was given a task to analyze the putative transgenic plants where preliminary studies were carried out to optimize conditions for enzyme and stress assays (Sarwar, 2007, 2013). Encouraged from preliminary results the putative transgenic plants were purified to homoplasmic level through sequential rounds of regeneration on spectinomycin-containing medium. Regenerated plants were subjected to genetic, enzyme, and stress analyses.

Here, we report overexpression of yeast *ArDH* gene in chloroplasts that confers tolerance to salt and drought at high levels than earlier reports. Further, protecting effects of arabinol on chloroplast proteins and membranes under stressful conditions are provided with reduced risk of outcrossing. To the best of our knowledge this is the first report on engineering stress tolerance through alterations in chloroplast metabolic pathway using *ArDH* gene in plants.

## Results

### Development of Transformation Vectors and Transgenic Plants

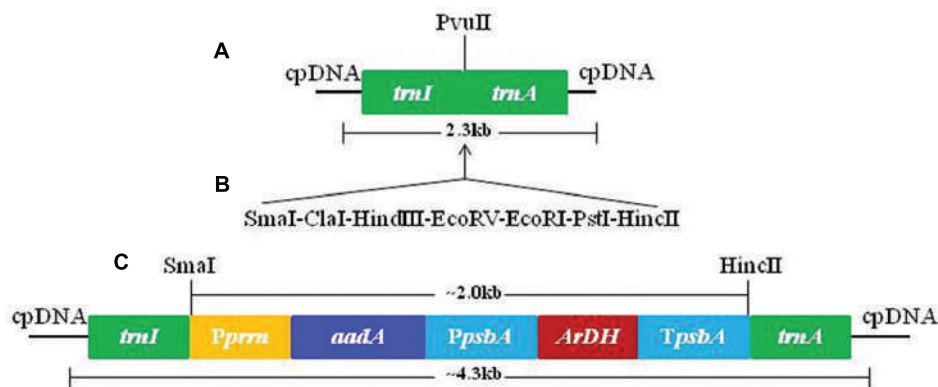
The chloroplast transformation vector pMSK83 was developed by a sequential process of amplification of DNA fragments from tobacco plastome regions and cloning in pTZ57 plasmid (Fermentas, Germany). The promoter was amplified from 16S rRNA operon to control *aadA* gene expression in chloroplasts (Khan et al., 2007). However, *ArDH* gene was tethered with a light regulated *psbA* promoter along with 5' and 3' untranslated regions (UTRs). The *psbA* in chloroplasts is light regulated and hence can be useful in transgene regulation at high levels. The *aadA* gene that confers resistance to both spectinomycin and streptomycin is used for selection of transformation events (Svab and Maliga, 1993; Khan and Maliga, 1999). The *ArDH* gene-containing expression cassette was cloned downstream of *aadA* gene in the chloroplast transformation vector (**Figure 1**). Hence, *aadA* gene is expressing without terminating sequences.

Fully expanded leaves from 6 to 8 weeks old tobacco plants were subjected to bombardments. After 48 h the bombarded leaves were chopped into 3 mm × 3 mm size pieces, which were then placed on spectinomycin (500 mg/l)-containing RMOP medium (Svab and Maliga, 1993; Khan and Maliga, 1999; Khan et al., 2007). Spectinomycin-resistant green shoots started appearing within 4–6 weeks of bombardments from bleached leaf sections on regeneration medium. The green shoots were transferred onto selective maintenance medium for proliferation. To purify the transplastomes to a genetically stable homoplasmic state, leaves from spectinomycin resistant plants were subjected to subsequent rounds of selection and regeneration. During the period the chloroplasts (both the WT and the transformed) and the plastome (the chloroplast genome both WT and the transformed) copies gradually sorted out. Homoplasmic shoots were recovered from leaf sections placed on regeneration medium with or without spectinomycin. From 35 bombardments, nine transgenic clones were recovered on selection and regeneration medium. These plants were rooted on MS medium and subjected to genetic analysis and seed setting for various experiments.

### Genetic Analysis Confirming Stable Integration of Transgene in to Plastome

Different sets of primers were used to analyze transgenic plants harboring *aadA* and *ArDH* genes under the control of chloroplast regulatory sequences (Khan et al., 2007). Spectinomycin-resistant





**FIGURE 1 | Sequential amplification, cloning, and development of pMSK83 chloroplast transformation vector. (A)** Tobacco flanks used for site-specific integration of transgenes. **(B)** Adopter was used to create multiple cloning sites to facilitate cloning. **(C)** Final tobacco chloroplast transformation vector with *aadA* and *ArDH* genes.

plants were analyzed using *aadA* and flanking sequence-specific primers for two reasons; one, confirming the integration of *ArDH* and *aadA* genes into the inverted repeat region. Two, to find out whether the resistance to spectinomycin was due to a mutation in the 16S rRNA gene (Fromm et al., 1987; Svab and Maliga, 1991) or due to the integration of transgenes into the nuclear genome through illegitimate recombination. A primer pair S19 and S20 recognizes sequences that flank the sites of integration on chloroplast genome (Figure 2, left). The primers A19 and A20 anneal to the *aadA* gene (selectable marker gene) and the primers D1 and D2 to *ArDH* gene and amplify fragments of 550 and 750 bp, respectively, (Figures 2A,B, right). The amplification of 3.0 kb fragment with primer pair A19 and S20 (Figure 2C, lanes C1,C2), and 2.3 kb with primer pair S19 and A20 (Figure 2D, lanes C1,C2) and absence of fragment from a non-transformed wild type (WT) plant confirms that the spectinomycin-resistant plants carry the *aadA* and *ArDH* genes in the inverted repeat region of the plastome because primers S19 and S20 are specific to inverted repeat regions of the plastome. As chloroplast transformation vector integrates the transgene cassettes into the inverted repeat regions; hence, to test the homoplasmy of the transplastome the sequences used as flanks were used as a probe. The presence of a single hybridizing fragment of ~6.0 kb in Southern blots confirms the homoplasmy of the transplastome, the plastome carrying *aadA* gene and the *ArDH* gene cassettes in the inverted repeats, compared with WT plant where only a 4.0 kb fragment is hybridized with the same probe, reflecting the plastome without insertions of transgenes.

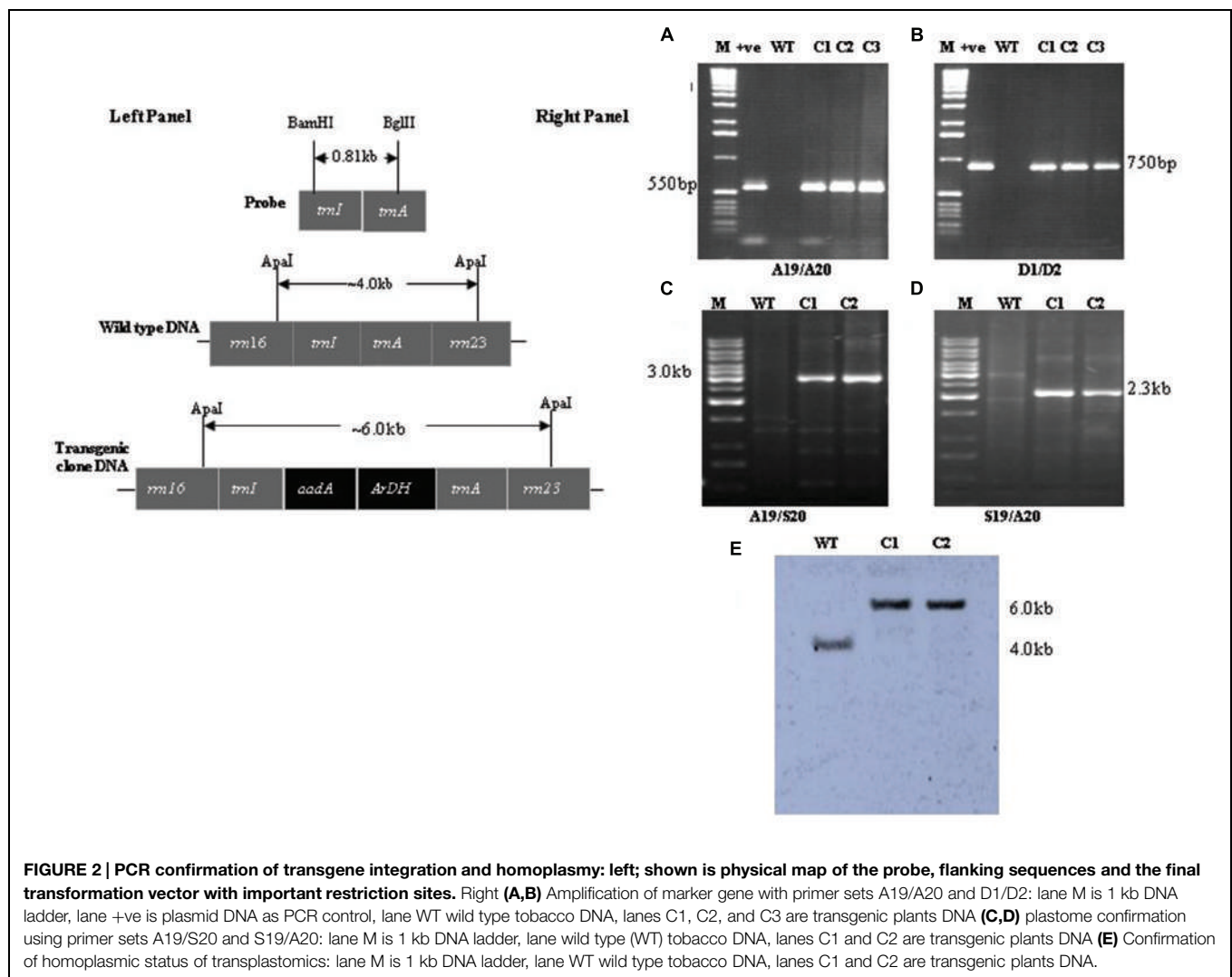
### Qualitative Analysis of *ArDH* Activity in Transgenic Plants

Arabitol dehydrogenase enzyme reduces NAD to NADH in the presence of arabitol in the solution and blue color is developed. The crude extracts prepared by homogenizing 0.25 g leaf tissue in 500  $\mu$ l homogenization buffer, as described in Section “Materials and Methods,” developed blue color depending upon the concentration of the expressed enzyme in the samples

from transplastomic plants, confirming the expression of the transgene in the plastid genome of tobacco. Nonetheless, no blue color development was detected in the leaf extracts from WT plants, entrusting original color (Figure 3 Sample No.2). The colorimetric test was repeated after every 4 weeks and color development was observed in all selected (T1, T2, T3, and T4; T stands for Transgenic) plants. Of these plants T4 exhibited deep color development. Therefore, clone T4 was multiplied and four plants namely; T4-1, T4-2, T4-3, and T4-4 were selected to measure the enzyme activity along with heteroplasmic plants.

### Quantitative Analysis of *ArDH* Activity in Transgenic Plants

Arabitol dehydrogenase activity was measured in crude extracts of leaves from wild-type and transgenic plants as previously described for yeast by Wong et al. (1995) and on heteroplasmic tobacco seedlings (Sarwar, 2007, 2013). The rate of reduction of NAD to NADH by ARDH enzyme in the presence of D-arabitol was measured by the reduction of MTT (3, 5 dimethylethiazol -2-yl 2, 5 dimethyle tetrazolium bromide) in the solution. Crude extracts from fully expanded leaves (Fourth from top to bottom) of transgenic chloroplast plants showed enzyme activity compared to non-transformed leaves (Figure 4). Enzyme activity was different in different plants (T1, T2, T3, and T4 samples) due to their heterogenetic nature though given readings are an average of three samples. The high ARDH enzyme activity was observed in fully expanded leaves of potted plants (T4 homoplasmic transgenic plants) compared to primary heteroplasmic transgenic plants, as described (Sarwar, 2007, 2013). This was due to two reasons: first, copy number for transgene was increased toward homoplasmy. Homoplasmic plants were recovered through multiple rounds on regeneration medium supplemented with spectinomycin (500 mg/L). Second, plants were subjected to analysis after the onset of light as *psbA* 5' UTR is light regulated (Staub and Maliga, 1994; Eibl et al., 1999). Hence, enzyme activity was enhanced under continuous light yet these levels are very low as expected this



may be because of minimal level of substrate availability in chloroplasts.

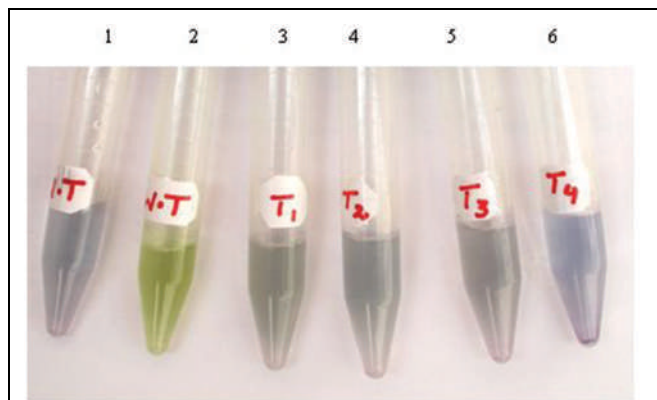
### Salt Tolerance in Transplastomic Plants

First generation heteroplasmic plants were grown in pots for seed setting and variable degrees of tolerance to NaCl were observed (Sarwar, 2007, 2013) hence, primary transgenic plants growing under *in vitro* conditions were subjected to a number of selection and regeneration rounds to purify transplastomes to homoplasmic level since the process requires 16–17 cell divisions (Moll et al., 1990; Khan and Maliga, 1999). Consequently, confirmed homoplasmic plants were recovered and grown in pots for seed setting. Nodal segments and seeds from homoplasmic transgenic and WT plants were subjected to various levels of NaCl. Plants developed from nodal segments were heterogeneous in growth hence were not used in salt tolerance assays, may give rise to inconsistent results. Therefore seeds of transgenic and WT plants were only used in subsequent assays. Seeds from transgenic plants were placed on solidified MS medium (Murashige and Skoog, 1962) supplemented with increasing

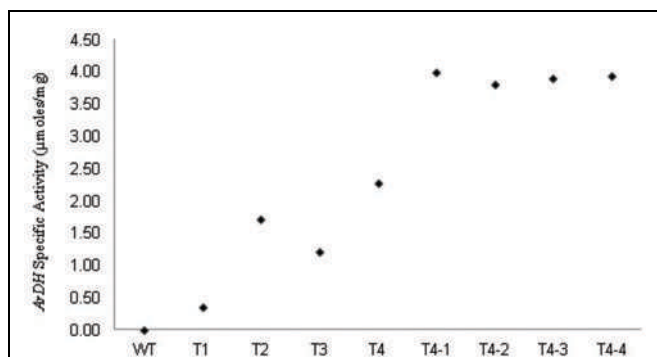
concentrations (100–600 mM) of NaCl in plastic Petri plates along with seeds of WT plant in growth room under standard temperature and light conditions. After attaining a reasonable height the plants were transplanted into magenta boxes at same levels of NaCl-containing solidified MS medium. Transgenic plants (T4-1 and T4-2) expressing the *ArDH* gene thrive well up to 350 mM NaCl (Figure 5A), whereas WT plants exhibited retarded growth with yellow phenotype, indicating that chloroplast-based expression of ARDH is adequate to confer high level of salinity tolerance in plants. Both transgenic and WT plants developed indistinguishable phenotype when grown in pots without salt stress (Figure 5B). As per literature, this appears to be the highest level of salt tolerance for tobacco, the plant with broader leaves and with more exposed surface area for transpiration.

### Drought Tolerance and Chlorophyll Contents in Transplastomic Plants

The homoplasmic transgenic plants exhibiting high enzyme activity and salt tolerance were tested for drought tolerance.

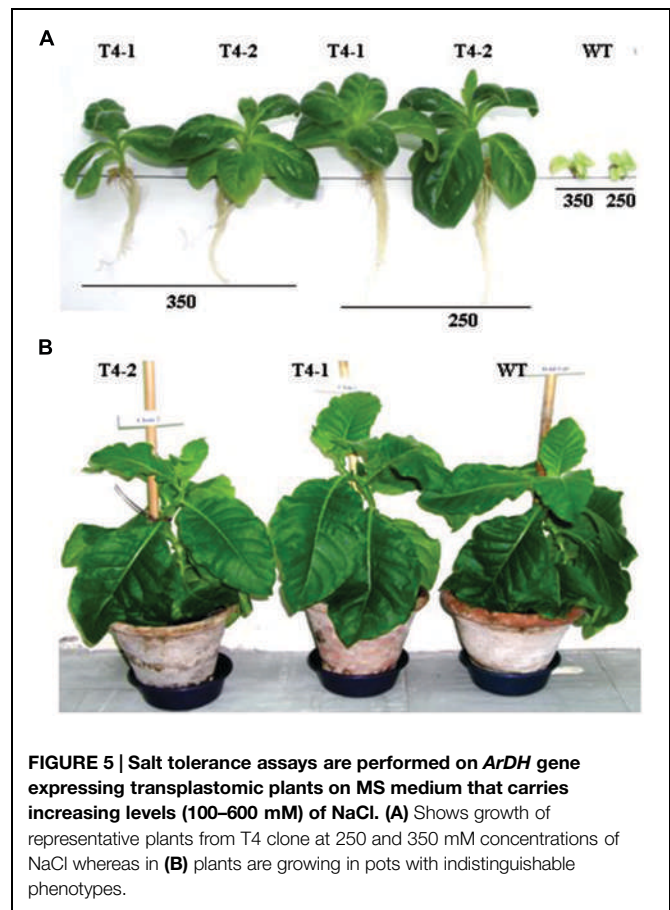


**FIGURE 3 | Color-based detection of *ArDH* gene expression in plant cells.** Tubes 1-2 are leaf extracts from WT plants with (tube 1) and without (tube 2) adding ARDH enzyme in the samples. Tubes 3-6 are transgenic plants that harbor *ArDH* gene in the transplastomes.



**FIGURE 4 | Levels of ARDH measured in heteroplasmic (T1, T2, and T3) and homoplasmic (T4; T4-1, T4-2, T4-3, and T4-4) plants under standard and continuous light.** Values shown represent the average value of the specific activity recorded in extracts from fourth leaf (from top to bottom) of tobacco plants. Values given for WT, T1, T2, T3, and T4 are from leaf extracts of plants grown under standard growth conditions (100 μmole photons  $m^{-2} s^{-1}$  – 16 h light, 8 h dark) at 25°C ± 0.2. Nevertheless, plants T4-1, T4-2, T4-3, and T4-4, which were regenerated from leaves of T4 transgenic plant were kept under continuous light for 7 days in pots. The enzyme activity recorded for T4-1, T4-2, T4-3, and T4-4 plants is not an average value as they are derived from same T4 clone.

Seeds from transgenic and WT plants were first germinated on solidified MS medium because seeds cannot be germinated in liquid medium. Then, 7 day-old seedlings were transferred to MS liquid medium containing increasing levels of polyethylene glycol, ranging from 1 to 6% in Magenta boxes, carrying plastic bridges to hold seedlings under *in vitro* conditions. As shown in **Figure 6A**, chloroplast transgenic seedlings grew variably in 5% PEG containing liquid MS medium in Magenta Boxes. Variable growth of plants was due to the genetically inconsistent (heteroplasmic) nature of plants. The inconsistent growth of heteroplasmic plants compared with uniform growth of homoplasmic plants in 5% PEG containing liquid MS medium clearly show that the increasing concentrations of the ARDH are advantageous to normal growth of transgenic plants under stress



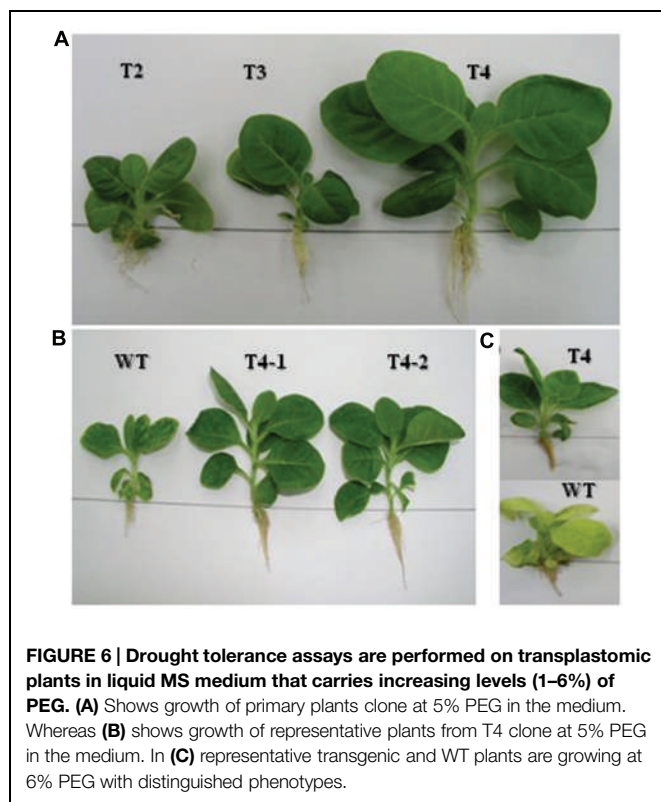
**FIGURE 5 | Salt tolerance assays are performed on *ArDH* gene expressing transplastomic plants on MS medium that carries increasing levels (100–600 mM) of NaCl. (A)** Shows growth of representative plants from T4 clone at 250 and 350 mM concentrations of NaCl whereas in **(B)** plants are growing in pots with indistinguishable phenotypes.

(**Figure 6B**). Transgenic T4 plant stayed green and developed as a normal plant in MS liquid medium that was supplemented with 6% PEG whereas control plant exhibited loss of chlorophyll and growth retardation, ultimately died (**Figure 6C**). Loss of chlorophyll in WT plant indicates breakdown of chloroplast thylakoid membranes due to osmotic stress caused by PEG. Whereas presence of chlorophyll in transgenic chloroplasts indicates the integrity of thylakoid membranes, even in the presence of very high concentration (6%) of PEG (**Figure 6**), clearly demonstrating the benefit of the expressed gene in transgenic chloroplasts. It was impossible to measure and compare the chlorophyll contents of transgenics with WT plants under stress conditions because WT plants bleached on PEG containing medium. The WT and transgenic plants were phenotypically indistinguishable in pots when grown without stress (**Figure 5B**).

## Discussion

Osmoprotectant concentrations to the levels of 200 mM or more are osmotically significant; subsequently have essential role in water uptake under stress by maintaining cell turgor as well as the driving gradient (Rhodes and Samaras, 1994). Amongst osmoprotectants reported in the literature, glycine betaine, mannitol, and proline occur commonly in plants,





whereas trehalose occurs rarely in plants but arabinol is found only in bacteria and yeast. Enzymes responsible for most of these and some other compounds, used to engineer plants for stress tolerance, have been studied under laboratory conditions. Under stress conditions, glycine betaine protects the sub-cellular structures, transcriptional, and translational machineries and also intervenes in the refolding of enzymes as a molecular chaperone (Wani et al., 2013). In our studies we have expressed an NAD-dependent D-ArDH gene from *Candida albicans* in chloroplasts to develop tolerance in tobacco to environmental stresses, for example salinity and drought. As ArDH reduced NAD in the presence of arabinol and chloroplasts remained green due to the accumulation of chlorophyll under stress hence, it is likely that salinity and drought tolerance in transgenic plants is due to the developed arabinol. Further, green color and normal growth of plants under stress conditions clearly demonstrate the customary functioning of transgenic plant chloroplasts. Hence, this is the first report to express ArDH gene in plant chloroplasts that reduces D-ribulose into D-arabinol. An *E. coli* gene, *at1D*, that converts non-plant-metabolizable arabinol into metabolizable xylulose in the medium, was expressed in rice in order to develop a positive selection system (Lafayette et al., 2005) since rice cannot metabolize D-arabinol (Stein et al., 1997). In another report, betaine aldehyde dehydrogenase gene was expressed to accumulate betaine in carrot plastids where improvement in water stress tolerance has been claimed (Kumar et al., 2004) since betaine occur commonly in plants.

High ARDH enzyme activity was observed in fully expanded leaves of potted plants (T4 plants) compared to primary

transgenic plants when exposed to continuous light as *psbA* 5' UTR is light regulated. Transgenically it was confirmed that accumulation of transprotein is in response to light and is controlled via *cis*-acting regulatory elements in the untranslated region of the *psbA* mRNA because reduction in accumulation was observed when these plants were transferred to dark but no reduction in mRNA levels was observed (Staub and Maliga, 1993). Transprotein accumulation was recorded as high as 135- to 200-fold (Staub and Maliga, 1994) in response to light, however, deletion of sequences from 5' UTR were resulted in fourfold decrease in translational efficiency (Eibl et al., 1999) but no change in translational efficiency was observed after exchanging the *psbA* 3' UTR (Staub and Maliga, 1994; Eibl et al., 1999), only mRNA levels were decreased, confirming that the *psbA* 5' UTR is light responsive (Eibl et al., 1999). We expressed ArDH gene under *psbA* promoter along with 5' UTR and an enhanced enzyme activity was observed. Though substrate specificity is unknown yet in plants but based on yeast, it is likely that ARDH reduces D-ribulose to D-arabinol where D-ribulose is derived by dephosphorylating D-ribulose-5-PO<sub>4</sub> in the oxidized pentose pathway in plant chloroplasts. But phosphorylation of D-ribulose-5-PO<sub>4</sub> results in D-ribulose-1,5-bisPO<sub>4</sub> that enters into Calvin cycle (Figure 7).

When transplastomic plants were observed tolerant to very high levels of NaCl (300–350 mM) and were green in color without any pleiotropic effects, then 7 day-old transplastomic and non-transformed control seedlings were grown in PEG-containing liquid MS medium. The transplastomic seedlings grew normal whereas control seedlings exhibited loss of chlorophyll and growth retardation. Loss of chlorophyll in WT plants indicates breakdown of chloroplast thylakoid membranes due to osmotic stress induced by PEG. But the presence of chlorophyll in transgenic chloroplasts indicates the integrity of thylakoid membranes, even in the presence of high concentrations of PEG, clearly demonstrating the advantage of expressed gene in transgenic chloroplasts.

Accumulation of arabinol in chloroplasts is not measured this, then, is one aspect that calls for further investigation. Yet, it is believed that accumulation in smaller quantities is adequate to protect chloroplasts from salt and drought stresses and larger quantities are advantageous for commercial applications.

## Materials and Methods

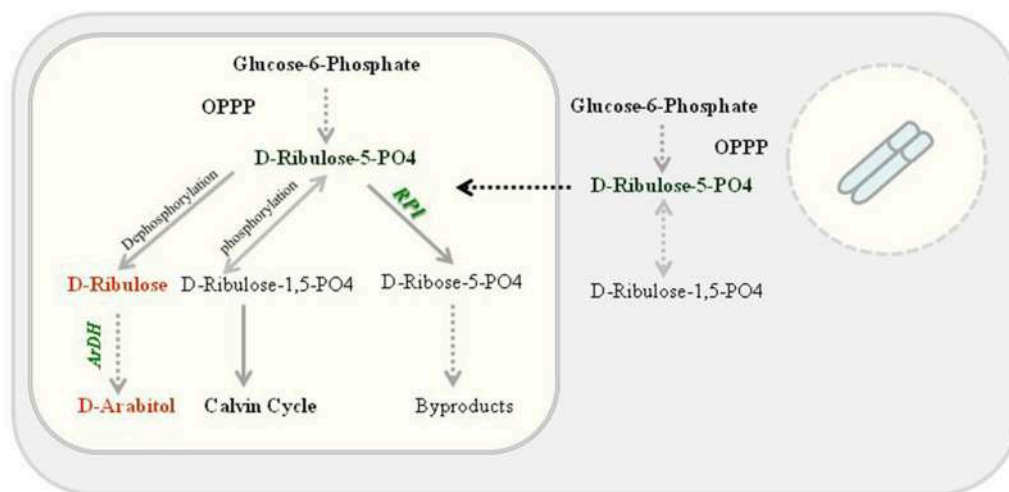
### Plant Material and Growth Conditions

*Nicotiana tabacum*, var. Petit Havana, was grown aseptically on 0.7% phyta-agar-solidified MS salts, pH 5.8, containing 3% sucrose at 25°C ± 0.2 under 100 μmole photons m<sup>-2</sup> s<sup>-1</sup> (16 h light, 8 h dark). Fully expanded leaves of 4–6 week-old plants were used for chloroplast transformation using microprojectile bombardments.

### Development of Chloroplast Transformation Vector

To develop transgenic chloroplasts, transformation vector was developed as described (Khan et al., 2007). The flanking





**FIGURE 7 | Proposed D-arabitol pathway in tobacco chloroplasts.** ARDH reduces D-ribulose to D-arabitol where D-ribulose is derived by dephosphorylating D-ribulose-5-PO<sub>4</sub> in the oxidized pentose pathway. Nevertheless, phosphorylation of D-ribulose-5-PO<sub>4</sub> results in D-ribulose-1,5-bisPO<sub>4</sub> that enters into Calvin cycle.

sequences were amplified using primers 5'-GATATCAAAA CCGTCCTCAGTTCGGATTGC-3' and 5'-GATATCCACGAG TTGGAGATAAGCGGA-3'). The underlined sequences are the created *EcoRV* sites, which were used to restrict the cloned PCR product from the TA cloning vector (MBI Fermentas, Italy) and to ligate it into the pBluescript II (MBI Fermentas, Italy) plasmid, opened with *PvuII* restriction enzyme. An adopter carrying unique restriction sites was ligated into the insertion site (*PvuII*) for subsequent cloning of selection and expression cassettes. The *psbA* promoter and 5'UTR were amplified using primers 5'AAGCTTACTAGCATATCGAAATTCT-3' and 5'GAATTCATATGAAAATCTTGGTT-3' and the PCR-amplified fragment was cloned into TA cloning vector (MBI Fermentas, Italy). The *psbA* 3'UTR was amplified using primers, 5'-TCTAGAATCTAGATC GTGC-3' and 5'-GAGCTCGGTGACCCTTGTATG-3' and cloned downstream of the promoter and 5'UTR of *psbA*. The *ArDH* gene was amplified from *Candida albicans* and cloned into the *psbA* cassette. The expression cassette carrying *ArDH* gene was cloned downstream to *aadA* gene that is tethered with 16SrRNA operon promoter to generate the final transformation vector, pMSK83. Hence, both genes were expressed under separate promoters, independently but sharing the terminating sequence, 3'UTR of *psbA*.

### Chloroplast Transformation and Selection of Transgenics

The transformation vector pMSK83 was coated onto the surface of 0.6  $\mu$  gold particles to transform tobacco chloroplast using optimized protocols (Svab and Maliga, 1993; Khan and Maliga, 1999; Daniell et al., 2002; Bock and Khan, 2004). Fully expanded tobacco leaves from 4 to 6 week-old plants were used and the transformation was carried out using a PDS 1000 helium gun (BIO-RAD). Bombarded leaves were chopped into small pieces of 3 mm  $\times$  3 mm size after 48 h of bombardments,

which were placed on spectinomycin (500 mg/l)-containing RMOP medium (Svab and Maliga, 1993; Khan and Maliga, 1999). Spectinomycin resistant shoots were recovered from bleached leaf pieces on RMOP medium (Khan and Maliga, 1999) and rooted on solidified MS medium (Murashige and Skoog, 1962).

### Genomic Analysis of Transgenic Plants to Determine the Homoplasmy

Total cellular DNA from WT as well as transgenic plants was isolated using the hexadecyltrimethyl ammonium bromide (CTAB) DNA extraction method (Rogers and Bendich, 1985) with modifications and was used as template in PCR reactions. The integration of transgenes carrying selection and expression cassettes into the plastome was confirmed using *aadA*- and flanking sequences-specific primers and probes, as described in results section. The homoplasmy for transgenes integration was confirmed by Southern blotting using flanking sequences as a probe.

### Enzyme Assays

Arabitol dehydrogenase enzyme assay was carried out as described by Wong et al. (1993) with modifications where standard curve was developed by using different concentrations of BSA and the standard factor was calculated as 0.01  $\mu$ g/mL/min. Crude extracts were prepared from plants that were grown under *in vitro* conditions at 25°C  $\pm$  02 and 100  $\mu$ mole photons  $m^{-2} s^{-1}$  (16 h light, 8 h dark) as well as from plants that were kept under continuous light for 7 days. The crude extracts were prepared by homogenizing 0.25 g tissues from fourth fully expanded leaf (from top to bottom) on ice (at  $\sim$ 04°C) in 500  $\mu$ l homogenization buffer. The buffer contains 35 mM HEPES (*N*-2-hydroxyethylpiperazine- *N*9-2-ethanesulfonic acid; pH 8.5), 0.2 mM NAD or NADP, 0.2 mM phenazine methosulfate, 0.4 mM MTT and 12.5 mM D-arabitol. Spectrophotometer was used

to measure the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) at wavelength (A578). The enzyme activity was calculated in  $\mu\text{moles/ml/min}$  (The amount of the enzyme necessary to convert 1  $\mu\text{mole}$  of substrate per min at 25°C) as described by Wong et al. (1993, 1995).

## Salinity and Drought Tolerance Assays

Nodal segments and seeds from transgenic and WT plants were subjected to various levels of NaCl (Sigma) and polyethylene glycol (MW, 8000, Sigma). Plants developed from nodal segments were variable in size hence seeds from transgenic and WT plants were tested on solidified MS medium supplemented with increasing intensities (100–600 mM) of NaCl. Seedlings were raised from seeds of both transgenic and non-transgenic WT plants to grow in

liquid MS medium in magenta boxes under standard light-dark conditions. The MS medium was supplemented with different concentrations (1–6%) of polyethylene glycol. The seedlings subjected to shock episodes were analyzed for stress tolerance.

## Acknowledgments

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# An engineered lipid remodeling system using a galactolipid synthase promoter during phosphate starvation enhances oil accumulation in plants

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Inorganic phosphate (Pi) depletion is a serious problem for plant growth. Membrane lipid remodeling is a defense mechanism that plants use to survive Pi-depleted conditions. During Pi starvation, phospholipids are degraded to supply Pi for other essential biological processes, whereas galactolipid synthesis in plastids is up-regulated via the transcriptional activation of *monogalactosyldiacylglycerol synthase 3 (MGD3)*. Thus, the produced galactolipids are transferred to extraplastidial membranes to substitute for phospholipids. We found that, Pi starvation induced oil accumulation in the vegetative tissues of various seed plants without activating the transcription of enzymes involved in the later steps of triacylglycerol (TAG) biosynthesis. Moreover, the *Arabidopsis* starchless phosphoglucomutase mutant, *pgm-1*, accumulated higher TAG levels than did wild-type plants under Pi-depleted conditions. We generated transgenic plants that expressed a key gene involved in TAG synthesis using the Pi deficiency-responsive *MGD3* promoter in wild-type and *pgm-1* backgrounds. During Pi starvation, the transgenic plants accumulated higher TAG amounts compared with the non-transgenic plants, suggesting that the Pi deficiency-responsive promoter of galactolipid synthase in plastids may be useful for producing transgenic plants that accumulate more oil under Pi-depleted conditions.

**Keywords:** monogalactosyldiacylglycerol, galactolipid, promoter, triacylglycerol, phosphate starvation, starch

## Introduction

Plants possess various mechanisms, including membrane lipid remodeling (Essigmann et al., 1998; Härtel et al., 2000; Andersson et al., 2003, 2005; Jouhet et al., 2004; Nakamura, 2013; Shimojima et al., 2013), to adapt to inorganic phosphate (Pi)-limited conditions, which are distinct from those related to nitrogen (N)-limited conditions. During Pi deficiency, phospholipids in the biological membranes are degraded and the phosphorus generated via phospholipid breakdown is used for other essential biological processes in the cell.



To compensate for the lack of phospholipids in the membranes under Pi-limited conditions, galactolipid synthesis in the outer envelope membranes of plastids is up-regulated (Awai et al., 2001; Kobayashi et al., 2004). Plants have two species of galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). Under normal growth conditions, both MGDG and DGDG are produced and remain in plastids as components of the thylakoid membrane (Benning and Ohta, 2005; Shimajima and Ohta, 2011). However, under Pi-depleted conditions the amount of DGDG doubles and is exported to the extraplastidial membranes by a yet unknown mechanism to substitute for the major phospholipid, phosphatidylcholine. In plastids, DGDG is mainly produced by an additional transfer of a galactose moiety to MGDG by DGDG synthases (DGD1 and DGD2) on the outer envelope membrane (Härtel et al., 2000; Kelly and Dörmann, 2002; Kelly et al., 2003). A previous analysis of an *Arabidopsis* mutant clearly showed that MGDG synthesis on the outer envelope membrane performed by type B MGDG synthases MGD2 and MGD3 has an important role in increasing the DGDG content during Pi depletion (Kobayashi et al., 2009). An *Arabidopsis* *mgd3* knock-out mutant showed a severe growth defect during Pi depletion, whereas an *mgd2* knock-out did not show a significant growth difference compared with wild type (WT; Kobayashi et al., 2009). Thus, between the two isoforms of type B MGDG synthase, MGD3 is predominantly involved in lipid remodeling during Pi starvation.

Type B MGDG synthase genes are widely conserved in seed plant genomes, suggesting that the enhancement of galactolipid synthesis under Pi starvation have been widely conserved in higher plants for adaptation to Pi-poor environments (Russo et al., 2007; Tjellström et al., 2008; Lambers et al., 2012; Yuzawa et al., 2012). Based on these previous findings, we hypothesized that a *MGD3* promoter might be useful for efficiently expressing introduced genes in the shoots and roots of plants in response to Pi starvation.

Plant storage lipids, triacylglycerols (TAGs), can be used as feedstock for the production of biodiesel or highly valuable fatty acids (Durrett et al., 2008; Dyer and Mullen, 2008; Riediger et al., 2009; Lu et al., 2011). However, most plant TAGs are synthesized and stored in seeds, which constitute a small portion of the total plant biomass. Although, TAGs are also synthesized in non-seed and vegetative tissues, such as leaves, the amount of TAG in vegetative tissues is usually very low (Chapman and Ohlrogge, 2012; Chapman et al., 2013). Many techniques have been used to increase TAG levels in vegetative tissues (Chapman and Ohlrogge, 2012; Chapman et al., 2013), and those approaches were based on knowledge obtained from TAG synthesis and breakdown in seeds. Most experiments involved ectopically overexpressing genes involved in TAG synthesis and knocking down/out genes involved in TAG breakdown. The transcription factors *LEAFY COTYLEDON1* and 2 (*LEC1* and 2) are involved in seed maturation and TAG biosynthesis, respectively (Santos Mendoza et al., 2005; Mu et al., 2008). In *Arabidopsis*, the overexpression of *LEC1* or *LEC2* in WT plants and of *LEC2* in the fatty acid-breakdown mutant *COMATOSE* leads to TAG accumulation in vegetative tissues

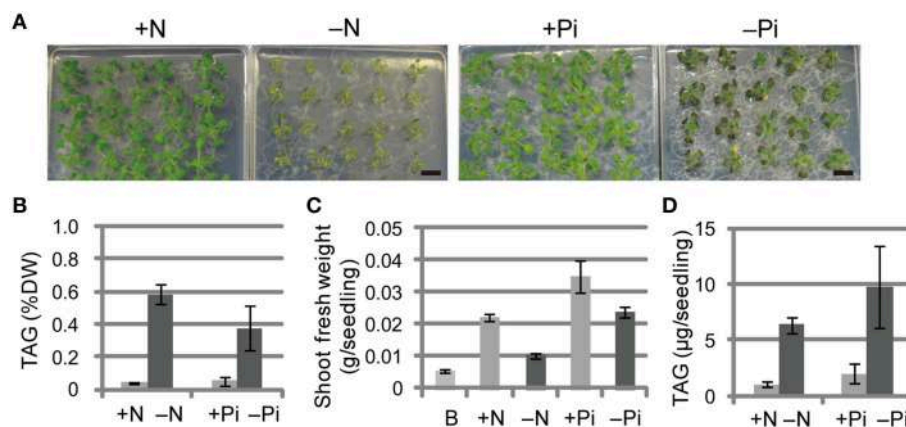
(Santos Mendoza et al., 2005; Mu et al., 2008; Slocombe et al., 2009; Kim et al., 2015). Transgenic tobacco that overexpresses *DIACYLGLYCEROL ACYLTRANSFERASE 1* (*DGAT1*) and *LEC2* also accumulates TAG in its leaves (Andrianov et al., 2010). Sanjaya et al. (2011) elevated TAG levels in vegetative tissues by (i) suppressing *APS1*, which encodes the small subunit of ADP-glucose pyrophosphorylase, which catalyzes the first step of starch biosynthesis, producing a starchless mutant and by (ii) overexpressing the transcription factor *WRINKLED1* (*WRI1*), which regulates TAG synthesis in *Arabidopsis* seeds and shoots. The ectopic overexpression of *Chlamydomonas reinhardtii* DGAT in *Arabidopsis* also elevates TAG levels in leaves (Sanjaya et al., 2013). The *Arabidopsis* COMPARATIVE GENE IDENTIFICATION-58 (CGI-58) homolog controls TAG breakdown exclusively in vegetative tissues by interacting with PEROXISOMAL ABC-TRANSPORTER 1 (PXA1), and the knockout mutant accumulates higher TAG levels in vegetative tissues compared with WT plants (James et al., 2010; Park et al., 2013). TAG levels in *Arabidopsis* vegetative tissues are also increased when *DGAT1* and *WRI1* are constitutively overexpressed in *sdp1* knockout mutant plants, which are defective in TAG breakdown under nutrient-sufficient conditions (Kelly et al., 2013). The overexpression of *PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE 1* (*PDAT1*) with oleosin in *Arabidopsis* is also effective for enhancing TAG levels in leaves (Fan et al., 2013). Recently, Vanhercke et al. (2014) succeeded in producing transgenic *Nicotiana tabacum* (tobacco) in which TAG comprised >15% of the leaf dry weight by co-expressing three genes, *oleosin*, *DGAT1*, and *WRI1*, without severely affecting plant development. Thus, the previous reports clearly showed that genetically engineered vegetative tissues have the potential to store relatively high levels of TAGs.

Here, as an application of the lipid remodeling system during Pi starvation, we produced transgenic *Arabidopsis* plants that express high levels of TAG synthesis genes under the control of the *MGD3* promoter. We analyzed the effects of this approach on plant growth and the TAG content in vegetative tissues to evaluate its efficiency in producing more oil in plant vegetative tissues.

## Results

### Pi Depletion Increases TAG Levels in *Arabidopsis* WT Plants

We first compared the phenotypes and TAG levels of WT plants grown under N-depleted and Pi-depleted conditions (Figure 1). The seedlings grown under N-depleted conditions were relatively more chlorotic than seedlings grown under Pi-depleted conditions (Figure 1A), and the TAG levels in N-depleted plants after 7 d were 1.5-fold higher than in the Pi-depleted plants after 10 d (Figure 1B), suggesting that the higher accumulation of TAG during N depletion was a consequence of the rapid breakdown of photosynthetic membranes. Consistent with the phenotypes shown in Figure 1A, WT seedlings grown under N-depleted conditions for 7 d were very small, and



**FIGURE 1 | Growth phenotypes and TAG accumulation in shoots of WT *Arabidopsis* plants grown under Pi- or N-depleted conditions.** WT plants (10 d old) were transferred to MS agar containing 1% (w/v) sucrose with (+Pi; 1 mM) or without (−Pi; 0 mM) Pi for 10 d, or with (+N; 3.5 mM) or without (−N; 0 mM) N for 7 d. **(A)** Growth under Pi-depleted or N-depleted

conditions. Bars = 1 cm. **(B)** TAG levels in shoots under Pi-depleted or N-depleted conditions. DW, dry weight. **(C)** Shoot fresh weight under Pi-depleted or N-depleted conditions. B, before transfer to Pi- or N-depleted conditions. **(D)** TAG content in shoots per seedling under N- or Pi-depleted conditions. Data are the means ± SD from three independent experiments.

their shoot fresh weight was half that of plants grown under N-sufficient or Pi-depleted conditions (Figure 1C). The TAG content per seedling in WT shoots under Pi-depleted conditions was ~1.5-fold higher than that of WT shoots under N-depleted conditions (Figure 1D).

### Pi Limitation Induces TAG Accumulation in Vegetative Tissues of Various Seed Plants

We also measured TAG levels in the roots of WT *Arabidopsis* plants grown under Pi-sufficient and Pi-depleted conditions. TAG levels in WT plants grown under Pi-depleted conditions were 5- to 6-fold higher in shoots and 1.5- to 2-fold higher in roots compared with those of plants grown under Pi-sufficient conditions (Figure 2A). It should be noted that we have also presented additional data here for shoot TAG levels for comparison with root TAG levels.

We used electron microscopy to assess the impact of low Pi availability on the accumulation of oil droplets in leaf mesophyll cells. Pi starvation resulted in many large starch granules within chloroplasts and oil droplets outside of chloroplasts, whereas no oil droplets were observed inside chloroplasts under these experimental conditions (Figure 2B; Supplementary Figure 1). At Pi levels between 0.1 and 1 mM, TAG levels were similar in plant leaves; however, when the Pi level was <0.1 mM the TAG levels increased, with 0.033 mM Pi producing the highest TAG level (Figure 2C). Thus, a Pi concentration of ≤0.033 mM was required to observe the low Pi-dependent TAG accumulation in leaves. We also analyzed the TAG levels of other plant species under Pi-depleted conditions. Increased TAG levels upon Pi starvation also occurred in tomato (*Solanum lycopersicum* L.; Figure 2D; Supplementary Figure 2A), tobacco (*N. tabacum*; Figure 2E), and barnyard grass (*Echinochloa crus-galli*; Figure 2F; Supplementary Figure 2B), suggesting that the phenomenon is widely conserved among seed plants, including monocots.

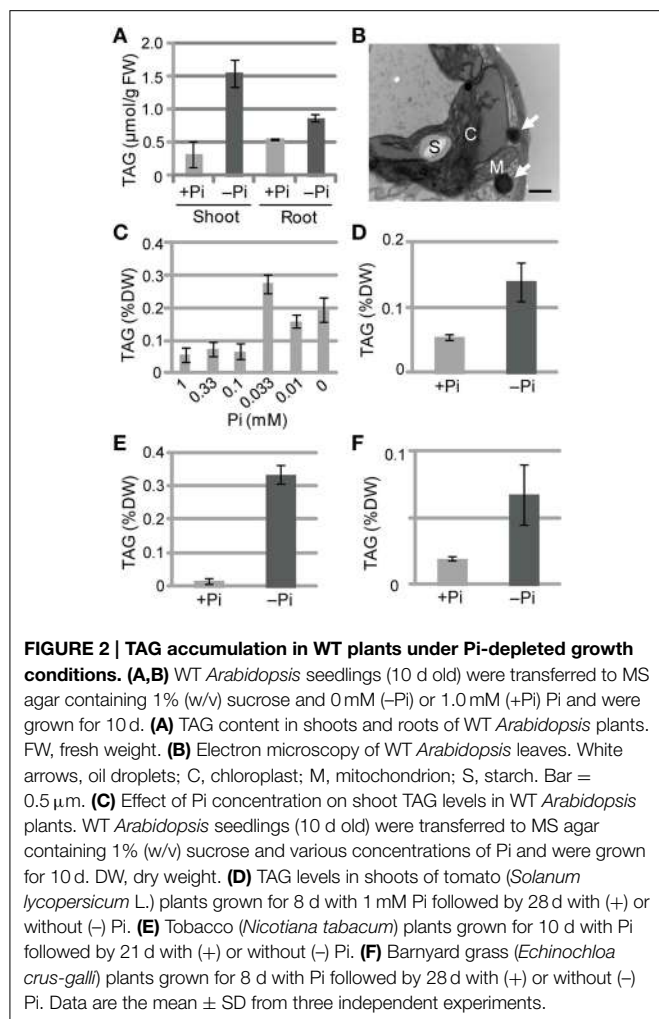
### The Vegetative Tissues of the Starchless Phosphoglucosyltransferase Mutant *pgm-1* Accumulate Higher Levels of TAGs Under Pi-depleted Conditions

Under Pi-depleted growth conditions, starch accumulates in leaf chloroplasts (Nielsen et al., 1998). Under nutrient-sufficient growth conditions, mutant *Arabidopsis* plants with low starch levels accumulate more TAGs in their vegetative tissues than WT plants (Sanjaya et al., 2011). To test whether the same pool of carbon sources was used for starch and oil synthesis in leaves under Pi-depleted conditions, we examined *Arabidopsis pgm-1* mutants, which lack almost all of the transitory starch in leaves because of a point mutation in the plastidic phosphoglucosyltransferase gene (Caspar et al., 1985; Periappuram et al., 2000). Although, the shoots of *pgm-1* plants accumulated more anthocyanin than did WT plants under both Pi-sufficient and Pi-depleted conditions, their fresh weights were similar under both conditions (Figures 3A,B).

Lipid droplets in leaf mesophyll cells of WT and *pgm-1* plants grown under Pi-depleted conditions were visualized using electron microscopy (Figure 3C, upper panels) and also with a neutral lipid-selective fluorescent dye, Nile red (Figure 3C, lower panels, green). The number of lipid droplets was higher in *pgm-1* than in WT plants, and, based on electron microscopy observations, these lipid droplets were likely to be located outside of the chloroplasts (Figure 3D).

### Pi Starvation-induced TAG Accumulation Occurs without the Transcriptional Activation of the Key Steps in TAG Biosynthesis

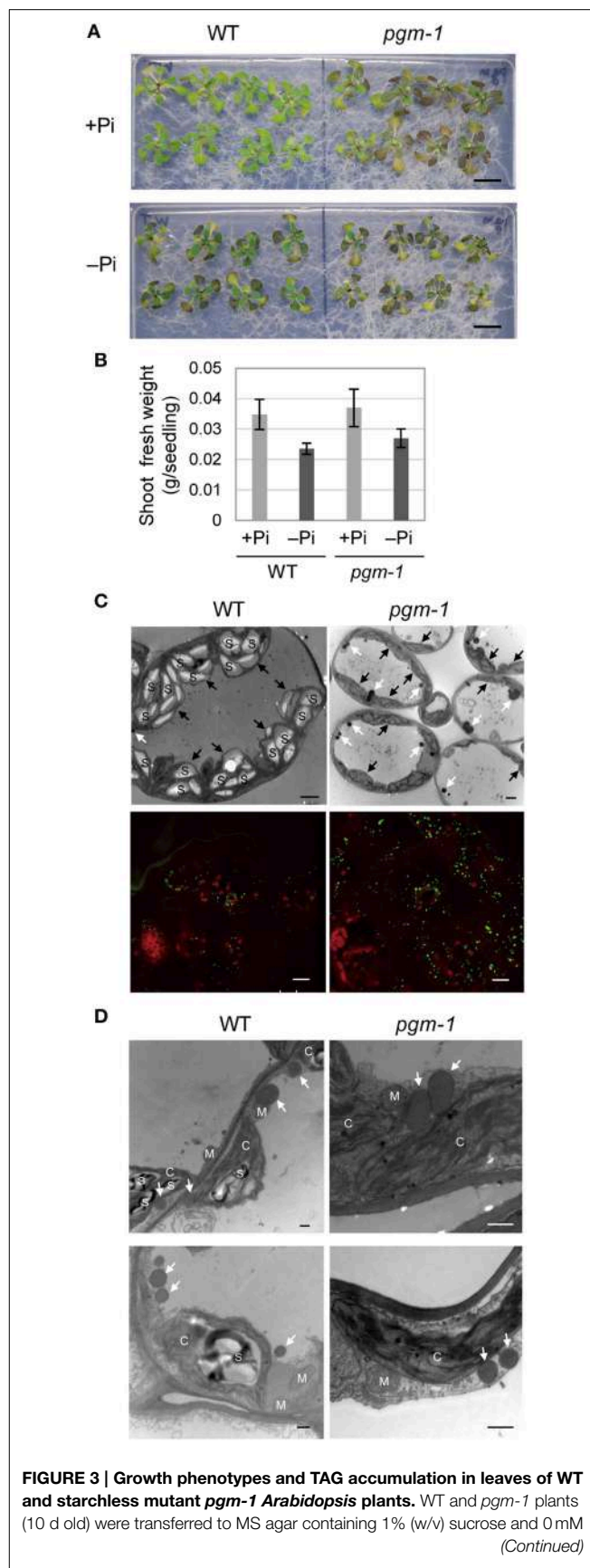
TAG accumulation during senescence is related to the transcriptional up-regulation of *DGAT1* (Kaup et al., 2002). TAG accumulation during N starvation occurs with the concomitant induction of the genes involved in TAG synthesis and accumulation, such as *DGAT1* and *OLEOSIN1* (Yang



et al., 2011). Thus, we analyzed the expression of the key TAG biosynthetic genes *DGAT1*, *DGAT2*, and *PDAT1* in WT and *pgm-1* plants under Pi-sufficient and Pi-depleted conditions (Figures 4A–C). Distinct from TAG accumulation during senescence or N starvation, the high TAG accumulation in WT and *pgm-1* plants under Pi-depleted conditions (Figure 3C) did not correlate with the transcriptional up-regulation of these genes (Figures 4A–C). The expression levels of these genes in WT roots under Pi-sufficient and Pi-depleted conditions were also analyzed and were clearly shown to be unchanged or decreased during Pi starvation (Figure 4D). These results suggested that the overexpression of these genes under Pi-depleted conditions might further increase TAG levels in the leaves and roots of WT and *pgm-1* mutants.

### Pi Starvation–induced Overexpression of *DGAT1*, *DGAT2*, or *PDAT1* Does Not Affect Plant Biomass

Previously, our group analyzed the promoter region of *Arabidopsis* *MGD3* (*ProMGD3*), which encodes a key enzyme in membrane lipid remodeling under Pi-depleted conditions.



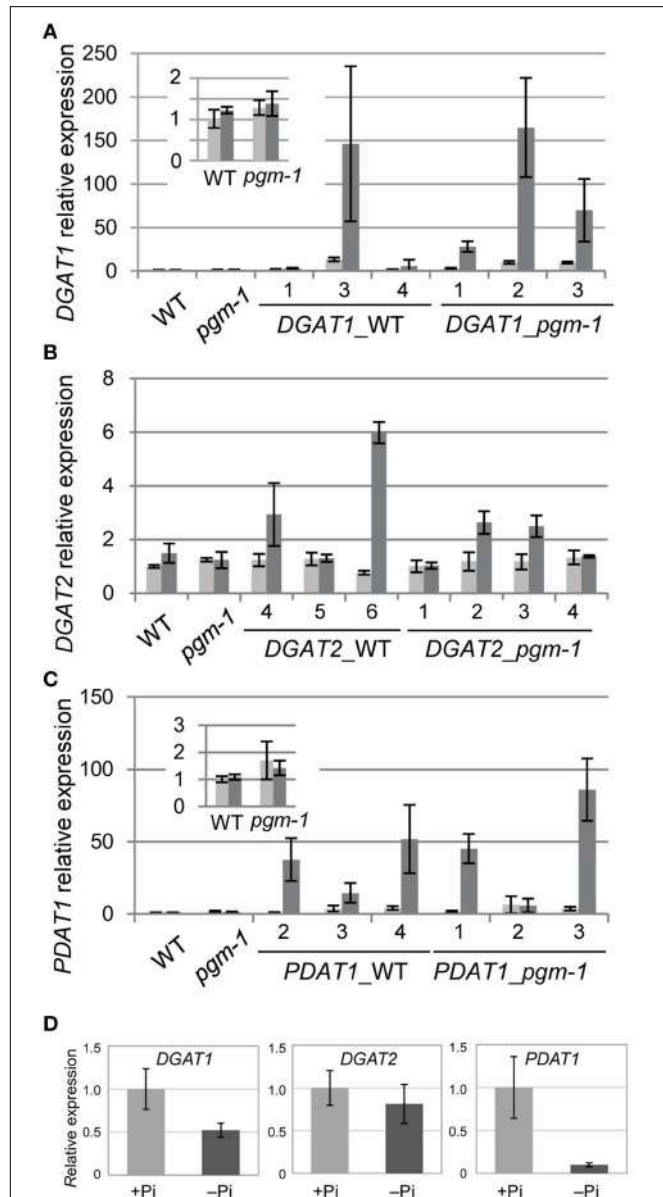


**FIGURE 3 | Continued**

(–Pi) or 1 mM (+Pi) Pi and were grown for 10 d. **(A)** Growth under Pi-sufficient (+Pi) or Pi-depleted (–Pi) conditions. Bars = 1.0 cm. **(B)** Shoot fresh weight of seedlings grown under Pi-sufficient and Pi-depleted conditions. **(C)** Oil droplets in leaf mesophyll cells under Pi-depleted conditions. Electron microscopy of leaf mesophyll cells (upper panels; white arrows, oil droplets; black arrows, chloroplasts; S, starch) and representative confocal fluorescence micrographs of leaves (lower panels) showing chloroplasts (red) and oil droplets stained with Nile red (green). Bars = 2  $\mu$ m in upper images and 10  $\mu$ m in lower images. **(D)** Electron microscopy of leaf mesophyll cells in WT and *pgm-1* plants grown under Pi-depleted conditions. White arrows indicate oil droplets. S, starch; M, mitochondrion; C, chloroplast. Bars = 0.5  $\mu$ m.

We showed a significant increase in *MGD3* expression levels in shoots and roots of WT plants upon Pi starvation (Awai et al., 2001; Kobayashi et al., 2004). To express TAG synthesis genes under Pi starvation, we produced transgenic *Arabidopsis* plants in WT and *pgm-1* backgrounds harboring the construct *ProMGD3:DGAT1* (designated as *DGAT1\_WT* and *DGAT1\_pgm-1*, respectively), *ProMGD3:DGAT2* (designated as *DGAT2\_WT* and *DGAT2\_pgm-1*, respectively), or *ProMGD3:PDAT1* (designated as *PDAT1\_WT* and *PDAT1\_pgm-1*, respectively). In shoots, compared with WT or *pgm-1* plants, *DGAT1* expression in *DGAT1\_WT* line 3 and *DGAT1\_pgm-1* line 2 were markedly higher under Pi-sufficient (~10- to 14-fold higher) and Pi-depleted (~140- to 170-fold higher) conditions (**Figure 4A**). In transgenic plants harboring *ProMGD3:DGAT2*, *DGAT2* expression in *DGAT2\_WT* line 6 was ~6-fold higher than that in WT and *pgm-1* plants under Pi-depleted conditions, whereas expression under Pi-sufficient conditions was similar to that of WT and *pgm-1* plants (**Figure 4B**). Among three lines of *DGAT2\_pgm-1* transgenic plants, *DGAT2* expression levels in lines 2 and 3 were only slightly higher than that in WT and *pgm-1* plants under Pi-depleted conditions (~2.5-fold higher; **Figure 4B**). *PDAT1* expression levels in *PDAT1\_WT* line 4 and *PDAT1\_pgm-1* line 3 were ~2.5-fold higher under Pi-sufficient conditions and markedly higher under Pi-depleted conditions (~50- and ~80-fold higher, respectively) compared with WT and *pgm-1* plants (**Figure 4C**).

Under both Pi conditions, growth phenotypes and shoot fresh weights of *DGAT1\_WT* line 3 and *DGAT1\_pgm-1* line 2 were similar to those of WT and *pgm-1* plants (**Figures 3A, 5A,D**). *DGAT2\_WT* line 6 and *DGAT2\_pgm-1* line 2 accumulated slightly less anthocyanin than did WT and *pgm-1* plants under both Pi conditions (**Figures 3A, 5B,D**). The shoot fresh weight of *DGAT2\_WT* line 6 grown under Pi-sufficient conditions was greater than those of WT and *pgm-1* plants, although under Pi-depleted conditions the fresh weight was similar to those of WT and *pgm-1* plants (**Figure 5D**). The growth phenotype of *PDAT1\_WT* line 4 was similar to that of *PDAT1\_pgm-1* line 3 under both Pi conditions but differed from those of WT and *pgm-1* plants (**Figures 3A, 5C**). Under Pi-depleted conditions, seedlings of both lines were yellowish and accumulated markedly less anthocyanin than did WT and *pgm-1* plants (**Figures 3A, 5C**). Moreover, the fresh weight of *PDAT1\_pgm-1* line 3 was

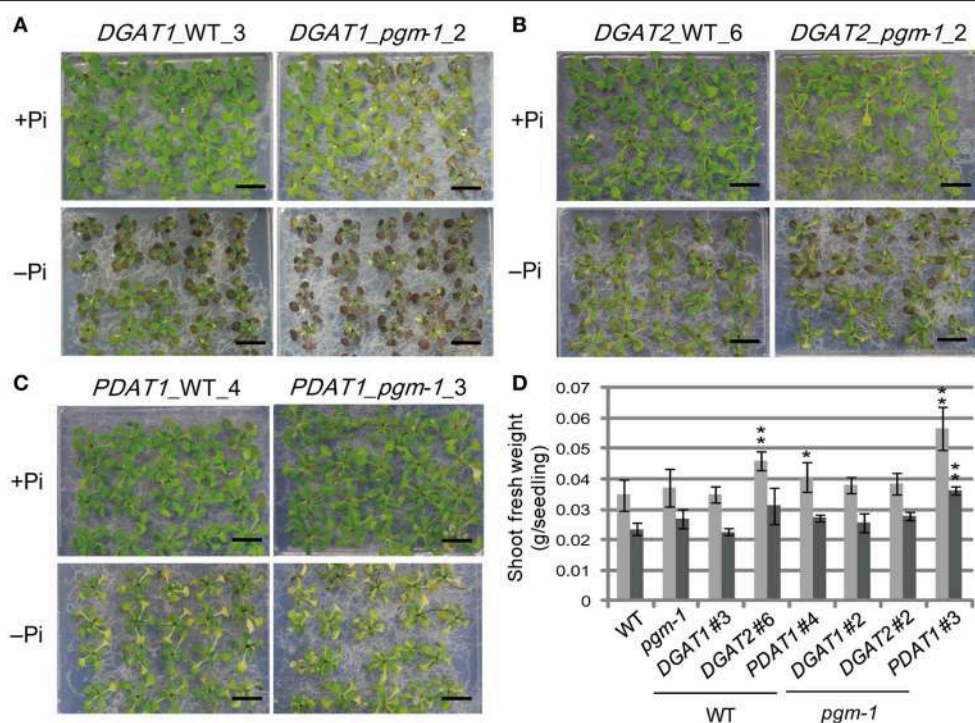


**FIGURE 4 | Quantitative reverse transcription-PCR analysis of *DGAT1*, *DGAT2*, and *PDAT1* expression in WT, *pgm-1*, and transgenic *Arabidopsis* plants under Pi-sufficient or Pi-depleted conditions.**

Seedlings (10 d old) of transgenic lines were transferred to MS medium containing 1% (w/v) sucrose, and 0 mM Pi (dark gray bars) or 1 mM Pi (light gray bars) and were grown for 10 d. **(A)** *DGAT1*, **(B)** *DGAT2*, and **(C)** *PDAT1* mRNA levels in shoots of WT, *pgm-1*, and transgenic lines in the WT and *pgm-1* backgrounds are shown. Line numbers are indicated for each transgenic strain. The expression level of each gene is relative to that in WT under Pi-sufficient conditions. Data are the mean  $\pm$  SD from three independent experiments. **(D)** mRNA levels of *DGAT1*, *DGAT2*, and *PDAT1* in roots of WT plants under Pi-sufficient (+Pi) and Pi-depleted (–Pi) conditions. The expression level of each gene is relative to that in WT under Pi-sufficient conditions. Data are the mean  $\pm$  SD from three independent experiments.

significantly greater than those of WT and *pgm-1* plants under both Pi conditions (**Figure 5D**). Taken together, the shoot fresh weights of all of the transgenic plants was similar to, or higher





**FIGURE 5 | Growth phenotypes and fresh weight of transgenic lines under Pi-sufficient and Pi-depleted conditions.** Seedlings (10 d old) of transgenic lines were transferred to MS agar containing 1% (w/v) sucrose and 0 mM (–Pi) or 1 mM (+Pi) Pi and were grown for 10 d. Growth phenotypes of (A) *DGAT1\_WT\_3* and *DGAT1\_pgm-1\_2*, (B) *DGAT2\_WT\_6*

and *DGAT2\_pgm-1\_2*, and (C) *PDAT1\_WT\_4* and *PDAT1\_pgm-1\_3*. Bars = 1 cm. (D) Shoot fresh weights of seedlings grown under Pi-sufficient (light gray) and Pi-depleted (dark gray) conditions. Data are the mean  $\pm$  SD from three independent experiments; \* $P < 0.05$  or \*\* $P < 0.01$  for t-test vs. WT under Pi-sufficient conditions.

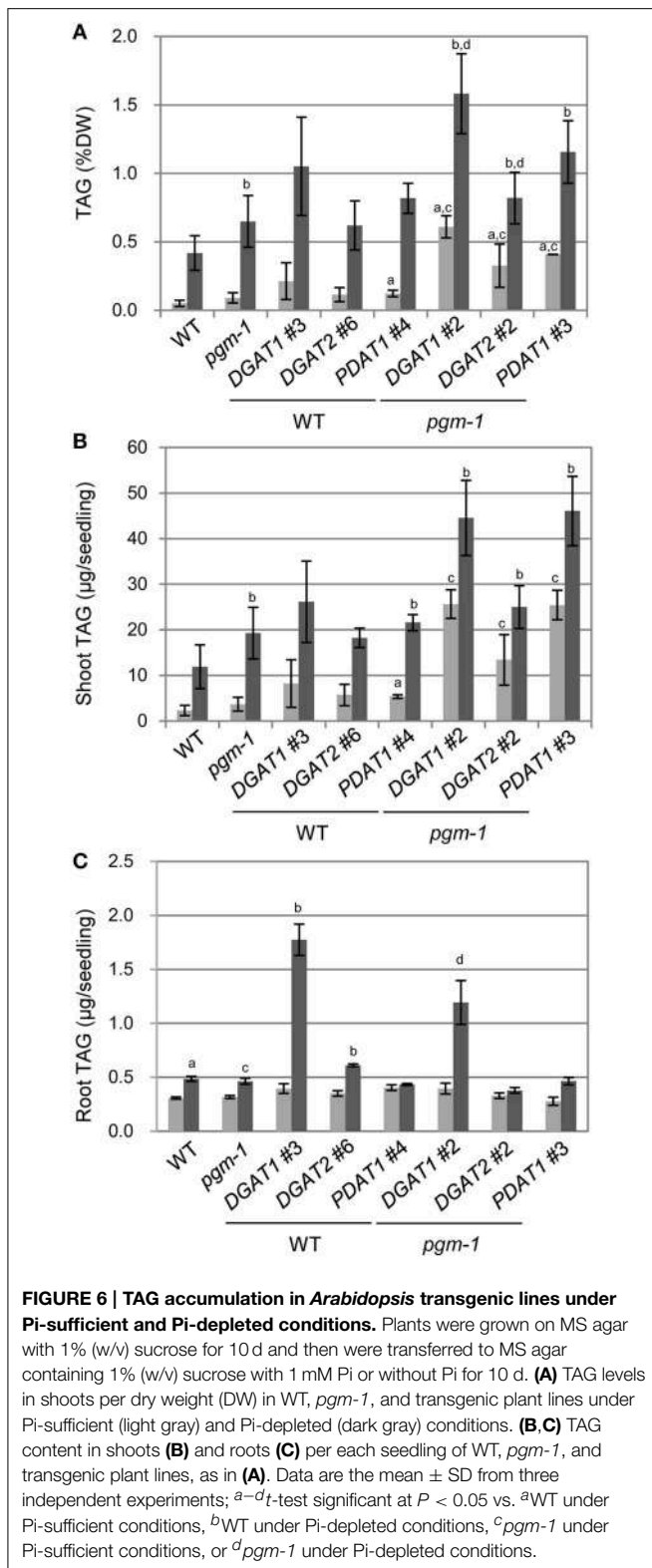
than, those of WT and *pgm-1* plants under both Pi conditions (Figure 5D).

### Pi Starvation–induced Overexpression of *DGAT1*, *DGAT2*, and *PDAT1* Enhances TAG Accumulation in Vegetative Tissues

We analyzed TAG levels in vegetative tissues of WT, *pgm-1*, and transgenic plants (Figure 6). Compared with WT plants under Pi-sufficient conditions, TAG levels in *pgm-1* under Pi-sufficient and Pi-depleted conditions were 1.8-fold and 13-fold higher, respectively (Figure 6A). Moreover, TAG levels in *DGAT1\_pgm-1* line 2, *DGAT2\_pgm-1* line 2, and *PDAT1\_pgm-1* line 3 transgenic lines under Pi-depleted conditions were 26-, 16-, and 23-fold higher, respectively, than WT under Pi-sufficient conditions (Figure 6A). Moreover, the TAG content in shoots per seedling of *pgm-1*, *DGAT1\_pgm-1* line 2, *DGAT2\_pgm-1* line 2, and *PDAT1\_pgm-1* line 3 transgenic lines under Pi-depleted conditions was 9.5-, 19-, 12-, and 23-fold higher, respectively, than that of WT seedlings under Pi-sufficient conditions, and the TAG content was 1.8-, 3.5-, 2.3-, and 4.3-fold higher, respectively, than that of WT seedlings under Pi-depleted conditions (Figure 6B).

We also measured the TAG content in roots per seedling in six transgenic lines under Pi-sufficient and Pi-depleted conditions (Figure 6C). The overexpression of these three genes had

different effects in roots than in shoots. First, the TAG content in roots per seedling of WT and *pgm-1* was comparable under both Pi conditions (Figure 6C). Second, *DGAT1* overexpression in WT and *pgm-1* plants was the most effective way to produce more TAGs under Pi-depleted conditions compared with *DGAT2*- or *PDAT1*-containing transgenic plants in the same background (Figure 6C). Under Pi-depleted conditions, the TAG content in *DGAT1\_WT* line 3 and *DGAT1\_pgm-1* line 2 increased to 5.8-fold and 4-fold of the levels in WT and *pgm-1*, respectively (Figure 6C). However, in the other transgenic plants, the TAG content was comparable with that in the non-transformed backgrounds, WT and *pgm-1* (Figure 6C). Thus, using the starchless mutant background during Pi starvation in combination with the overexpression of *DGAT1*, *DGAT2*, or *PDAT1* was the most efficient way to increase TAG levels in shoots (Figures 6A,B), but for roots, the use of the *pgm-1* background for transgenic plants, relative to WT, had a negative effect on increasing TAG levels. Although, the level of TAG in *DGAT1\_pgm-1* roots increased by 4-fold under Pi-depleted conditions compared with that in *pgm-1* under Pi-sufficient conditions, the TAG level in the roots of *pgm-1* and the other *pgm-1* background transgenic plants was similar to, or smaller than, that in WT and the other WT background transgenic plants (Figure 6C). Thus, our results clearly showed that the Pi starvation–induced overexpression of *DGAT1* or *PDAT1* in the



## Discussion

Plants increase TAG levels in vegetative tissues during senescence and under several stresses such as freezing, drought stress, or oxidative stress (Sakaki et al., 1990a,b,c; Kaup et al., 2002; Gaude et al., 2007; Moellering et al., 2010; Lippold et al., 2012; Troncoso-Ponce et al., 2013). Among the essential macronutrients for plants, N starvation is a well-known trigger for TAG synthesis in leaves (Gaude et al., 2007; Yang et al., 2011; Lippold et al., 2012). However, N starvation promotes senescence and degradation of chlorophyll, causing a severe reduction in photosynthetic activity, and thus results in smaller seedlings as compared with well-fertilized plants (Yang et al., 2011). We showed that Pi starvation alters TAG levels in seedlings and compared the effect with N starvation (Figures 1, 2). Although, N starvation also results in TAG accumulation, the biomass of plants grown under N-depleted conditions was markedly smaller than that under Pi-depleted conditions (Figure 1C). Moreover, chloroplast ultrastructure was not severely affected, during Pi deficiency except for an increase in the number of starch granules in the stroma (Supplementary Figure 1). Taken together, these results suggest that either N or Pi starvation results in TAG accumulation in leaves, but the damage to plants is relatively more severe during N starvation. Indeed, *Arabidopsis* WT plants under Pi-depleted conditions maintain ~85% of their photosynthetic activity as compared with plants grown under Pi-sufficient conditions (Kobayashi et al., 2009). These results also suggest that both types of nutrient deficiency result in TAG accumulation in leaves, but the pathways and the mechanisms of TAG synthesis might differ slightly.

Because, TAG levels were increased in both shoots and roots, we first thought that the TAG synthesis genes were up-regulated under Pi-depleted conditions. Unexpectedly, the expression levels of three major TAG synthesis genes, *DGAT1*, *DGAT2*, and *PDAT1*, were not significantly increased in WT shoots under Pi-depleted conditions (Figures 4A–C). Moreover, in WT roots, although expression levels of *DGAT2* remained unchanged under both Pi conditions, those of *DGAT1* and *PDAT1* under Pi-depleted conditions were significantly decreased compared with those under Pi-sufficient conditions (Figure 4D). These results suggested that enhanced TAG accumulation under Pi-depleted conditions was not correlated with the transcriptional up-regulation of TAG synthesis genes, as was also suggested by Pant et al. (2015), but could be due to the down-regulation of genes involved in TAG breakdown or homeostasis during Pi depletion (James et al., 2010; Kelly et al., 2013; Park et al., 2013). Currently, the molecular mechanisms behind Pi starvation-induced TAG accumulation are unclear but are under investigation.

We clearly showed that the Pi starvation-inducible promoter *ProMGD3* is a useful engineering tool for producing transgenic plants that accumulate TAG in shoots in response to Pi starvation; however, it was only partially successful in roots. As the roots of *DGAT1*-WT accumulated significant levels of TAG in response to Pi starvation (Figure 6C), the reason for unchanged TAG levels in *DGAT2*- or *PDAT1*-containing transgenic plants might be due to the decreased availability of the preferred substrates in roots compared with shoots under

*pgm-1* mutant background is the most efficient way to increase TAG levels in shoots, but *DGAT1* overexpression in WT is effective for enhancing TAG accumulation in roots.

Pi-depleted conditions. Indeed, the substrate preferences of *Arabidopsis* *DGAT1* and *DGAT2* were reported to be different (Zhou et al., 2013; Ayme et al., 2014). As for the fatty acid composition of TAGs in roots of WT, *pgm-1*, and transgenic plants under Pi-depleted conditions, both *DGAT1*-WT and *DGAT1*-*pgm-1* showed notable increases in the 18:1 ratio and decreases in the 18:3 ratio compared with those in the other plants (Supplementary Figure 3). However, for the fatty acid composition of TAGs in shoots, an increase in the 18:1 ratio and a decrease in the 18:3 ratio were observed only in *DGAT1*-containing transgenic plants under Pi-depleted conditions (Supplementary Figure 4). The fatty acid composition of TAGs in *DGAT1*-containing transgenic plants was in agreement with the substrate preference, 18:1 over 18:3, of *DGAT1* (Zhou et al., 2013). Thus, these transgenic plants might also be useful for further analyzing the differences in TAG accumulation mechanisms and the availability of substrates in shoots and roots under Pi starvation.

In this study, we enhanced TAG accumulation in vegetative tissues using the promoter of a plastid-localized galactolipid synthase gene in combination with the lipid remodeling system under Pi starvation, but the amount of TAG was still low compared with the levels seen in previous studies. The highest TAG levels in shoot of our transgenic plants was ~1.5 % D.W. as shown in Figure 6A. Fan et al. (2013) showed that coexpression of *PDAT1* with oleosin in wild-type background and *tgdl-1* mutant background boost leaf TAG content by up to 6.4–8.6% of the dry weight, respectively. Kelly et al. (2013) showed that in transgenic plants constitutively coexpressing *WRINKLED1* and *DGAT1* in *sdp1* mutant background, the accumulation of TAG in roots, stems, and leaves was elevated to levels ranging from 5 to 8% of dry weight. Thus, all the other works were performed by engineering multiple genes involved in TAG accumulation or degradation such as co-overexpression of oleosin or knock-out of *SDP1*. Given that we only introduced single gene into the transgenic plants, we still have chance to elevate TAG levels in leaves by engineering multiple genes using our system. Moreover, we are currently investigating whether this system can be applied to crop plants, and we are determining the best soil growth conditions for enhanced TAG accumulation without extreme plant growth retardation. Although, we showed its ability to produce TAG only in vegetative tissues, this system can be applied to producing other useful industrial compounds by introducing the corresponding synthesis genes in place of the TAG synthesis genes. Thus, we hope that the system will be used in many industrial applications in the near future.

## Materials and Methods

### Plant Material and Growth Conditions

Seeds of *pgm-1* were obtained from the Arabidopsis Biological Resource Center. Surface-sterilized seeds of WT *A. thaliana* (Columbia-0), the starchless mutant *pgm-1*, and transgenic mutant lines were incubated at 4°C in darkness for 3 d prior to plating on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 0.8% (w/v) agar (MS agar) supplemented with 1% (w/v) sucrose and were then incubated

at 23°C under continuous white light (40–50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for all growth conditions. *Arabidopsis*, tomato (*S. lycopersicum* L.), barnyard grass (*E. crus-galli*), and tobacco (*N. tabacum*) seeds were grown on solidified MS agar supplemented with 1% (w/v) sucrose for 8 d (tomato, barnyard grass) or 10 d (*Arabidopsis* and tobacco) and then were grown for another 10 d (*Arabidopsis*), 21 d (tobacco), or 28 d (tomato and barnyard grass) on solidified Pi-sufficient (1 mM Pi) or Pi-depleted (0 mM Pi) medium (Härtel et al., 2000) supplemented with 1% (w/v) sucrose, or for 7 d on solidified N-sufficient (3.5 mM N) or N-depleted (0 mM N) medium supplemented with 1% (w/v) sucrose, with  $\text{KNO}_3$  and  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  replaced with  $\text{KCl}_2$  and  $\text{CaCl}_2$ , respectively.

### Electron Microscopy

Leaf segments were fixed with 2% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde in 0.067 M sodium phosphate buffer (pH 7.4) for 2 h at room temperature and then for 16 h at 4°C. Samples were then washed six times in the sodium phosphate buffer for 10 min each at room temperature. They were post-fixed with 2% (w/v) osmium tetroxide in 0.067 M sodium phosphate (pH 7.4) for 2 h at room temperature. The fixed samples were dehydrated in a graded ethanol series and embedded in epoxy resin mixture (Quetol 651 mixture; Nissin EM). Ultrathin 70-nm sections were cut with a diamond knife on a Leica Ultracut UCT ultramicrotome and were transferred onto copper grids. The sections were stained with 2% (w/v) uranyl acetate for 15 min followed by 0.4% (w/v) lead citrate for 5 min at room temperature. The specimens were observed on a Hitachi H-7500 transmission electron microscope at an accelerating voltage of 80 kV.

### Lipid Analysis

Total lipid was extracted from tissues as described by Bligh and Dyer (1959). The polar membrane lipids were separated by two-dimensional thin-layer chromatography (Kobayashi et al., 2007). TAGs were separated by one-dimensional thin-layer chromatography using the solvent system of hexane/diethyl ether/acetic acid (160:40:4, v/v/v). Lipids on silica gel plates were visualized with 0.01% (w/v) primuline in 80% (v/v) acetone under UV light. Lipids isolated from silica gel plates were methylated, and fatty acid methyl esters were quantified by gas chromatography using pentadecanoic acid as an internal standard (Kobayashi et al., 2006).

### Imaging Lipid Droplets *In situ*

Leaves were vacuum-fixed in 4% (w/v) paraformaldehyde in 50 mM PIPES buffer (pH 7.0) and stained with 50  $\mu\text{g ml}^{-1}$  Nile red (Sigma) to selectively visualize lipid droplets *in situ* (Greenspan et al., 1985). The Nile Red signal and chlorophyll autofluorescence were observed using a confocal laser-scanning microscope (TCS SE; Leica) with an argon laser for excitation at 488 nm, a 556- to 580-nm filter for detection of the Nile Red signal, and a 718- to 749-nm filter for detection of chlorophyll fluorescence. Images were merged and pseudocolored using Leica confocal software.



## Generation and Selection of Transgenic Plants

To obtain transgenic plants, the complete coding sequences of *DGAT1*, *DGAT2*, and *PDAT1* from *A. thaliana* were amplified from the reverse transcript of WT leaf total RNA. The primers used were *DGAT1\_FW* (5' CGCCCGGGTATGGCGA TTTTGGATTCTGCTGGC 3'), *DGAT1\_RV* (5' GCGAGC TCTCATGACATCGATCCTTTTCGGTTC 3'); *DGAT2\_FW* (5' GCCCGGGTATGGGTGGTTCAGAGAGTTCCGAG 3'), *DGAT2\_RV* (5' GCGAGCTCTCAAAGAATTTTCAGCTC AAGATC 3'); and *PDAT1\_FW* (5' CGCCCGGGTATGCCCC TATCATCGGAAAAAG 3'), *PDAT1\_RV* (5' GCGAGCTCTCAC AGCTTCAGGTCAATACGCTC 3'). Each amplified fragment was cloned into the pZER cloning vector (Life Technologies). To remove the *Sac* I site in the *DGAT1* coding sequence, the obtained vector was subjected to a Quikchange Lightning reaction (Qiagen) using the following primers: *DGAT1\_c845t\_fw* (5' GTCTCCTACTACGTTAGCTTGAAGAGCTTGGCATATTTTC 3') and *DGAT1\_c845t\_rv* (5' GAAATATGCCAAGCT CTTCAAGCTAACGTAGTAGGAGAC3'). Vectors were subjected to restriction analysis and DNA sequencing to confirm the presence of the expected sequences. Each *DGAT1*, *DGAT2*, and *PDAT1* fragment was digested with *Sma* I and *Sac* I and independently ligated into the *Sma* I and *Sac* I sites of plasmid *atMGD3::GUS/pBI101* (Kobayashi et al., 2004). All of the *Arabidopsis* transformants described here were produced using a modified version of the floral dip method (Clough and Bent, 1998) and were selected on MS agar containing 50 µg ml<sup>-1</sup> kanamycin.

## Quantitative Reverse Transcription-PCR

Total RNA was isolated from three independent plant samples using the SV Total RNA Isolation System (Promega). Reverse transcription was performed using the PrimeScript RT reagent kit (TaKaRa Bio). cDNA amplification was carried out using SYBR PreMix Ex Taq (TaKaRa Bio). Signal detection and quantification were performed in duplicate using the Thermal Cycler Dice Real Time System (TaKaRa Bio). Quantitative PCR determination of *DGAT1*, *DGAT2*, and *PDAT1* transcripts was carried out using the *Arabidopsis* *UBQ10* transcript levels for normalization (Narise et al., 2010). Expression levels were obtained from at least three replicates. The gene-specific primers used were as follows: *DGAT1\_fw* (5' GAGAGAG

AGTCCACTTAGCTC 3'), *DGAT1\_rv* (5' CGTTCTGATCAAC CAACCATAC 3'); *DGAT2\_fw* (5' TCCAGCCTAATCG TGCCTATG 3'), *DGAT2\_rv* (5' GGGAGTGTAGAATATAGC ACTAC 3'); *PDAT1\_fw* (5' AGGCAAACAATGCGCTGATGG 3'), *PDAT1\_rv* (5' TGTCAAGTGACATGTGTTCCAC 3'); *UBQ10\_fw* (5' GGCCTTGATAATCCCTGATGAATAAG 3'), *UBQ10\_rv* (5' AAAGAGATAACAGGAACGGAAACA TAGT 3').

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00664>

**Supplementary Figure 1 | Electron microscopy of leaf mesophyll cells in WT *Arabidopsis* plants.** Plants were grown on MS agar with 1% (w/v) sucrose for 10 d and then were transferred to MS agar containing 1% (w/v) sucrose without (0 mM) Pi for 10 d. White arrows indicate oil droplets. S, starch; M, mitochondrion. Bars = (A) 10 µm and (B) 0.5 µm.

**Supplementary Figure 2 | TAG levels in various plants under Pi-depleted conditions.** (A,B) TAG levels in tomato (*S. lycopersicum* L.) shoots (A) and barnyard grass (*E. crus-galli*) (B) grown for 13 d with Pi and then for 31 d with (+) or without (−) Pi. For all conditions: +Pi, 1 mM; −Pi, 0 mM. (C,D) Fatty acid profiles of TAGs in shoots of tomato (C) and barnyard grass (D). Data are the mean ± SD from three independent experiments.

**Supplementary Figure 3 | Fatty acid composition of TAG in roots of WT, *pgm-1*, and transgenic plant lines grown under Pi-sufficient and Pi-depleted conditions.** Plants were grown on MS agar with 1% (w/v) sucrose for 10 d and then were transferred to MS agar containing 1% (w/v) sucrose with (A) or without (B) Pi for 10 d. Data are the mean ± SD from three independent experiments.

**Supplementary Figure 4 | Fatty acid composition of TAG in shoots of WT, *pgm-1*, and transgenic plant lines.** Plants were grown under Pi-sufficient (gray) and Pi-depleted (black) conditions in all of the figures, except the WT (N) figure, in which plants were grown under N-sufficient (gray) and N-depleted (black) conditions. Data are the mean ± SD from three independent experiments.

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# Plastids: The Green Frontiers for Vaccine Production

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Infectious diseases pose an increasing risk to health, especially in developing countries. Vaccines are available to either cure or prevent many of these diseases. However, there are certain limitations related to these vaccines, mainly the costs, which make these vaccines mostly unaffordable for people in resource poor countries. These costs are mainly related to production and purification of the products manufactured from fermenter-based systems. Plastid biotechnology has become an attractive platform to produce biopharmaceuticals in large amounts and cost-effectively. This is mainly due to high copy number of plastids DNA in mature chloroplasts, a characteristic particularly important for vaccine production in large amounts. An additional advantage lies in the maternal inheritance of plastids in most plant species, which addresses the regulatory concerns related to transgenic plants. These and many other aspects of plastids will be discussed in the present review, especially those that particularly make these green biofactories an attractive platform for vaccine production. A summary of recent vaccine antigens against different human diseases expressed in plastids will also be presented.

**Keywords:** molecular farming, biopharmaceuticals, plant-based vaccines, plastids, infectious diseases, developing countries, cost-effective vaccines

## INTRODUCTION

Increase in the rate of infectious diseases is an escalating problem in both developed and developing world. There are number of factors that play role in the incidence rate of infectious diseases. Among these, main factors are global warming, lack of healthcare facilities, and costly preventive measures or treatments. With increase in global warming, threat of infectious diseases is also rising. Data from last 10 years show increase in the incidence of diseases and projections also predict more rise in future (Altizer et al., 2013). In developed countries, enhanced rate of infectious diseases may possibly be prevented by the availability of good healthcare facilities, cleaner resources and clean environment. However, in developing countries, where 2.2 billion people lived on less than US \$2 a day in 2011 (World Bank, 2015), spread of infectious agents could be faster due to the reason that a large population is not able to afford costs related to treatments of these diseases. In addition, poor sanitation, malnutrition, use of unclean water and lack of precautionary measures at government levels are additional major contributors to the increase in the risks of infectious diseases.

Considering above mentioned factors, there are number of levels at which disease spread could be controlled. Two major levels include prevention and cure. Many diseases can be stopped more effectively by taking preventive measures. However, for eradicating existing infections and to control massive outbreaks of some infectious agents, cure is preferable. Keeping in view the scenario

of developing countries, it is particularly important that these preventive/treatment strategies should be affordable and cost-effective. Use of vaccines can be an effective strategy that can be either used as prophylactic (before the onset of disease) or therapeutic (after the onset of disease). There are number of platforms that are used for antigen-based vaccine production, mainly mammalian cell culture based and fermentation-based systems. However, many concerns are related to the vaccines that are in market. Most important of these are cost, stability, safety, and efficacy. Hence, alternate strategies needs to be opted to cover the shortcomings of vaccines in use.

## WHY CHLOROPLASTS?

There are various advantages of plant-based expression systems that are generally related to plants and some relevant to chloroplast-based expression only. In general, in relation to cost, a major advantage is at production level, i.e., the up scaling of plants to as much area as needed. If land is available, large number of plants can be grown by using inexpensive resources. This is the major level where cost is reduced in comparison to fermenters or bioreactors where establishing and running of a total new setup for up scaling is very costly. There are certain other advantages that are particularly related to chloroplast-based expression of vaccine antigens. Chloroplasts are organelles of plants present in leaves and other green parts that carry out photosynthesis. In recent biotechnological innovative era, chloroplasts have been explored for the expression of foreign proteins, industrially and pharmaceutically important compounds such as antibodies, growth factors, enzymes, hormones, cytokines, and antigens (Daniell et al., 2009; Lössl and Waheed, 2011). Previously, various aspects of plant/chloroplast-based pharmaceutical compounds have been extensively reviewed (Bock, 2014; Rybicki, 2014; Abiri et al., 2015; Chan and Daniell, 2015; Fahad et al., 2015; Salazar-González et al., 2015). Here we give an outline of chloroplast transformation and certain characteristics of plastids that are particularly important for the production of antigen-based vaccines against human diseases at low costs. We will review different aspects of chloroplasts for vaccine production particularly relevant to cost, stability, safety, and efficacy.

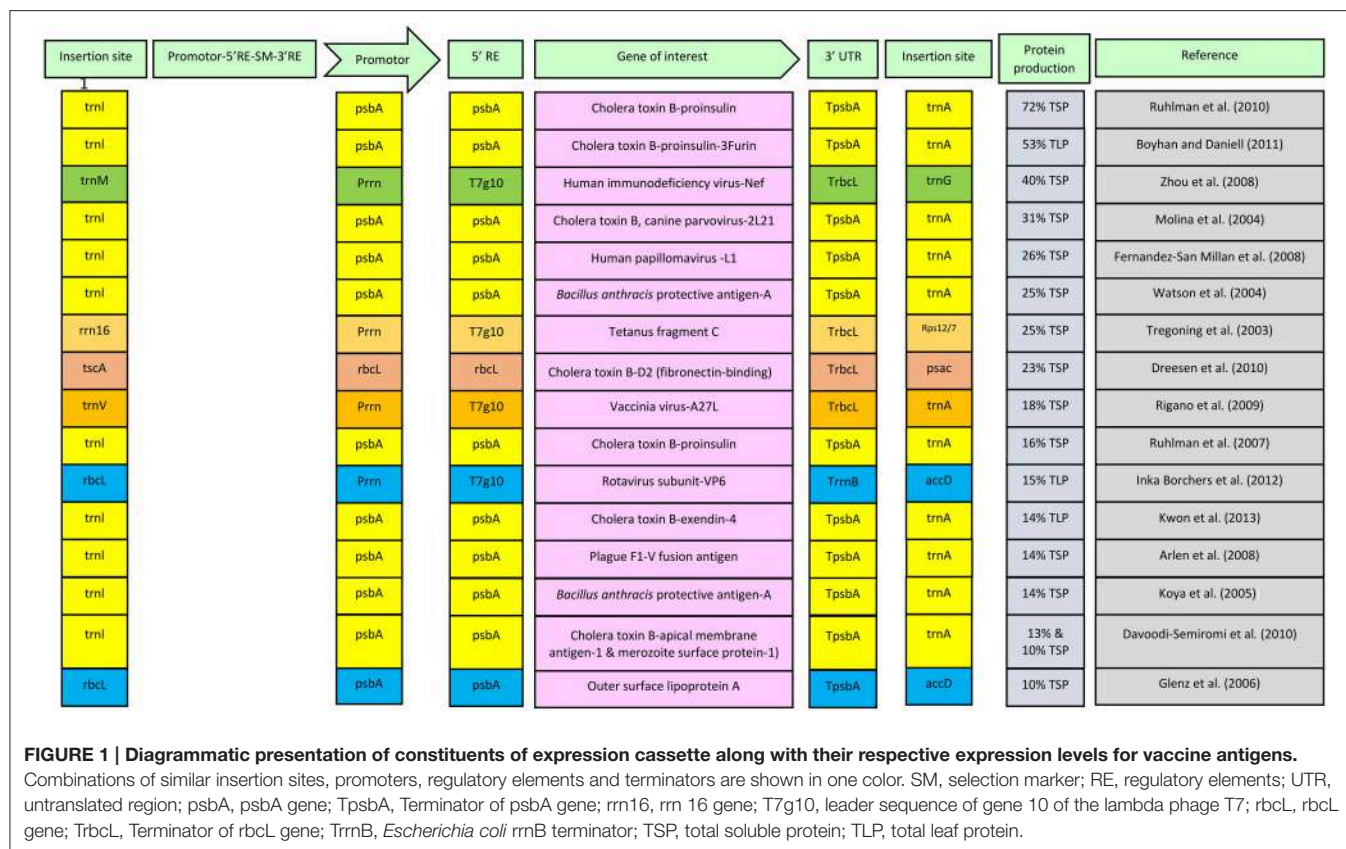
## PLASTID TRANSFORMATION

There are mainly two methods that are used for plastid genome transformation: polyethylene glycol (PEG)-mediated transformation and gene gun-mediated transformation. First method is inexpensive and involves the isolation of protoplasts that are later transformed in the presence of PEG. However, the protoplast isolation is tricky and the protocols for protoplast isolation and regeneration are not optimized for many edible plant species. Transformation via particle delivery system (PDS) is expensive, both in the expenses of biolistic gun (although it is one time cost) and gold particles which are mostly used for the delivery of foreign DNA into plastomes. Despite, biolistic delivery is the most widely used method for chloroplast transformation and protocols are very well established (Verma et al., 2008; Abdel-Ghany et al., 2015). Different strategies have

been adopted over time to enhance the foreign protein expression in chloroplasts attaining a very high amount of 72% of total leaf protein (TLP) from tobacco leaves (Ruhlman et al., 2010). These strategies include use of 5' and 3' untranslated regions or regulatory elements, use of active promoter, N-terminal fusion of a stabilizing peptide sequence and insertion site. Although there are very few reports showing very high expression of proteins in plastids, the expression levels in most studies of plastid transformation cross minimum level required for a feasible large scale commercial production, i.e., 1% of total soluble protein (TSP) or 50 µg/g fresh leaf tissue (Rybicki, 2009, 2010).

Integration of expression cassette in plastid genome takes place via homologous recombination. Flanking sequences used for insertion on right and left sides are amplified from the species which is to be transformed and an expression cassette is constructed through various cloning steps (Verma et al., 2008; Bock, 2015). Choosing the insertion site for integration of expression cassette within plastomes is one of the important parameters for enhanced expression of transgenes. Different insertion sites can lead to different levels of expression. Two important parameters that should be kept in mind while choosing the insertion sites are their location in actively transcribed region and within the inverted repeat region of plastid genome (Verma et al., 2008). Insertion site can also have some negative effects on plants. Hence, to ensure safety of plant and enhanced expression of a vaccine antigen in plastomes, care should be taken in choosing the insertion site. Different insertion sites along with respective expression levels are shown in **Figure 1**. Since the flanking sequences are amplified from the plastome of the target species, a resulting species-specific vector is likely to express more in specific species for which it is designed. It is known that certain level of sequence homology exists between chloroplast genomes of different plant species (Bisaro and Siegel, 1980). On this basis, concept of universal vector was presented and it was proposed that a universal vector, having flanking sequences that are conserved in most plant species, can be constructed and utilized for transforming the plastomes of many related plant species (Verma and Daniell, 2007). However, using a universal vector may result in low expression. Hence to achieve high expression, a species-specific vector should be the choice. Nevertheless, it may be a technical challenge to develop species-specific vector for each particular species to be transformed because of lack of chloroplast genome sequence. A typical expression cassette of a transformation vector that is inserted in plastomes is shown in **Figure 1**. In this figure different components of expression cassette along with their respective yields of foreign proteins are also given. Data is given for those reports where the expression level is up to 10% or above of total soluble protein (TSP) or total leaf protein (TLP). Figure shows that highest expression for a vaccine antigen, which is 72% of TLP (Ruhlman et al., 2010), was achieved by using insertion sites *trnI* and *trnA* under the control of *psbA* promoter with 5' regulatory elements from *psbA* gene. Most reports in the figure showing high expression of transgenes in plastids use this cassette containing *trnI* and *trnA* as insertion sites, promoter, 5' regulatory elements and terminator from *psbA* gene. Hence it can be concluded that this expression cassette





can serve as standard for attaining a high level expression in chloroplasts.

After transformation, several rounds of selection and regeneration are required on selection medium containing appropriate antibiotic to regenerate the homoplasmic transplastomic plants (Verma et al., 2008; Ahmad and Mukhtar, 2013). Homoplasmy refers to a state of plant when all chloroplasts are transformed and no wild type untransformed copy of plastid genome is left. However, to achieve homoplasmy, 1–2 extra regeneration cycles on selection medium are necessary. In comparison to mature leaves, very young leaves with immature chloroplasts have low plastome copy number. If these leaves are used for transformation, homoplasmy can be more quickly attained during regeneration phase. Normally, revised medium for organogenesis of plants (RMOP) is used, supplemented with appropriate concentration of hormones to promote callogenesis and shooting. For selection, antibiotic is added in the medium. Only those plants regenerate on selection medium which contain antibiotic resistant gene inside, i.e., transformed. Complete transplastomic plants are regenerated under aseptic controlled conditions and acclimatized to green house for further growth.

## STABLE GENETIC RESOURCE

Once a complete transplastomic plant is regenerated via tissue culture, seeds are collected for growing next generation. These seeds can now serve as a stable genetic resource, an important

outcome of stable genetic transformation. The costs involved in developing such resource are only one time, whether the developmental experimental procedures are carried out in academic research laboratories or at industrial scale. Currently, most of the research work regarding developing initial platforms and experimentations to develop techniques for enhancing the expression levels of foreign proteins in plants is carried out in academic research labs. Stable genetic resource in the form of seeds can be preserved and grown at any place where the vaccine production is required, exploiting a significant advantage of plants, i.e., “grown at site.” This aspect has potential to circumvent the costs related to transportations and cooling chain in those cases when vaccines are produced elsewhere and need to be transported to the target areas. Hence, it will ultimately reduce the cost of final product in the market.

## POLYPLOIDY AND VERY HIGH EXPRESSION OF FOREIGN PROTEINS

An average tobacco leaf contains almost 100 chloroplasts per cell and in each chloroplast there are approximately 100 chloroplast genomes. In total, this makes 10,000 chloroplast genomes in one cell (Maliga, 2002; Koop et al., 2007). Theoretically, if all chloroplasts are transformed in each and every cell of every leaf in a plant, this makes very high expression of a foreign protein possible. In reality, a very high expression has been achieved, reaching up to 72% of total leaf protein (TSP) and 70% of total

soluble protein (TSP), reported by Ruhlman et al. (2010) and Oey et al. (2009), respectively. For vaccine production at a cost-effective rate, this very high expression can play a key role. Higher the expression lesser will be the cost of final product because more product will be produced from less resources. The potential oral delivery of plant-based vaccines, which if possible in reality, will also greatly reduce the costs due to elimination of costly downstream processing. However, this does not seem to be a reality at the present stage due to number of limitations (for detailed discussion on this topic see Rybicki, 2009). In contrast, if the purification of protein has to be done then cost reduction is relevant only at production level, which is estimated to be about 31% (Rybicki, 2009). In such case, using the chloroplast-based expression and homoplasmy in which all plastomes are in transformed state, can lead to very high expression. This feature is expected to further reduce costs at production level because more protein will be produced per kilogram weight of plant. However, this also depends upon a number of other factors such as use of plants with wide leaves and having high biomass. In contrast to this characteristic of plastids, nuclear transformation, where mostly 1–2 copies of transgenes are mostly inserted in the nuclear genome, results in low yield (an average of 0.01–0.4% of TSP; Daniell et al., 2001a). Thus, for vaccine production, a high protein yielding platform such as chloroplasts needs to be opted. Various antigen-based vaccine candidates have been expressed in chloroplasts against number of human diseases. A detailed list has been previously published by our group (Lössl and Waheed, 2011). Reports published onwards are summarized in **Table 1**.

## ABSENCE OF EPIGENETIC EFFECTS

Plastids are double membrane organelles that originated from prokaryotic symbionts and largely retained their characteristics of prokaryotes. Presence of double membrane enables the compartmentalized production of foreign proteins, thus retaining the proteins inside. Due to this reason, there are less chances of affecting the plant physiology by interfering with cellular metabolic pathways of plants. Related to their prokaryotic nature, an important fact is the absence of gene silencing and other epigenetic effects in plastids. Till date, there is no report of these effects taking place in plastids. This characteristic of the green organelles ensures the stable and continued expression of transgenes in chloroplasts. In contrast, nuclear genome is susceptible to epigenetic effects which affect the yield of foreign proteins.

## EXPRESSION OF MULTIGENES AS SINGLE OPERON

Vaccines are often accompanied with adjuvants that boost the effect of a given antigen (Guy, 2007). Both chemical-based and biological adjuvants can be used for this purpose. *Escherichia coli* heat-labile enterotoxin subunit B (LTB) and cholera toxin subunit B (CTB) are two biological adjuvants that can be given with vaccine antigens to enhance their immunogenicity. Here, another advantage of plastids can play a role for production of

vaccines coupled with biological adjuvants. Due to prokaryotic nature of plastids, multiple genes can be stacked as a single operon and co-expressed (Lössl and Waheed, 2011). Two or more genetic sequences can be stacked one by one and expressed under a single promoter. Utilizing this characteristic, coupled expression of a biological adjuvant with an antigen in chloroplasts can be achieved. In this way, costs related to separate production of adjuvants can be eliminated. In addition, direct coupling is believed to enhance the immunogenicity of antigens more than their separate administration (Guy, 2007; Sánchez and Holmgren, 2008) and it has been found that due to coupling of antigen with CTB, much strong response is achieved upon oral administration (Guo et al., 2012). This characteristic of plastids to express many coding sequences as single operon can also be utilized to develop bivalent to multivalent vaccines, in which two or more vaccine antigens against different diseases can be co-expressed. Development of multivalent vaccines, used to cure multiple infections with one vaccine formulation can be particularly important in case of patients with acquired immunodeficiency syndrome (AIDS) where multiple diseases may need to be cured at one time. This advantage of plastids is also applicable for those vaccines that need more than one epitope for their function.

Another advantage of using biological adjuvants and their direct coupling is potential safety and efficacy. Use of biological adjuvants will help to eliminate toxic chemical adjuvants such as aluminum hydroxide and aluminum phosphate which commonly cause many adverse side effects such as local irritation and carcinogenesis (Gupta and Siber, 1995; Sun et al., 2006). Thus, use of biological adjuvants addresses the safety concern. Additionally, direct coupling of adjuvants is advantageous in terms of efficacy of antigen. Direct coupling not only can enhance the immunogenicity of an antigen but also the adjuvanticity is presumed to be more pronounced in an adjuvant-antigen couple. Furthermore, if an adjuvant such as LTB is directly linked with an antigen, it can facilitate the entry of antigen through gut mucosa, bind to GM1-ganglioside receptors and aid in eliciting the protective immunity (Granell et al., 2010; Salyaev et al., 2010).

## SAFETY OF PLANTS/CHLOROPLAST-DERIVED VACCINES

Safety concerns arise at two levels related to plant-based vaccines: the environment safety and safety of final product for patients. To successfully launch these vaccines into market, both these concerns are essential to address in order to get the approval from competent authorities.

## MATERNAL INHERITANCE OF PLASTID GENOME

A major concern related to regulatory approval of plant-based pharmaceuticals is environment safety. Environment can be potentially contaminated by unwanted flow of transgenes, especially antibiotic resistant marker genes, to the wild species

**TABLE 1 | Different vaccine antigens against human diseases expressed via plastid genome since 2011.**

Vaccine Antigen (Disease)	Expression system	Maximum expression level	Immunological investigation	References
<b>VIRAL ANTIGENS</b>				
Dengue epitope region of E protein of DENV domain I and II (Dengue fever)	Lettuce	Not reported	Cross reaction of antibodies from the sera of dengue patients	Maldaner et al., 2013
Dengue-3 serotype capsid complete premembrane (prM) and truncated envelope (E) protein prM/E (Dengue fever)	Lettuce	Not reported	Not tested	Kanagaraj et al., 2011
Mutated human papillomavirus (HPV)-16 oncoprotein E7 (cervical cancer)	<i>Chlamydomonas reinhardtii</i>	Not reported	Vaccination in mice with algae extracts showed high level of E7-specific antibodies but low activation of E7-specific CD8+ cells	Vlasák et al., 2013
Mutated, attenuated E7 oncoprotein (E7GGG), alone or as a fusion with affinity tags (His6 or FLAG) (cervical cancer)	<i>Chlamydomonas reinhardtii</i>	0.12% of total soluble protein (TSP)	Induction of specific anti-E7 IgGs and E7-specific T-cell proliferation detected in C57BL/6 mice vaccinated with total <i>Chlamydomonas</i> extract and with affinity-purified protein	Demurtas et al., 2013
E7 translationally fused with $\beta$ -glucuronidase	Tobacco	GUS-E7 showed expression between 30 and 40 times higher than previously reported for unfused E7 (0.1% of TSP)	Not tested	Morgenfeld et al., 2014
Modified HPV-16 L1 gene fused with glutathione-S-transferase (GST) GST-L1_2xCysM (cervical cancer)	Tobacco	Not detected	Not tested	Hassan et al., 2014
Synthetic gene encoding a C4V3 recombinant protein (HIV)	Tobacco	~25 $\mu$ g/g of fresh weight	Plant-derived C4V3 has elicited both systemic and mucosal antibody responses in BALB/c mice, as well as CD4+ T cell proliferation responses	Rubio-Infante et al., 2012
Multi-epitopic protein (Multi-HIV) carrying several neutralizing epitopes from both gp120 and gp41 (AIDS)	Tobacco	Protein accumulation levels up to 16 $\mu$ g/g of fresh tobacco biomass	Multi-HIV protein was able to elicit humoral responses in mice when orally administered	Rosales-Mendoza et al., 2014
HIV-1 capsid protein p24 alone and in fusion with the negative regulatory protein Nef (p24-Nef) (AIDS)	Tobacco	P24 up to ~4% and p24-Nef up to ~40% of TSP	Subcutaneous immunization with purified chloroplast-derived p24 elicited a strong antigen-specific serum IgG response. Oral administration of a partially purified chloroplast-derived p24-Nef fusion protein, used as a booster after subcutaneous injection with either p24 or Nef, also elicited strong antigen-specific serum IgG responses	McCabe et al., 2008; Zhou et al., 2008; Gonzalez-Rabade et al., 2011
Rotavirus VP6 gene (gastroenteritis)	Tobacco	> 15% of total leaf protein (TLP)	Not tested	Inka Borchers et al., 2012
<b>BACTERIAL ANTIGENS</b>				
Cholera toxin subunit B (CTB) fused with acid alpha glucosidase (GAA) CTB-GAA (Cholera, Pompe disease)	Tobacco	Between 0.13 and 0.21% of TLP	CTB-GAA fusion protein significantly suppressed immunoglobulin formation against GAA in Pompe mice	Su et al., 2015a
CTB fused with <i>Mycobacterium tuberculosis</i> antigens ESAT-6 and Mtb72F (a fusion polyprotein from two TB antigens, Mtb32 and Mtb39) (Cholera, TB)	Tobacco Lettuce	Maximum expression was 7.5% of TSP in mature tobacco leaves for CTB-ESAT-6	Hemolysis assay with purified CTB-ESAT6 protein showed partial hemolysis of red blood cells confirming the functionality of ESAT-6	Lakshmi et al., 2013

(Continued)

TABLE 1 | Continued

Vaccine Antigen (Disease)	Expression system	Maximum expression level	Immunological investigation	References
Major membrane protein I (mmpI) from <i>Mycobacterium leprae</i> fused with LTB (TB)	Tobacco	Not reported	Not tested	Hassan et al., 2013
EspA and Tir/Intimin antigens from enterohemorrhagic <i>E. coli</i> O157:H7 (hemorrhagic colitis)	Tobacco	Up to 1.4% of TSP	Upon oral administration of tobacco plant leaves high IgG and IgA specific antibodies were detected in serum and feces of mice	Karimi et al., 2013
Domain IV of <i>Bacillus anthracis</i> protective antigen gene [PA(dIV)] (Anthrax)	Tobacco	5.3% of TSP	Antibody titers of $>10^4$ were induced upon intraperitoneal (ip) and oral immunizations with plant derived PA(dIV). Mice challenged with <i>B. anthracis</i> showed 60% and 40% protection upon ip and oral immunization with adjuvanted plant PA(dIV)	Gorantala et al., 2011
Anthrax protective antigen (PV) (Anthrax)	Tobacco	2.5–4% of TSP	Intraperitoneal and oral immunization with plant PA in murine model indicated high serum PA specific IgG and IgA antibody titers. Oral immunization experiments demonstrated generation of immunoprotective response in mice	Gorantala et al., 2014
<b>PROTOZOAN ANTIGENS</b>				
<i>Plasmodium falciparum</i> surface protein 25 (Pfs25) and 28 (Pfs28) (Malaria)	<i>Chlamydomonas reinhardtii</i>	0.5 and 0.2% of TSP, respectively	Antibodies to algae-produced Pfs25 were bound to the surface of <i>in vitro</i> cultured <i>P. falciparum</i> sexual stage parasites and exhibited transmission blocking activity	Gregory et al., 2012
<i>Plasmodium falciparum</i> surface protein (Pfs25) fused to the $\beta$ subunit of the cholera toxin (CtxB) (Malaria)	<i>Chlamydomonas reinhardtii</i>	0.09% TSP	Algae produced CtxB-Pfs25 elicited CtxB-specific serum IgG antibodies and both CtxB- and Pfs25-specific secretory IgA antibodies	Gregory et al., 2013
<i>Toxoplasma gondii</i> surface antigen of (SAG1), alone and in fusion with heat shock Protein of <i>Leishmania infantum</i> (LiHsp83) SAG1, chLiHsp83-SAG1 (Toxoplasmosis)	Tobacco	0.1–0.2 $\mu$ g/g fresh weight	Human seropositive samples reacted with chloroplast-derived SAG1, oral immunization in mice elicited significant reduction of the cyst burden	Albarracín et al., 2015
<i>T. gondii</i> GRA4 antigen (Toxoplasmosis)	Tobacco	0.2% of total protein	Oral immunization with chGRA4 resulted in a decrease of 59% in the brain cyst load of mice compared to control mice. ChGRA4 immunization elicited both mucosal immune responses	Yácono et al., 2012
Sexual stage antigenic surface protein Pfs48/45 antigen of <i>Plasmodium</i> (Malaria)	<i>Chlamydomonas reinhardtii</i>	Not reported	Not tested	Jones et al., 2013
<b>AUTOANTIGENS</b>				
Human proinsulin (A, B, C peptides) containing three furin cleavage sites fused with CTB (CTB-PFx3) (Diabetes type-1)	Tobacco Lettuce	47% of TLP in tobacco 53% of TLP in lettuce	Oral delivery of unprocessed proinsulin bioencapsulated in plant cells or injectable delivery into mice showed reduction in blood glucose levels similar to processed commercial insulin	Boyhan and Daniell, 2011

(Continued)



TABLE 1 | Continued

Vaccine Antigen (Disease)	Expression system	Maximum expression level	Immunological investigation	References
Human proinsulin gene fused with protein A (Diabetes type-1)	Tobacco	0.2% of TSP	Not tested	Yarbakht et al., 2015
Exendin-4 (EX4) fused with CTB (Diabetes type-2)	Tobacco	14.3% of TLP	Upon oral delivery in mice CTB-EX4 stimulated insulin secretion similar to the intraperitoneal injection of commercial EX4	Kwon et al., 2013

via pollen or to the environment or food chain vertically as well as horizontally. Certain aspects of environment safety concerns can be addressed by opting plastid transformation. In most plant species, plastids follow maternal inheritance pattern, i.e., not transferred through pollen. This is a major advantage of plastids that should aid in addressing the regulatory concerns related to genetically modified plants (GMPs) because there will be no or very negligible outflow of plastid DNA paternally (Daniell, 2007; Ruf et al., 2007; Svab and Maliga, 2007). In case of tobacco, there is an extra advantage that it is a non-food non-feed crop that is not very common as wild. Hence, chances of crossing transplastomic plants with any wild species are excluded. Generally, the risk of horizontal gene transfer from plants to microorganisms, particularly when transformed plants contain antibiotic resistance genes, is very low and there is no existing report of such incidence (Obembe et al., 2011). The risk goes more toward downside by the fact that plants naturally harbor many bacteria that contain antibiotic-resistant genes (Nielsen et al., 1998). Hence, it can be argued that horizontal gene transfer to soil bacteria is very negligible. Considering the advantage of chloroplast transformation related to transgene containment, it is very likely that field trials can be allowed in isolated areas at local level in developing countries after addressing the containment issues to the local authorities. Permission of field trials of transplastomic plants may be easier in case of non-food and/or non-feed crops such as tobacco, because in such case the risk of human food chain contamination is minimal. If safety concerns are addressed, field trials are allowed and have been done previously in various countries for plant/chloroplast derived recombinant proteins and biopharmaceuticals (Arlen et al., 2007; Hefferon, 2015).

Plant-based vaccines have the potential to be used for oral administration. However, a matter of concern is that whether the consumption of plant-made pharmaceuticals is safe for administration or not. In general, plants are safe for human consumption as plants are not host for human pathogens and many plant species such as lettuce are consumed in normal diet as raw. However, for consuming transgenic plants, safety needs to be addressed. There are several reports in which safety of plant-based pharmaceuticals have been shown in animal models via oral delivery against different diseases using raw plant material (for review see Lössl and Waheed, 2011; Chan and Daniell, 2015). However, in addition to product used for treatment, raw material also contains unwanted antibiotic resistant gene that raises concerns regarding safety and needs to be addressed in

humans. This problem can be covered by: using selection markers other than antibiotic resistant genes, excising the antibiotic resistant genes after selection (Iamtham and Day, 2000) or purifying the final product. Although purification will add to the cost, yet it will address an important regulatory concern. In context of purification of plant-based vaccines, it is pertinent to mention that these vaccines will require less stringent purification methods compared to fermenter-based systems.

## STABILITY OF PLANT/CHLOROPLAST-EXPRESSED PROTEINS

In developing countries, a major limitation can be the maintenance of cooling chain during storage or when delivering the vaccines to remote areas. This factor may additionally add to the costs related to the production of vaccines. Plant-based vaccines have a potential that these can be stored at room temperature in the form of dried material for longer period of time. There are many reports which show that the protein expressed in plants remained stable at room temperature or even at elevated temperatures for a longer period of time when stored as dried plant material. An algal chloroplast-derived vaccine antigen remained stable for 20 months at room temperature in lyophilized form and was comparably immunogenic when tested in comparison with the antigen stored at 4°C (Dreesen et al., 2010). In another report, transplastomic lettuce leaves expressing CTB fused with ESAT-6 (antigen from *Mycobacterium tuberculosis*) were lyophilized and stored for 6 months at room temperature (Lakshmi et al., 2013). After 6 months CTB-ESAT6 fusion protein was stable and preserved proper folding, disulfide bonds and assembly into pentamers. Lyophilization also increased the antigen concentration per gram of leaf tissue up to 22-fold. Gregory et al. (2013) demonstrated that CtxB-Pfs25 accumulated as a soluble, properly folded and functional protein within algal chloroplasts, and it was stable in freeze-dried alga cells at ambient temperatures. Kwon et al. (2013) expressed exendin-4 (EX4) as a cholera toxin B subunit (CTB) fusion protein. They observed that lyophilization of leaf material increased therapeutic protein concentration by 12- to 24-fold, extended their shelf life up to 15 months when stored at room temperature and eliminated microbes present in fresh leaves. In addition, the pentameric structure, disulphide bonds, and functionality of CTB-EX4 were well

preserved in lyophilized materials (Kwon et al., 2013). In a recent report Su et al. (2015a) showed that plastid-derived cholera toxin subunit B fused with acid alpha glucosidase (CTB-GAA) concentration was increased by lyophilization to 30-fold (up to 190 µg per g of freeze-dried leaf material). Same group (Su et al., 2015b) expressed coagulation factor IX (FIX) fused with CTB in commercial lettuce. They showed that CTB-FIX in lyophilized cells was stable with proper folding, disulfide bonds, and pentamer assembly when stored for approximately 2 years at ambient temperature. All these reports strengthen the fact that plant/chloroplast-produced biopharmaceuticals can be stored at room temperature for long periods of time in the form of lyophilized material. A vaccine may be very effective in the laboratory; however, its commercial potential can be limited unless suspension can be stabilized for storage and distribution. Alternative techniques such as establishment of a cold chain may cause the potential loss of vaccine stocks resulting from freezer failure and also adds to the costs when distributing frozen materials. Lyophilization (freeze-drying) is a well-established technique used in the pharmaceutical industry for stabilizing high-cost, labile bioproducts, such as vaccines (Adams, 2003). Firstly, plant-produced vaccines can be stored in the form of dried plant material. This will also increase the antigen concentration. Alternatively, plant-derived vaccines can be purified and lyophilized for storage at room temperatures and transported when required. This strategy will help to circumvent cooling chain in developing countries and thus eliminating the costs related to storage and transportation under cooling.

## WHAT NEXT?

It has been almost 14 years since the expression of first vaccine candidate antigen against human diseases in chloroplasts of *Nicotiana tabacum* (Daniell et al., 2001b). Since then lot of research has been done on different aspects of expression that has resulted in improved protocols and a very high expression level has been achieved in chloroplasts. In 80–90% of cases, tobacco is used for the chloroplast-based expression and comparatively very little research has been done on other plant species. For tobacco, excellent data is available to follow for chloroplast-based vaccine production. However, despite of all this research, not a single plant/chloroplast-based vaccine against human diseases has entered the market and hence humanity has not been benefited. What is needed but lacking is more industrial interest. Big pharma companies have already well-established platforms for the production of pharmaceutical compounds. A good amount of revenue is already generated from these systems. To adopt plant-based systems, pharmaceutical companies would need to invest money and resources to develop their own platforms. In addition, clinical trials and the ultimate regulatory approval would also require cost and time. In contrast, newly established pharma companies or small industries can adapt more quickly to plant-based systems because opting to new cost-effective systems will help these industries to compete and earn more compared to the expenditures. Another possible solution can be to establish and strengthen the research/academia-industry linkages. Research groups in collaboration with industry

can develop platforms that could be later taken over by the industries. Such collaborative projects can be funded by many organizations that are working for the control of diseases throughout the world. Since, many cost-effective vaccines are needed mainly in developing countries, initially platforms can be established directly in those countries where vaccines are mostly needed. Small pharmaceutical industries can be collaborated to run the projects directly in a developing country where the product is to be utilized. Growing of plants, harvesting, processing and packaging can be carried out locally that will be economical due to less labor and land costs as compared to developed countries. A possible attraction for the pharmaceutical companies can be an adequate amount of revenue that can be generated from the production of vaccines and its launch to previously uncovered areas. This will be especially valid in those cases where already marketed vaccines such as those against HPV are very expensive and hence the large market of developing world remains uncovered. An affordable alternative solution in such cases can bring a handsome amount of turnover to the companies. The involvement of local pharma will be necessary because production can be more cost-effective if manufactured locally rather than exporting from a distant industrially developed country. In this way costs related to transportation and maintenance of cooling chain will be circumvented. Another advantage of this strategy will be the strengthening of local small pharmaceutical industries that will also help in the economic uplift of low and middle income countries. To achieve this, technology can be established in the developed countries in the laboratories/industries. Thus, a stable genetic resource in the form of seeds can be maintained in developed countries where more funding is available for research. The technology can be initially patented where developed. Later, local pharmaceutical companies, local governments and/or humanitarian organizations can be engaged to set up the industrial scale production platforms. Involvement of local governments and organizations such as world health organization (WHO) can serve to utilize already existing setups of vaccine storage, transportation and administration. However, a local patent may be needed if license is already not obtained in developing country, for which some variations in the existing established protocols of expression may be needed. To accomplish these goals, already established tobacco prototypes may be taken and advanced to the industrial level. Two main advantages of adopting tobacco chloroplast transformation: non-edible nature of tobacco thus avoiding food chain contamination and lack of transgene transmission due to maternal inheritance of chloroplasts may facilitate to address biosafety concerns in the target developing country and thus making large scale production in the fields possible. Established protocols, high level of expression and high biomass are additional reasons that make this species ideal for vaccine production at large scale. Other possible alternative plant species may be lettuce due to its high biomass and broad leaf. Taking lettuce for industrial level production will be more realistic in achieving the successful patent due to less number of existing protocols.

Efficacy of plant-based vaccines has been very well shown in animal models. Large number of reports exists that show many

potential vaccine candidates and their high immunogenicity in different animal models (Rybicki, 2014). Now the need is to investigate immunogenicity and safety of some prominent vaccine candidates in humans. For this purpose, relevant medical groups and industries can be involved in the design of research projects so that resources and funding may become available for human clinical trials.

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## AUTHOR CONTRIBUTIONS

MW, JG developed the concept and drafted the manuscript. MW and HI drew the figure and finalized the table. BM and AL contributed toward the concept and revised the manuscript critically. All authors have read and approved the final version of manuscript.

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# Transgene Expression in Microalgae—From Tools to Applications

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Microalgae comprise a biodiverse group of photosynthetic organisms that reside in water sources and sediments. The green microalgae *Chlamydomonas reinhardtii* was adopted as a useful model organism for studying various physiological systems. Its ability to grow under both photosynthetic and heterotrophic conditions allows efficient growth of non-photosynthetic mutants, making *Chlamydomonas* a useful genetic tool to study photosynthesis. In addition, this green alga can grow as haploid or diploid cells, similar to yeast, providing a powerful genetic system. As a result, easy and efficient transformation systems have been developed for *Chlamydomonas*, targeting both the chloroplast and nuclear genomes. Since microalgae comprise a rich repertoire of species that offer variable advantages for biotech and biomed industries, gene transfer technologies were further developed for many microalgae to allow for the expression of foreign proteins of interest. Expressing foreign genes in the chloroplast enables the targeting of foreign DNA to specific sites by homologous recombination. Chloroplast transformation also allows for the introduction of genes encoding several enzymes from a complex pathway, possibly as an operon. Expressing foreign proteins in the chloroplast can also be achieved by introducing the target gene into the nuclear genome, with the protein product bearing a targeting signal that directs import of the transgene-product into the chloroplast, like other endogenous chloroplast proteins. Integration of foreign genes into the nuclear genome is mostly random, resulting in large variability between different clones, such that extensive screening is required. The use of different selection modalities is also described, with special emphasis on the use of herbicides and metabolic markers which are considered to be friendly to the environment, as compared to drug-resistance genes that are commonly used. Finally, despite the development of a wide range of transformation tools and approaches, expression of foreign genes in microalgae suffers from low efficiency. Thus, novel tools have appeared in recent years to deal with this problem. Finally, while *C. reinhardtii* was traditionally used as a model organism for the development of transformation systems and their subsequent improvement, similar technologies can be adapted for other microalgae that may have higher biotechnological value.

**Keywords:** *Chlamydomonas*, chloroplast transformation of algae, diatoms, microalgae, nuclear transformation of algae, red microalgae, selection markers

## INTRODUCTION

Microalgae first attracted the attention of the biotech industry several decades ago as a potential platform for extracting natural products, with further improvements in their production relying on advanced technologies. Microalgae provide a manufacturing platform for the expression of foreign genes due to their quick growth under photoautotrophic conditions and their low cost of maintenance, as compared to land plants, mammalian cells, yeast, and bacteria. In addition, many algal species have developed unique metabolic pathways that produce compounds of commercial value (Priyadarshani and Rath, 2012; Rasala and Mayfield, 2014). Certain algal species, such as *Dunaliella*, are adapted for growth under extreme conditions, in this case high salinity (Ben-Amotz and Avron, 1972), thereby reducing the risk of contamination.

The plethora of high-value chemicals that can be extracted from both cyanobacteria and eukaryote microalgae include compounds used in the food industry, medicine, and cosmetics. The possibility of advancing the use of microalgae for future development of biofuel production and additional health-related products has also been examined. Microalgae have been exploited for the production of food additives, such as  $\beta$ -carotene (Varela et al., 2015) and astaxanthine, as well as long-chain polyunsaturated fatty acids (PUFAs; Borowitzka, 2013; Sharon-Gojman et al., 2015). Another line of industrial exploitation makes use of cell-wall sulfated polysaccharides, mainly for the cosmetics industry (Arad and Levy-Ontman, 2010). However, competition from cheaper parallel compounds extracted from macroalgae reveals the need for improvements to microalgal systems. Efforts have long been directed at the industrial use of microalgal biomass for biofuel production, although the relatively high cost and low yield are of concern. Thus, biotechnological improvements combined with global market changes may, however, have major implications for the future use of microalgae as a biofuel source. Algae can also contribute in the development of novel and sensitive biosensors for environmental uses, such as monitoring pollutants in soil and water sources (Viji et al., 2014; Diaz et al., 2015). Finally, algae are an important component of the marine aquaculture, and can be used in ecological reef rehabilitation, especially in view of their ability to form symbiotic relationships with coral (Hagedorn and Carter, 2015).

Heterotrophic growth of microalgae is usually limited to bioreactors, whereas photoautotrophic growth can be carried out in open ponds. Still, the use of open ponds is restricted to extreme conditions (salt, temperature, pH) that allow exclusive growth of the organism of interest and avoid contamination by opportunistic organisms. This has been shown for *Dunaliella*, which is adopted to grow at high salt concentrations (Ben-Amotz and Avron, 1983), as well as for *Spirulina*, a photoautotrophic cyanobacteria that grows at high pH (Nolla-Ardevol et al., 2015).

In view of the above, genetic manipulations of microalgae can lead to major changes in microalgal biotechnology-based industries. Such manipulations include the transfer of biosynthetic cascades into organisms that are suitable for growth in open ponds, or alternatively, adapting organisms that produce

compounds of interest to grow under extreme conditions, thus reducing the risk of contamination. Recent developments in genome sequencing, combined with old and new systems for genetic manipulation, act synergistically to advance all aspects of microalgal research, both basic and applied. This review focuses on available systems and approaches for genetic manipulation of the chloroplast and nuclear genomes of microalgae, addressing unsolved issues, as well as points that await improvement.

Significant efforts have been invested in establishing tools that will allow for realization of the promise that algae hold for the production of high value bio-products, with most such tools having been originally developed for the model alga *Chlamydomonas reinhardtii* (Kindle et al., 1989). Transgene expression in *Chlamydomonas* was based on the accumulated identification of regulatory elements, such as promoters and untranslated regions (UTRs; Harris, 2009). Subsequently, successful nuclear transformation systems were also developed for ~25 microalgae species (see Tables 1, 2). In many cases, microalgal transformation resulted in stable expression of transgenes from either the nuclear or plastid genomes. The large amount of genomic and EST data from different algae contribute to the rich molecular toolbox currently available.

## CHLOROPLAST TRANSFORMATION SYSTEMS

### Technical Approaches Used for Chloroplast Transformation

Although stable transformation of microalgae was first developed for the chloroplast of *C. reinhardtii*, there are still fewer transformation systems that target the chloroplast, as compared to those targeting the microalgal nucleus. One great advantage of chloroplast transformation is that transgenes can be easily directed to integrate via homologous recombination, whereas nuclear transformation of microalgae usually results in random integration events. The development of the CRISPR-CAS9 system in microalgae may offer a solution for targeting transgenes into specific sites in the nuclear genome. The improvement of chloroplast transformation tools is also a dynamic field, as discussed elsewhere for microalgae (Purton, 2007; Purton et al., 2013) and higher plants (Bock, 2015).

The first stable transformation system for the chloroplast of *C. reinhardtii* was established using biolistic delivery. The foreign DNA was designed to rescue three mutants of the chloroplast *atpB* gene by homologous recombination of the transgenic marker into the target mutant strain and restore photosynthetic activity (Boynton et al., 1988). It was further shown that *Chlamydomonas* chloroplast transformation could be achieved by agitating cell wall-deficient cells with the DNA of interest in the presence of glass beads (Kindle et al., 1991; Economou et al., 2014; Rochaix et al., 2014). A chloroplast transformation system that was based on integration into the inverted repeat of the plastid genome using electroporation was also developed for *Phaeodactylum tricornutum* (Xie et al., 2014). Thus, similar technologies can be used for both chloroplast and

**TABLE 1 | List of selection markers and selection modes for chloroplast transformation.**

Type of selection marker	Selection gene	Gene product	Selection mode	Species/Genetic background	References
Antibiotic resistance	<i>aadA</i>	Aminoglycoside 3' adenylyltransferase	Resistance to Spectinomycin/Streptomycin	<i>Chlamydomonas reinhardtii</i>	Goldschmidt-Clermont, 1991
				<i>Euglena gracilis</i>	Doetsch et al., 2001
				<i>Haematococcus pluvialis</i>	Gutiérrez et al., 2012
	<i>aphA6</i>	Aminoglycoside 3' - transferase	Resistance to Kanamycin	<i>Chlamydomonas reinhardtii</i>	Bateman and Purton, 2000
	<i>ereB</i>	Erythromycin esterase	Resistance to Erythromycin	<i>Dunaliella tertiolecta</i>	Georgianna et al., 2013
	<i>rrnS</i> and <i>rrnL</i> point mutation	16S and 23S	Resistance to Spectinomycin, Streptomycin, Kanamycin and Erythromycin	<i>Chlamydomonas reinhardtii</i>	Newman et al., 1990
	<i>Cat</i>	Chloramphenicol acetyltransferase	Resistance to Chloramphenicol	<i>Phaeodactylum tricornutum</i>	Xie et al., 2014
Herbicide resistance	<i>psbA</i> mutant	Photosystem II protein D1	Resistance to 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU)	<i>Chlamydomonas reinhardtii</i>	Przibilla et al., 1991; Newman et al., 1992
	<i>ahas</i> (W492S)	Acetohydroxyacid synthase	Resistance to Sulfometuron methyl	<i>Porphyridium</i> sp.	Lapidot et al., 1999, 2002
	<i>Bar</i>	Phosphinothricin acetyltransferase	Tolerance to Glufosinate, or its ammonium salt DL-Phosphinothricin	<i>Parietochloris incisa</i> <i>Platymonas subcordiformis</i>	Grundman et al., 2012 Cui et al., 2014
Metabolic markers	<i>atpB</i>	$\beta$ subunit of ATP synthase	Photoautotrophic growth	<i>Chlamydomonas reinhardtii</i> cc-373 ( <i>atpB</i> -)	Boynton et al., 1988
	<i>nifH</i>	$\beta$ -glucuronidase	Photoautotrophic growth	<i>Chlamydomonas reinhardtii</i> ( <i>chlL</i> -)	Cheng et al., 2005
	<i>psbA</i>	Photosystem II protein D1	Photoautotrophic growth	<i>Chlamydomonas reinhardtii</i> FUD7	Michelet et al., 2011
	<i>tscA</i>	Small RNA that participates in trans-splicing of the <i>psaA</i> transcript	Photoautotrophic growth	<i>Chlamydomonas reinhardtii</i> ( <i>tscA</i> -)	Goldschmidt-Clermont, 1991; Kindle et al., 1991
	<i>arg9</i>	Acetylornithine aminotransferase	Arginine free media	<i>Chlamydomonas reinhardtii</i> ( <i>arg9</i> -)	Remacle et al., 2009

nuclear transformations. The various markers for chloroplast transformation systems available today are summarized in **Table 1**.

## Selection Systems

### Selection Markers for Chloroplast Transformation Based on Photoautotrophic Growth

*Chlamydomonas* offers the advantage of being able to grow under non-photosynthetic conditions, using acetate as a carbon energy source. Thus, using non-photosynthetic mutants as recipient

strains and recovery of their photosynthetic activity as a reporter system was used for reconstituting expression of the mutated *atpB* gene, encoding for ATP synthase (Boynton et al., 1988), and the *tscA* gene, encoding a small RNA that participates in trans-splicing of the *psaA* transcript (Goldschmidt-Clermont, 1991; Kindle et al., 1991).

Marker rotation is an approach that was originally aimed at examining whether the bacterial *nifH* gene from *Klebsiella pneumoniae* could replace the algal *ChlL* gene, which is responsible for chlorophyll biosynthesis in the dark. Both



**TABLE 2 | Commonly used nuclear control elements for constitutive or inducible transgene expression.**

Functional elements	Originally controlling expression of	Property	Source of element	Comments	Algal species used with this control element
CaMV35S promoter	35S Viral protein from the Cauliflower mosaic virus	Strong heterologous constitutive promoter which functions well in land plants and some algae species	Cauliflower mosaic virus	Contradictory data for <i>Chlamydomonas</i>	<i>Chlamydomonas reinhardtii</i> , Successful transformation: (Kumar et al., 2004)
				High efficiency for different <i>Chlorella</i> species, <i>Dunaliella</i> , diatoms, <i>Haematococcus</i> and <i>Nannochloropsis</i>	Unsuccessful transformation: (Day et al., 1990; Diaz-Santos et al., 2013) <i>Chlorella kessleri</i> , (El-Sheekh, 1999) <i>Chlorella ellipsoidea</i> , (Jarvis and Brown, 1991) <i>Chlorella vulgaris</i> , (Chow and Tung, 1999; Wang C. et al., 2007; Cha et al., 2012) <i>Dunaliella salina</i> (Tan et al., 2005) <i>Amphidinium</i> sp. and <i>Symbiodinium microadriaticum</i> , (ten Lohuis and Miller, 1998) <i>Phaeodactylum tricornutum</i> , (Sakaue et al., 2008) <i>Haematococcus pluvialis</i> , (Kathiresan et al., 2015) <i>Nannochloropsis</i> sp., (Cha et al., 2011)
RBCS2 promoter	Small subunit of the ribulose biphosphate carboxylase	Strong endogenous constitutive promoter	<i>Chlamydomonas reinhardtii</i>	Introducing the first intron of <i>RBCS2</i> into the coding region or fusing the HSP70A promoter upstream to the <i>RBCS2</i> promoter greatly improves the expression of transgenes	<i>Chlamydomonas reinhardtii</i> , (Stevens et al., 1996; Lumberras et al., 1998; Fuhrmann et al., 1999; Schroda et al., 2000) <i>Dunaliella salina</i> , (Sun et al., 2005) <i>Chlorella ellipsoidea</i> , (Kim et al., 2002) <i>Volvox carteri</i> , (Hallmann and Wodniok, 2006) <i>Pseudochoricystis ellipsoidea</i> , (Imamura et al., 2012) <i>Nannochloropsis</i> sp., (Chen et al., 2008; Li and Tsai, 2009)
			<i>Dunaliella salina</i>	Adding a nuclear matrix attachment regions (MAR) before the promoter region and after the terminator sequence was shown to increase transgene expression	<i>Dunaliella salina</i> (Wang T. Y. et al., 2007)
			<i>Dunaliella tertiolecta</i>	Only one transgenic line was recovered when used to transform <i>Dunaliella tertiolecta</i>	<i>Chlamydomonas reinhardtii</i> , (Walker et al., 2005a) <i>Dunaliella tertiolecta</i> , (Walker et al., 2005b)
			<i>Lobosphaera (Parietochloris) incisa</i>	The upstream region of the <i>Lobosphaera incisa</i> RBCS promoter (ranging from –1000 to –450) contains elements counteracting transformation or gene expression	<i>Chlamydomonas reinhardtii</i> , (Zorin et al., 2014)
					<i>Lobosphaera (Parietochloris) incisa</i> , (Zorin et al., 2014)

(Continued)

TABLE 2 | Continued

Functional elements	Originally controlling expression of	Property	Source of element	Comments	Algal species used with this control element
<i>PSAD</i> promoter	An abundant chloroplast protein of Photosystem I complex, encoded by the nuclear genome	A strong endogenous constitutive promoter	<i>Chlamydomonas reinhardtii</i>	Expression driven by the <i>PSAD</i> promoter can be enhanced by high light	<i>Chlamydomonas reinhardtii</i> , (Fischer and Rochaix, 2001)
<i>FCP</i> promoter	Fucoxanthin-chlorophyll binding protein of the light-harvesting antennae complexes	Strong Endogenous and constitutive promoter	<i>Phaeodactylum tricornutum</i>  <i>Cylindrotheca fusiformis</i>	<i>FCP</i> promoters (A-E) are capable of driving expression of the bacterial <i>ble</i> gene at levels sufficient to confer resistance to Zeocin  <i>Thalassiosira pseudonana</i> The transformation of <i>Cylindrotheca fusiformis</i> using a p <i>FCP</i> -based vector improved the transformation efficiency about four fold as compared to the P $\delta$ -containing vectors	<i>Phaeodactylum tricornutum</i> , (Apt et al., 1996; Falcatore et al., 1999; Zaslavskaja et al., 2000; Kilian and Kroth, 2005) <i>Thalassiosira weissflogii</i> , (Falcatore et al., 1999) <i>Cylindrotheca fusiformis</i> , (Poulsen and Kroger, 2005) <i>Thalassiosira pseudonana</i> , (Poulsen et al., 2006)
P $\delta$	$\epsilon$ Frustulin—member of the calcium-binding glycoproteins	Endogenous constitutive promoter	<i>Cylindrotheca fusiformis</i>	Used to drive functional expression of a membrane protein	<i>Cylindrotheca fusiformis</i> , (Fischer et al., 1999)
<i>Ubi1</i> - $\Omega$ promoter	Ubiquitin promoter fused with the TMV-omega translation enhancer element	Strong heterologous constitutive promoter	<i>Zea mays</i>	Highly efficient for transformation of <i>Chlorella ellipsoidea</i> and <i>Dunaliella salina</i> cells	<i>Chlorella ellipsoidea</i> , (Chen et al., 2001) <i>Dunaliella salina</i> , (Geng et al., 2003, 2004)
GAPDH	Glyceraldehyde -3-phosphate dehydrogenase	Endogenous constitutive promoter	<i>Dunaliella salina</i>	Used to drive expression of the heterologous gene encoding bialaphos resistance ( <i>bar</i> ) and of the N-terminal fragment of human canstatin	<i>Dunaliella salina</i> , (Jia et al., 2012)
<i>CABII-1</i>	Light-harvesting chlorophyll a/b-binding proteins of photosystem II	Endogenous promoter	<i>Chlamydomonas reinhardtii</i>	The <i>NIT1</i> gene expressed under the control of the <i>CABII-1</i> promoter was highly stimulated by light	<i>Chlamydomonas reinhardtii</i> , (Blankenship and Kindle, 1992)
<i>NIT1</i>	Nitrate reductase promoter	Strong inducible endogenous promoter	<i>Chlamydomonas reinhardtii</i>  <i>Chlorella ellipsoidea</i>  <i>Phaeodactylum tricornutum</i>  <i>Dunaliella salina</i> <i>Cylindrotheca fusiformis</i>  <i>Thalassiosira pseudonana</i> <i>Volvox carteri</i>	Expression of the nitrate reductase is switched off when cells are grown in the presence of ammonium ions and becomes switched on within 4 h when cells are transferred to a medium containing nitrate. An expression vector with the <i>NIT1</i> promoter is widely used in many studies	<i>Chlamydomonas reinhardtii</i> , (Ohresser et al., 1997; Koblenz and Lehtreck, 2005; Schmollinger et al., 2010) <i>Chlorella ellipsoidea</i> , (Wang et al., 2004) <i>Phaeodactylum tricornutum</i> , (Niu et al., 2012); <i>Chlorella vulgaris</i> , (Niu et al., 2011) <i>Dunaliella salina</i> , (Li et al., 2007, 2008) <i>Cylindrotheca fusiformis</i> , (Poulsen and Kroger, 2005); <i>Phaeodactylum tricornutum</i> , (Miyagawa et al., 2009) <i>Thalassiosira pseudonana</i> , (Poulsen et al., 2006) <i>Volvox carteri</i> , (von der Heyde et al., 2015)

(Continued)

TABLE 2 | Continued

Functional elements	Originally controlling expression of	Property	Source of element	Comments	Algal species used with this control element
LIP	Light-induced protein		<i>Dunaliella</i> sp.	The LIP promoter can be used for conditional gene expression in response to high light	<i>Chlamydomonas reinhardtii</i> , (Park et al., 2013; Baek et al., 2016)
B12-responsive element	B12-independent methionine synthase (METE)		<i>Chlamydomonas reinhardtii</i>	The B12-responsive element can repress expression of a reporter gene following the addition of B <sub>12</sub>	<i>Chlamydomonas reinhardtii</i> , (Helliwell et al., 2014)
TPP riboswitch	Thiamine pyrophosphate (TPP)	A riboswitch-regulated element based on the thiamine pyrophosphate	<i>Chlamydomonas reinhardtii</i>	The TPP riboswitch regulates expression in response to the presence or absence of TPP in the growth medium	<i>Chlamydomonas reinhardtii</i> , (Ramundo et al., 2013)
CYC6 promoter	Cytochrome c6	An inducible endogenous promoter		Metal-responsive element that is responsive to both nickel and cobalt ions and could be inhibited by EDTA	<i>Chlamydomonas reinhardtii</i> , (Quinn and Merchant, 1995)

genes show a remarkable similarity in their domain structure, suggesting that *nifH* could replace ChlL for binding to a [4Fe–4S] cluster, thereby directly introducing the nitrogenase Fe protein into the *Chlamydomonas* plastome. In addition to using this approach for investigating the nitrogenase-like complex in the chloroplast, it could serve as a platform for plastid engineering into a functional nitrogenase-containing organelle. Accordingly, *petB* (cytochrome b6) was initially replaced by the selection marker *aadA*, and non-photosynthetic transformants were selected. A second round of transformation with the mutant strain restored the *petB* gene product and, moreover, introduced *nifH* (or *uidA*, encoding  $\beta$ -glucuronidase), allowing selection based on the ability to grow under photoautotrophic conditions (Cheng et al., 2005).

The use of FUD7 as a recipient strain offered a selection mechanism based on the ability of the transformed algae to grow under photoautotrophic conditions. FUD7 is a mutant that carries a deletion between exon 1 of *psbA* and the 5S gene. Thus, a cassette that reinstalls the open reading frame of *psbA*, along with a tagged gene of interest flanked by 3'UTR sequences, was established (Michelet et al., 2011).

### Selection Markers for Chloroplast Transformation Based on Metabolic Enzymes

Metabolic selection of transformed cells provides a great advantage over the use of genes encoding for resistance to antibiotics and herbicides, as metabolic selection is considered to be more environmental friendly. Metabolic selection is common in yeast and animal cells, especially since many markers offer positive and negative selections. For instance, acetylornithine aminotransferase (ARG9) is a key enzyme in the metabolic pathway of arginine. It is encoded in the nucleus of *C. reinhardtii*,

with the protein translocating into the chloroplast. A mutation in ARG9 resulted in an auxotrophic phenotype, such that the algal cells could only grow if arginine was added to the medium. The *Arabidopsis thaliana* ARG9 gene, known for its high A/T content that is typical of the chloroplast genome (Nakamura et al., 2000), was expressed in the plastid of a *Chlamydomonas* mutant strain that was originally deficient of ARG9 expression. The foreign ARG9 gene, now encoded by the chloroplast genome, was able to rescue the auxotrophic phenotype and restore arginine synthesis. This elegant approach created a metabolic selection system for chloroplast transformation in *Chlamydomonas* (Remacle et al., 2009).

### Selection Markers for Chloroplast Transformation Based on Resistance to Antibiotics

Mutations in the sequence of the 16S (*rrnS*) and 23S (*rrnL*) rRNA that confer resistance to spectinomycin, streptomycin, kanamycin and erythromycin in *C. reinhardtii* (Harris et al., 1989) were previously used to establish an antibiotic-based selective marker that exchanged wild type RNA with the gene encoding resistant RNA (Newman et al., 1990). Later, resistance to spectinomycin was conferred by introducing the bacterial-derived *aadA* gene into the chloroplast genome, encoding aminoglycoside 3' adenylyl transferase. This gene is still the most frequently used marker for chloroplast transformations in algae and higher plants. The *aadA-rbcL* expression cassette was adapted and used to transform the genome of the *Haematococcus pluvialis* chloroplast (Gutiérrez et al., 2012).

An additional bacterial gene, *aphA6*, encoding aminoglycoside (3') transferase that confers resistance to aminoglycoside antibiotics was used with *Chlamydomonas reinhardtii*. The combination of *aadA* with *aphA6* extended

the possibility for expressing multiple foreign genes in the chloroplast (Bateman and Purton, 2000). In 2013, the group of Stephan Mayfield constructed a cassette for *Dunaliella tertiolecta* chloroplast transformation. The construct that encodes both the gene of interest and the erythromycin esterase gene, *ereB*, was successfully introduced into *D. tertiolecta* cells via particle bombardment (Georgianna et al., 2013). A chloroplast-based expression system developed for the diatom *P. tricornutum* uses the chloramphenicol acetyltransferase (CAT) gene as a selection marker, as it confers resistance to chloramphenicol. This cassette promoted the expression of a foreign gene, along with the selection marker (Xie et al., 2014).

Finally, there is a strong demand for marker removal systems due to biotechnological concerns and commercial requests. Such a system was first established for *Chlamydomonas* by designing a dedicated cassette for promoting a recombination event that allows for removal of the selection marker, once the selection pressure is removed. The additional foreign gene in this cassette was maintained in the genome despite the removal of the selection gene marker because it was located outside of the sequence repeats that drive the recombination event (Fischer et al., 1996).

### Selection Markers for Chloroplast Transformation Based on Resistance to Herbicides

A mutation in the fifth exon of the *psbA* coding region conferred resistance of *Chlamydomonas* cells to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a herbicide that blocks the electron transfer pathway in PSII. Thus, DCMU resistance was initially used as a selection marker for understanding the occurrence of integration hotspots in the chloroplast genome (Newman et al., 1992). A different mutation in *psbA* also provides the cells with herbicide resistance to metribuzin (Przibilla et al., 1991).

The herbicide sulfometuron methyl (SMM) that inhibits growth of bacteria, yeast, algae and plants is commonly used as a selection marker in plants and algae. The target of SMM is the gene encoding acetohydroxyacid synthase (AHAS), an enzyme involved in the biosynthesis of branched amino acids. Since AHAS is encoded by the chloroplast genome of the red microalgae *Porphyridium* sp., it was found to be an ideal marker for chloroplast transformation. As such, a naturally occurring W492S mutation in the algal AHAS gene that confers resistance to SMM was used to establish a transformation system for *Porphyridium* sp. (Lapidot et al., 1999, 2002). During evolution, the gene encoding AHAS moved to the nucleus in land plants and green algae (Mazur et al., 1987), therefore allowing for its use as a selection marker in nuclear transformations in plants (Li et al., 1992; Ott et al., 1996) and green algae (Kovar et al., 2002). The AHAS-encoding gene was also later used as a transformation marker for the green microalgae *Lobosphaera* (*Parietochloris*) *incisa*, an algae with added value for biotechnological purposes (Grundman et al., 2012). In this case, the endogenous gene was first cloned, the W605S mutation in the active site of the enzyme that confers resistance to SMM was introduced, and the mutated gene was used for nuclear transformation.

Another chloroplast transformation system based on resistance to herbicides was developed for *Platymonas*

*subcordiformis* using the bacterial *bar* gene encoding phosphinothricin acetyltransferase, which confers tolerance to glufosinate, or its ammonium salt, DL-phosphinothricin. This latter compound is the active ingredient in several herbicides, including the widely used Basta. The *bar* gene was also described as a selection marker in tobacco (Lutz et al., 2001). The use of this system in algae resulted in the development of a useful tool and a suitable selection system for this algae that is not sensitive to most commonly used antibiotics, such as spectinomycin, streptomycin or kanamycin (Cui et al., 2014).

Finally, as will be discussed below (see Section Biotechnological Exploitation of Microalgae), antibiotic and herbicide markers may raise biosafety concerns when genetic engineering is recruited for biotechnological purposes. Thus, selections involving metabolic markers that offer functional complementation of a missing endogenous gene product provide a clear advantage.

### Regulatory Elements: Promoters, UTRs, and Codon Optimization

Efficient expression of foreign genes in algal chloroplasts is usually best obtained through the use of endogenous regulatory elements, derived from genes that are abundantly expressed. Thus, promoters that drive expression of the large subunit of ribulose biphosphate carboxylase/oxygenase (*rbcL*), the D1 protein of the photosystem II reaction center (*psbA*), and the Y subunit of ATP synthase (*atpA*), were recruited for this purpose. The highest expression of soluble GUS was recorded for the *atpA* promoter and the 5'UTR (Ishikura et al., 1999). In another study, the *atpA* and *psbD* promoters and 5'UTRs were shown to drive the highest expression of a GFP reporter gene, when compared to the promoter and 5'UTRs of the *rbcL* and *psbA* genes. The presence of a 3'UTR derived from the different genes was required but had little effect on the accumulation of the transcript and foreign protein (Barnes et al., 2005). In later studies, the Goldschmidt-Clermont group demonstrated that the use of the *psaA*-exon1, its promoter and its 5'UTR increased foreign protein expression levels in the chloroplast. The authors also suggested that the variable expression observed among the different transformants that were derived from the same construct was related to the number and location of the recombination events (Michelet et al., 2011).

For a long time, foreign gene expression in the chloroplast suffered from low yields. With this in mind, limiting factors that could explain the low expression were extensively sought. It should be noted that gene expression in the chloroplast is tightly regulated at the translational level, and is less sensitive to transcriptional regulation. This was demonstrated in studies that inhibited the accumulation of specific chloroplast transcripts (by 90%) without affecting their translation rates. Gene dosage also proved to have little effect on the level of proteins encoded by these genes (Eberhard et al., 2002). The bottleneck in transgene expression from the chloroplast genome was addressed by the Mayfield group in 2007. They demonstrated that instead of targeting the expression cassette to the inverted repeats of the chloroplast genome, which yielded a low expression level of 0.5%



relative to the total protein content in the cell, direct replacement of the *psbA* gene with a foreign coding region increased the production yield of the foreign protein to >5%. The authors suggested that the increased level of expression was due to a lack of competition encountered by regulatory factors (Mayfield and Schultz, 2004; Manuell et al., 2007).

Further improvements increased the level of transgene expression in the chloroplast of *C. reinhardtii* up to 20% of the total soluble protein content by focusing on optimization of codon usage and inhibition of ATP-dependent proteases. In addition, the toxicity of the transgene product may have a dramatic effect on its level of expression, and finally, large screens are usually required in order to isolate clones that efficiently express a transgene, possibly due to transformation-associated genotypic modifications that occur due to the random insertion of the foreign gene into the nuclear genome. Thus, the type of promoter and UTRs used are not the only factors that affect expression levels. The site of integration in the chloroplast, as well as accompanying random integration events in the nucleus, may also affect expression (Surzycki et al., 2009).

Chloroplast transformation systems that were developed for other algal species revealed differences with those developed for *Chlamydomonas*. For example, chloroplast transformation of *Euglena* revealed that the cassette containing the *aadA* gene flanked by the endogenous *psbA* promoter/5'UTR and 3'UTR generated drug resistant clones in which the DNA was maintained on a high molecular weight episomal element. Additional experiments using the *aadA* cassette introduced into the independently expressed *Euglena gracilis* *psbK* operon demonstrated proper splicing of the group III intron derived from the operon (Doetsch et al., 2001). Episomal elements carrying resistance genes are also common in non-photosynthetic kinetoplastids, such as *Leishmania* (Kapler et al., 1990), although the algal case describes a stable chloroplast episome.

## Inducible Expression in the Chloroplast

Inducible expression offers considerable advantages, especially when the product of the transgene may interfere with cell growth or be toxic. NAC2 is essential for stabilization of the *psbD* mRNA (Kuchka et al., 1989), binding a unique target site in the *psbD* 5'UTR, and regulating the expression of any foreign gene under control of the *psbD* 5'UTR. The group of Rochaix introduced the NAC2 gene into *Chlamydomonas* cells under control of the cytochrome C<sub>6</sub> promoter, which can be induced upon depletion of copper ions, by exposure to anaerobic conditions and in the presence of nickel ions (Merchant and Bogorad, 1987; Nickelsen et al., 1994; Quinn et al., 2002, 2003; Surzycki et al., 2007; Rochaix et al., 2014). Thus, manipulating the NAC2 gene to be induced by any of these conditions resulted in a similar pattern of regulation of a foreign gene that was driven by the *psbD* 5'UTR (Rochaix et al., 2014).

## NUCLEAR TRANSFORMATION

Nuclear expression of transgenes in microalgae offers several advantages, including targeting of foreign proteins for expression

in organelles, such as the chloroplast, and protein glycosylation and/or additional post-translational modifications, as well as secretion (León-Bañares et al., 2004). These advantages are especially required for the exploitation of microalgae for industrial production for recombinant proteins, especially in view of the known difficulties to obtain efficient expression of foreign genes in microalgae (Eichler-Stahlberg et al., 2009). A collection of protocols for nuclear transformation, combined with the availability of several constitutive or inducible promoters and the availability of multiple selectable markers, offers a multitude of approaches for expression of transgenes in the nucleus.

## Gene Delivery Methods

### Generation of Protoplasts

The algal cell wall represents a physical barrier preventing the entry of foreign DNA through the cell membrane. Thus, many protocols for transformation rely on the use of protoplasts, which are cell wall-deficient. However, in many cases, generating protoplasts is a major bottleneck due to the diverse composition of the cell wall in different algae species, which, in many cases, remain poorly characterized (Popper and Tuohy, 2010). The *Chlamydomonas* cell wall is built from glycoproteins and contains little cellulose or chitin. As such, polysaccharide-degrading enzymes are ineffective, with protoplasts instead being generated by incubation with autolysins, namely hydroxyl-proline-specific proteases that are active during gametogenesis and mating (Jaenicke et al., 1987; Imam and Snell, 1988). Unlike *Chlamydomonas*, the cell wall in *Chlorella* consists of sugar polymers that can be degraded by sugar digesting-enzymes (Takeda, 1991). Thus, protoplasts of different *Chlorella* species can be generated by incubation with different sugar-degrading enzyme mixes (Braun and Aach, 1975; Yamada and Sakaguchi, 1982; Afi et al., 1996). Protoplasts from red and brown algae have been prepared using numerous cell wall-digesting enzymes extracted from marine mollusks or echinoderms (Liu et al., 1984; Cheney et al., 1986; Reddy et al., 2010).

### Biolistic Delivery

The most frequently used method of gene delivery is biolistic transformation, also referred to as micro-projectile bombardment. This method utilizes DNA-coated gold or tungsten micro-particles that are delivered through a particle delivery system at high velocity into algal cells, surpassing the physiological barrier of the cell wall. Successful transformation by this approach have been reported for *C. reinhardtii* (Kindle et al., 1989), *Dunaliella salina* (Tan et al., 2005), *Haematococcus pluvialis* (Steinbrenner and Sandmann, 2006), and diatoms (Dunahay et al., 1995; Apt et al., 1996; Falcioratore et al., 1999; Zaslavskaya et al., 2000).

### Glass Beads Method

A simple method that is often used for gene delivery is based on agitating protoplasts or cell wall-deficient mutants in the presence of glass beads, polyethylene glycol (PEG) and foreign DNA (Kindle et al., 1989). Successful transformations by this method were reported for *C. reinhardtii* (Kindle et al., 1989)

and *Chlorella ellipsoidea* (Jarvis and Brown, 1991). In the case of *C. reinhardtii*, linearized plasmids usually yield higher transformation frequencies than supercoiled DNA when using glass beads or silicon carbide whiskers to mediate DNA entry (Kindle, 1990; Dunahay, 1993).

### Electroporation

Applying an electric pulse is a commonly used method to introduce DNA into cells. This technique was used to transform microalgal protoplasts, cell wall-deficient mutants, and other thin walled algal cells. It was used to transform *C. reinhardtii* (Brown et al., 1991; Shimogawara et al., 1998; Yamano et al., 2013), *D. salina* (Walker et al., 2005b), *Chlorella vulgaris* (Chow and Tung, 1999), *Scenedesmus obliquus* (Guo et al., 2013), and *Nannochloropsis* sp. (Kilian et al., 2011). The use of cell wall-deficient strains improves transformation efficiency, with successful transformation using this methodology having been reported for *Lobosphaera* (Zorin et al., 2014). However, successful transformation without using cell wall mutants was also shown in *P. tricornutum*, either by electroporation (Niu et al., 2012; Zhang and Hu, 2014) or multi-pulse electroporation (Miyahara et al., 2013). In all these cases, the cells were grown without silica, which probably influenced cell wall structure. Overall, reducing cell wall thickness improves the transformation of microalga by electroporation.

### Agrobacterium-Based Transformation of Microalgae

The highly popular system for nuclear transformation of land plants using *Agrobacterium tumefaciens* was also adapted for transformation of microalga, using the pCambia transformation vector. Several microalgal species were transformed by *Agrobacterium*, including *Chlamydomonas* (Kumar et al., 2004; Pratheesh et al., 2014), as well as other algae of biotechnological value, such as *H. pluvialis* (Kathiresan et al., 2015), *Schizochytrium* (Cheng et al., 2012), *Isochrysis galbana*, and *Isochrysis* sp. (Prasad et al., 2014).

## Nuclear Promoters and Control Elements

### Gene Promoters

Efficient expression of foreign genes is achieved under the control of strong promoters. These are often derived from viruses, especially in land plants (Sanger et al., 1990), or from highly abundant endogenous genes, such as the small subunit of Rubisco (Goldschmidt-Clermont and Rahire, 1986). The use of a Cauliflower Mosaic Virus (CaMV35S) heterologous promoter that functions well in land plants gave inconsistent results when used to transform different algae species. Whereas, this promoter could drive the expression of reporter genes or chimeric genes in *D. salina* (Tan et al., 2005), *Chlorella kessleri* (El-Sheekh, 1999), and the dinoflagellates *Amphidinium* sp. and *Symbiodinium microadriaticum* (ten Lohuis and Miller, 1998; see Table 2) it gave contradictory results in *C. ellipsoidea* (Jarvis and Brown, 1991; Kim et al., 2002) and *C. reinhardtii* (Day et al., 1990; Blankenship and Kindle, 1992; Kumar et al., 2004; Diaz-Santos et al., 2013).

The use of strong endogenous promoters is recommended for nuclear transformation in algae, since heterologous promoters

did not have an advantage, although they were active (Diaz-Santos et al., 2013). Among the endogenous regulatory elements, the *RBCS2* promoter that drives expression of the small subunit of Rubisco, and *PSAD* (an abundant chloroplast protein of the Photosystem I complex) from *C. reinhardtii* drove efficient expression of transgenes (Stevens et al., 1996; Lumbreras et al., 1998; Fuhrmann et al., 1999; Fischer and Rochaix, 2001). Improved expression of transgenes was demonstrated in *C. reinhardtii* when the *RBCS2* promoter was fused with the *HSP70A* (heat shock protein 70A) promoter, which acts as a transcriptional enhancer when placed upstream of the *RBCS2* promoter (Schroda et al., 2000). Driving expression by the *HSP70A/RBCS2* regulatory elements has become highly recommended for nuclear transformation of *Chlamydomonas*.

Other endogenous promoters have been used to drive the expression in other algal species, such as the promoter of the fucoxanthin-chlorophyll binding protein (*FCP*; Falciatore et al., 1999; Zaslavskaja et al., 2000; Poulsen et al., 2006). In the unicellular green alga *Lobosphaera (Parietochloris) incisa*, the endogenous *RBCS* promoter was used to drive expression of the *ble* gene, thus developing a platform for future successful engineering of this alga, which has great biotechnological potential due to its long-chain polyunsaturated fatty acid (PUFA) metabolism (Zorin et al., 2014).

Elements in the promoter region may occasionally have a negative effect on the expression of a transgene. Expression of the *ble* gene was silenced in 80% of transformants when it was driven from the *RBCS* promoter alone. However, when it was introduced under the control of the *HSP70A/RBCS* tandem promoter, silencing occurred in only 36% of transformed cells (Schroda et al., 2002). Modifying the *HSP70A/RBCS2* promoter so that it contained four copies of the first *RBCS2* gene intron between the *HSP70A* and *RBCS2* promoters significantly increased the expression of the downstream gene (Rasala et al., 2012).

A recent study described the use of the constitutive endogenous promoter that drives expression of the Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), for expression of the bacterial gene encoding bialaphos resistance (*bar*) and of the N-terminal fragment of human canstatin in *D. salina* (Jia et al., 2012).

A search for efficient endogenous promoters was performed by a “promoter trapping approach” in *C. reinhardtii*, by which a promoter-less selectable marker gene was randomly integrated into the nuclear genome (von der Heyde et al., 2015). The appearance of drug-resistant colonies indicated that the selectable marker gene integrated near a strong promoter. This approach, combined with the growing amount of species whose genomes have been sequenced, should enable the isolation of strong endogenous promoters in other algae as well.

### Inclusion of Splicing Signals

Another point of concern is the inclusion of signals for RNA processing in the algal nuclei, since only processed mRNAs can exit the nucleus through the nucleopores. Thus, expression cassettes that contain an endogenous intron were prepared for improvement of transgene expression (Lumbreras et al., 1998; Fischer and Rochaix, 2001; Kovar et al., 2002).

## Optimization of Codon Usage

The absence of tRNAs that are compatible with the codon usage of a given gene can dramatically affect expression of a transgene (Heitzer et al., 2007). Differences in codon usage were recorded between various microalgal species, as well as for the chloroplast and nuclear genomes of the same species (León-Bañares et al., 2004) and between organelles (Jarvis et al., 1992). Therefore, codon optimization of exogenous genes can significantly improve protein expression (Zaslavskaja et al., 2000; Franklin et al., 2002).

## Inducible Expression in the Nucleus

The biotechnological exploitation of constitutive promoters for the effective expression of toxic compounds for industrial or pharmaceutical uses or for establishing silencing systems may occasionally be problematic. Hence, introducing inducible expression systems offers considerable advantages. Overall, the use of inducible systems is also attractive for large-scale production of recombinant proteins in microalgae, as these enable the cells to first reach an optimal concentration before expression of the transgene is initiated.

Nitrogen metabolism is based on the exploitation of ammonium ions. However, some algae can adsorb nitrate and convert it into ammonium with the help of nitrate reductase (Fernandez et al., 1989; Berges, 1997). The promoter of the nitrate reductase gene that is switched on and off in response to the presence of nitrate or ammonium ions (Poulsen and Kroger, 2005) was able to promote the inducible expression of a reporter gene in *Cylindrotheca fusiformis* (Poulsen and Kroger, 2005), *C. reinhardtii* (Schmollinger et al., 2010), *C. ellipsoidea* (Wang et al., 2004), *D. salina* (Li et al., 2007, 2008), *Volvox carteri* (von der Heyde et al., 2015), and *Phaeodactylum triornutum* (Niu et al., 2012).

The use of light for inducing expression in microalgae has long been pursued, initially by the use of the *CABII-1* promoter that was shown to respond to changing light conditions and stimulate expression of the *NIT1* gene under light in *Chlamydomonas* (Blankenship and Kindle, 1992). More recently, the light-regulated elements of the light-inducible protein (*LIP*) from *Dunaliella* was mapped to the flanking 400 bp upstream sequences of the *LIP* gene, which contains triplicates of light-responsive motifs. These were shown to induce expression of a heterologous reporter gene in *Chlamydomonas* (Park et al., 2013; Baek et al., 2016).

The metal-responsive *CYC6* promoter that is repressed by copper was used for inducible gene expression in *C. reinhardtii* (Quinn and Merchant, 1995). The copper response element (*CuRE*) is responsive to both nickel and cobalt ions, and could be inhibited by EDTA (Quinn et al., 2003). Expression from this inducible system was improved by the addition of the first intron of the *RBCS2* gene to the *CYC6* promoter (Ferrante et al., 2011). In several marine and freshwater algae species, a control element located upstream of the  $B_{12}$ -independent methionine synthase (*METE*) gene could repress the expression of a reporter gene following addition of  $B_{12}$  (Helliwell et al., 2014). The strong repressible nature and high sensitivity of the  $B_{12}$ -responsive element can, therefore, be used

as another promising gene expression tool for biotechnological applications.

Yet another element that was used to drive transgene expression is based on riboswitch biology (Mandal and Breaker, 2004). A riboswitch-regulated element based on the thiamine pyrophosphate (TPP) biosynthesis was identified in the *THIC* genes of a variety of organisms (Breaker, 2012), including *Arabidopsis*, where it affected *THIC* mRNA stability by alternative splicing of the primary mRNA (Bocobza et al., 2007; Wachter et al., 2007; Bocobza and Aharoni, 2014). This TPP riboswitch was later used for generating an inducible expression system in *Arabidopsis* (Bocobza et al., 2013) and in *C. reinhardtii* (Ramundo et al., 2013). Using the TPP system, Ramundo and colleagues were able to conditionally repress expression of the *rpoA* or *rps12* genes in the chloroplast, affecting their organelle transcription or translation, respectively. Although riboswitch elements are common in prokaryotes, and have been used to generate inducible expression systems in bacteria (Neupert and Bock, 2009), the only riboswitch to be identified in higher eukaryotes was the TPP riboswitch, described above. The use of riboswitch-based regulation was recently used to develop a system for increasing expression of chloroplast genes in *Arabidopsis*, via an RNA amplification-based system that strongly improves the efficiency of riboswitches (Emadpour et al., 2015). A summary of the constitutive and inducible expression systems is given in Table 2.

## Nuclear Selectable Marker Genes

Stable transformation is based on the use of a proper selection marker. These include genes that confer resistance to antibiotics or herbicides, and various metabolic enzymes that control growth under specific nutritional conditions. A collection of nuclear selective genes are listed in Table 3.

## Selection of Nuclear Transformants Based on Auxotrophic Growth

Auxotroph genes can be used as selection markers for rescuing the phenotype of specific mutants that restrict growth under minimal conditions. The *NIT1* gene, encoding nitrate reductase, promotes growth in the presence of nitrates as a nitrogen source (Fernandez et al., 1989), and was introduced for the nuclear transformation of the *NIT1* mutant of *C. reinhardtii* (Kindle et al., 1989). This useful selection system was also applied for many other algal species (see Table 3). Another metabolic marker is the *ARG7* gene, encoding argininosuccinate lyase. It was used to rescue arginine-requiring *ARG7* mutants to prototrophy (Debuchy et al., 1989; Haring and Beck, 1997), and is commonly used for nuclear transformation of *C. reinhardtii*. The *ARG7* mutant strain has been widely used for transformation of *Chlamydomonas*, mostly for basic research. A similar approach for use with other algae would be welcome, although a mutant that does not express argininosuccinate lyase must first be isolated. Another selection marker is based on *NIC7*, encoding quinolinate synthetase that is required for NAD biosynthesis. A plasmid carrying this gene could rescue a mutation in the *C. reinhardtii* *NIC1* gene (Ferris,

**TABLE 3 | List of selection markers and selection modes for nuclear transformation.**

Type of selection marker	Selection gene	Gene product	Selection mode	Species/Genetic background	References
Antibiotic resistance	<i>Ble</i>	Phleomycin-binding protein	Resistance to Zeocine/Phleomycin	<i>Chlamydomonas reinhardtii</i> <i>Dunaliella salina</i> <i>Volvox carteri</i> <i>Chlorella ellipsoidea</i> <i>Phaeodactylum tricomutum</i>  <i>Cylindrotheca fusiformis</i>  <i>Nannochloropsis</i> sp. <i>Nannochloropsis granulate/gaditana/oculata/oceanica/salina</i>	Stevens et al., 1996; Guo et al., 2013 Sun et al., 2005 Hallmann and Rappel, 1999 Kim et al., 2002 Apt et al., 1996; Falciatore et al., 1999; Zaslavskaja et al., 2001; De Riso et al., 2009; Kira et al., 2015 Fischer et al., 1999; Poulsen and Kroger, 2005 Kilian et al., 2011 Li et al., 2014
	<i>aphVIII</i>	Aminoglycoside 3'-phosphotransferase	Resistance to Paromomycin	<i>Chlamydomonas reinhardtii</i>  <i>Gonium pectoral</i> <i>Eudorina elegans</i>	Sizova et al., 2001  Lerche and Hallmann, 2009 Lerche and Hallmann, 2013
	<i>aadA</i>	Aminoglycoside 3'-adenylyltransferase	Resistance to Spectinomycin/Streptomycin	<i>Chlamydomonas reinhardtii</i>	Cerutti et al., 1997b
	<i>aph7</i>	Aminoglycoside phosphotransferase	Resistance to Hygromycin	<i>Chlamydomonas reinhardtii</i>  <i>Haematococcus pluvialis</i> <i>Volvox carteri</i> <i>Chlorella vulgaris</i> <i>Laminaria japonica</i>	Berthold et al., 2002  Kathiresan et al., 2015 Jakobiak et al., 2004 Chow and Tung, 1999 Qin et al., 1999
	<i>nptII</i>	Neomycin phosphotransferase	Resistance to Neomycin	<i>Chlamydomonas reinhardtii</i>  <i>Chlorella sorokiniana</i> , <i>Chlorella vulgaris</i> <i>Amphidinium</i> sp., and <i>Symbiodinium microadriaticum</i> <i>Cyclotella cryptica</i> , <i>Navicula saprophila</i> <i>Phaeodactylum tricomutum</i>	Hall et al., 1993  Hawkins and Nakamura, 1999 ten Lohuis and Miller, 1998  Dunahay et al., 1995
	<i>cat</i>	Chloramphenicol acetyltransferase	Resistance to Chloramphenicol	<i>Dunaliella salina</i>  <i>Chlorella vulgaris</i>	Zaslavskaja et al., 2000 Geng et al., 2004; Sun et al., 2008 Niu et al., 2011
	<i>CRY1-1</i>	Cytosolic ribosomal protein S14	Resistance to Emetine	<i>Chlamydomonas reinhardtii</i>	Nelson et al., 1994; Neupert et al., 2009
Herbicide resistance	<i>GAT</i>	Glyphosate aminotransferase	Resistance to Glyphosate	<i>Chlamydomonas reinhardtii</i>	Bruggeman et al., 2014
	<i>ALS</i>	Acetolacetate synthase	Resistance to Sulfometuron methyl	<i>Chlamydomonas reinhardtii</i>  <i>Porphyridium</i> sp. <i>Parietochloris incisa</i>	Kovar et al., 2002  Lapidot et al., 2002 Grundman et al., 2012
	<i>PDS1</i>	Phytoene desaturase	Resistance to Norflurazon	<i>Haematococcus pluvialis</i>  <i>Chlorella zofingiensis</i>	Steinbrenner and Sandmann, 2006; Sharon-Gojman et al., 2015 Huang et al., 2008; Liu et al., 2014

(Continued)



TABLE 3 | Continued

Type of selection marker	Selection gene	Gene product	Selection mode	Species/Genetic background	References
Metabolic markers	<i>NIT1</i>	Nitrate reductase	Growth in the presence of nitrate salt	<i>Chlamydomonas reinhardtii</i> ( <i>nit1</i> -) <i>Volvox carteri</i> ( <i>nit1</i> -)	Kindle et al., 1989 Schiedmeier et al., 1994; Hallmann and Sumper, 1996
				<i>Dunaliella viridis</i> ( <i>nit1</i> -)	Sun et al., 2006
				<i>Chlorella sorokiniana</i> ( <i>nit1</i> -)	Dawson et al., 1997
				<i>Chlorella ellipsoidea</i> ( <i>nit1</i> -)	Bai et al., 2013
	<i>ARG7</i>	Argininosuccinate lyase	Growth in Arginine free media	<i>Chlamydomonas reinhardtii</i> ( <i>arg7</i> -)	Debuchy et al., 1989; Haring and Beck, 1997; Molnar et al., 2009
	<i>NIC7</i>	Quinolinate synthetase	Growth in Nicotinamide free media	<i>Chlamydomonas reinhardtii</i> ( <i>nic7</i> -)	Ferris, 1995; Adam et al., 2006; Lin et al., 2010
	<i>OEE1</i>	Oxygen-evolving enhancer protein1	Photoautotrophic growth	<i>Chlamydomonas reinhardtii</i> ( <i>oeel</i> -)	Mayfield and Kindle, 1990

1995; Lin et al., 2010), allowing growth in the absence of nicotinamide.

### Selection of Nuclear Transformants Based on Resistance to Antibiotics

As with animal cell systems, numerous antibiotics genes have been successfully used as selection markers of microalgae. The *ble* gene that was originally isolated from *Streptoalloteichus hindustanus*, confers resistance to zeomycin and phleomycin (Sugiyama et al., 1994) and was used for generating transgenic clones of different algal species (see Table 3). The synthetic aminoglycoside adenyltransferase *aadA* gene confers resistance to spectinomycin and streptomycin (Svab and Maliga, 1993). It was originally used for chloroplast transformations but was further adopted for the nuclear system in *Chlamydomonas* (Cerutti et al., 1997b), and *H. pluvialis* (Gutiérrez et al., 2012). The aminoglycoside phosphotransferase genes *aphVIII* (*aphH*) from *Streptomycesrimosus* and *aph7* from *Streptomyces hygroscopicus* confer resistance to paromomycin (Sizova et al., 2001) and hygromycin B, respectively. These were used to select drug-resistant algae in several species (see Table 3). The bacterial *nptII* gene encoding neomycin phosphotransferase was proven to be relatively inefficient in *C. reinhardtii* (Bingham et al., 1989; Hall et al., 1993). Codon optimization of the *nptII* gene, however, led to its improved function as a nuclear selection marker (Barahimipour et al., 2016). This selection marker was more successful when used for transforming other algal strains (see Table 3), as well as seed plants (Elghabi et al., 2011). The use of the *cat* gene, encoding CAT that confers resistance to chloramphenicol, has also been widely used (Table 3). Finally, the mutated version of the *C. reinhardtii* gene encoding ribosomal protein S14 (*CRY1*) confers resistance to emetine and cryptopleurine, and was adopted as a dominant selection marker (Nelson et al., 1994). Indeed, most antibiotic resistance genes have been successfully and routinely used for algal transformations (see Table 3). Nonetheless, their use as

selection markers raises both health and ecological concerns for large-scale production in plants and microalgae.

### Selection of Nuclear Transgenic Algae Based on Herbicide Resistance

The demand for production of plants and microalgae that are free of antibiotic resistance markers encouraged the use of herbicide-resistance markers, such as acetohydroxyacid synthase (*ALS/AHAS*) that confers resistance to sulfometuron methyl (SMM), phytoene desaturase (*PDS*) that generates resistance to the bleaching herbicide norflurazon, and glyphosate acetyltransferase (*GAT*) that contributes to resistance against glyphosate (Malik et al., 1989).

*ALS/AHAS* catalyzes the first step in the biosynthesis of the branched-chain amino acids, valine, leucine, and isoleucine (Kishore and Shah, 1988; Chipman et al., 1998). It is the target enzyme of the SMM herbicide that effectively inhibits growth of bacteria, yeast, plants, and algae. A mutant form of the gene encoding *ALS/AHAS* was used as a dominant selectable marker in the green algae *Lobosphaera (Parietochloris) incisa* (Grundman et al., 2012), based on experience gained from transformation of the chloroplast of the unicellular red alga *Porphyridium* sp. (Lapidot et al., 2002), and the green algae *C. reinhardtii* (Kovar et al., 2002).

*PDS* functions in the carotenoid biosynthesis pathway. Inhibition of this enzyme causes degradation of chlorophyll and the chloroplast membrane, as well as photo-bleaching of green tissues (Boger and Sandmann, 1998). Point mutations in *PDS* confer enhanced resistance to the bleaching herbicide norflurazon in the green microalgae *H. pluvialis* (Steinbrenner and Sandmann, 2006; Sharon-Gojman et al., 2015) and *Chlorella zofingiensis* (Huang et al., 2008; Liu et al., 2014). The use of *GAT* that confers increased tolerance to glyphosate is less recommended due to the reduced growth of *GAT*-expressing transformants in response to high concentrations of glyphosate, which were required to inhibit wild type cells (Bruggeman et al., 2014).

## Targeting of Foreign Proteins to the Chloroplast

Targeting foreign proteins to sub-cellular compartments can affect their expression yield through protein folding, assembly and post-translational modifications. For example, the chloroplast provides an oxidizing environment that promotes the formation of disulfide bridges, and an abundance of chaperones that are required for the folding of soluble proteins after their import. The ability to properly fold and generate native disulfide bridges is fundamental for generating functional proteins and protein complexes, thus making the chloroplast an attractive organelle for the expression of recombinant proteins. This was demonstrated in *C. reinhardtii* by the production of the disulfide-containing Pfs25 protein from *Plasmodium falciparum*, which blocks transmission of the malaria parasite (Gregory et al., 2012). The importance of disulfide bond formation was also demonstrated by monitoring the expression and assembly of several fully active antibodies against pathogenic agents (Mayfield et al., 2003; Mayfield and Franklin, 2005).

Another advantage of organelle-specific expression is that targeting of nuclear-expressed proteins to chloroplasts can reduce the danger of proteolysis (Barnes et al., 2005; Doran, 2006; Mayfield et al., 2007). However, it still remains unclear why the ability to accumulate high levels of exogenous proteins in the chloroplast is heterogeneous. The plastid contains three large families of proteases, each of bacterial origin. These include the ATP-dependent Zn-metallo protease FtsH family (Adam et al., 2006; Liu et al., 2010), the ATP-independent Deg/HtrA family of serine endopeptidases (Huesgen et al., 2009; Sun et al., 2010) and the ATP-dependent serine-type Clp family (Adam et al., 2006). The chloroplast proteases can cleave exogenous proteins, as demonstrated in *C. reinhardtii* by the use of cyanide m-chlorophenylhydrazine (CCCP), which uncouples chloroplast and mitochondrial energy production. The presence of CCCP reduced degradation of the model protein VP28 from the White Spot Syndrome Virus three-fold expressed in the chloroplast, demonstrating that ATP-dependent proteases are involved in degrading this protein (Surzycki et al., 2009). Most chloroplast proteases are encoded in the nucleus, except for ClpP1. Thus, attempts to limit proteolysis of foreign genes expressed in the chloroplast can be controlled by knockdown technologies, such as RNAi, an approach that still requires further improvement in microalgae. Attempts to reduce expression of chloroplast-encoded ClpP by riboswitch control resulted in malfunctioning cells (Ramundo et al., 2014). However, deletion of the gene encoding ClpP protease from the chloroplast was achieved in *A. thaliana* (Zheng et al., 2006; Stanne et al., 2009), indicating that this approach could be feasible for microalgae as well.

Protein toxicity should also be considered when expression of foreign proteins in photosynthetic organisms is attempted. For example, the toxic effect of avidin, when expressed in the cytosol of transgenic tobacco plants, can be surpassed when this molecule is targeted to the vacuole (Murray et al., 2002). This also holds true for the cholera toxin-B subunit that is toxic to tobacco cells when expressed in the cytosol but not in the chloroplast (Daniell et al., 2001). Similar aspects should be considered when expressing foreign genes in microalgae.

Attempts to identify a consensus sequence shared by chloroplast and mitochondrial targeting peptides (cTPs and mTPs, respectively) were only partially successful (Habib et al., 2007; Huang et al., 2009). For this reason, data-driven machine learning techniques were developed (Schneider and Fechner, 2004), leading to the development of several programs, such as TargetP and Predotar (Emanuelsson et al., 2000; Emanuelsson, 2002; Small et al., 2004), that try to predict targeting peptides (TPs) for land plants. ChloroP is another program that predicts cleavage sites of TPs (Emanuelsson et al., 1999). These programs are based on the detection of an N-terminal targeting sequence that is shared between different targeted polypeptides. However, since green algae diverged from land plants over 725–1200 million years ago (Becker and Marin, 2009), their organelle import machineries, as well as their TPs, differ substantially from those of land plants. As such, most prediction programs are less reliable when used to predict the localization of algal proteins (Patron and Waller, 2007). With this in mind, a new algorithm, PredAlgo, that identifies cTPs/mTPs in green algae was developed. It is based on the accumulation of a large dataset from large- and small-scale proteomic studies of *Chlamydomonas* organelles (<https://giavap-genomes.ibpc.fr/cgi-bin/predalgotdb.perl?page=main>; Tardif et al., 2012).

A recent study, based on the genomes of *P. tricornutum* and *Thalassiosira pseudonana*, provides a detailed analysis of TP motifs that target nuclear-encoded proteins to chloroplasts in diatoms and algae with secondary plastids of the red lineage (i.e., dinoflagellates, cryptophytes, and stramenopiles; Gruber et al., 2015). Organisms that belong to these phyla are of great interest to algae-based industry.

## Targeting Transgene Products for Expression in Specific Organelles or Secretion

Targeting a transgene protein product for secretion is a common strategy for avoiding its degradation. A dedicated vector was designed in which luciferase expression in *C. reinhardtii* was greatly improved (up to 84%) by fusing the transgene with the previously identified secretion element of carbonic anhydrase (Lauersen et al., 2013a). This approach was further implemented for the production of secreted ice-binding protein, a protein of considerable industrial value (Lauersen et al., 2013b, 2015).

The targeting of nuclear transformation proteins to different cellular compartments is achieved by the addition of a TP-encoding sequence at the 5'-end of the transgene. TPs are recognized by the import machineries, which direct import into the proper organelle. Vectors that efficiently and specifically target transgene products to different compartments were generated (Rasala et al., 2014). Such vectors introduce TPs to the nucleus, mitochondria, chloroplast and ER using the nuclear localization signal (NLS) from simian virus 40, the N-terminal mTP from the nuclear gene encoding the alpha subunit of the mitochondrial ATP synthase, the N-terminal cTP from the photosystem I reaction center subunit II (encoded by *psaD*), and the ER-transit sequence from either BiP1 or ARS1, respectively. The TP that targets proteins to peroxisomes was also identified and was further shown to target a GFP transgene to the peroxisome of *C. reinhardtii* (Hayashi and Shinozaki, 2012).

In some cases, protein tagging is required for downstream applications, such as pull-down assays, immunoprecipitation, or protein purification. The introduction of such tags could interfere with the targeting signals. To prevent the obscuring of subcellular targeting signals, it is important to identify the TP of the native protein. In *Chlamydomonas*, endogenous proteins are usually tagged at their C-termini, since TPs are most frequently found at the N-terminus (Franzen et al., 1990; Patron and Waller, 2007).

## Increasing the Efficiency of Transgene Nuclear Expression

While chloroplast transformation toolkits have been established for several microalgae, similar tools for nuclear-based protein expression remain under-developed, as for land plants. The reasons that lead to the low expression of transgenes from the nuclear genome could be varied, including the effects of position on integration events, epigenetic-derived transgene silencing, and difficulties related to variable codon usage systems (Jinkerson and Jonikas, 2015). Although the precise mechanism of insertion of DNA into the genome is unknown, it involves ligation of the transforming DNA at a site of double-stranded genomic DNA break, an event that occurs randomly throughout the genome with little sequence specificity, via the non-homologous end joining repair pathway (Kindle et al., 1989; Mayfield and Kindle, 1990; Zhang et al., 2014). In some cases, the transformed DNA integrates as a whole cassette, although truncated versions, fragments, or multiple cassettes that result from enzymatic cleavage can also be inserted (Zhang et al., 2014). Random integration events can sometimes result in “position effects,” in which the level of transgene expression is influenced by the surrounding genomic regions (Leon and Fernandez, 2007). It is generally accepted that screening of a large number of transformants in search of a high expressing clone is necessary (Hallmann, 2007).

## Generation of Strains for Improved Transgene Expression

Despite the great advances made in developing systems for algal transformation, with *C. reinhardtii* serving as a fully sequenced model organism (Blaby et al., 2014), transgene expression using different methodologies and approaches remained limited until specific mutant strains capable of increased expression were isolated (Neupert et al., 2009). The basic assumption was that transgene expression could be affected by epigenetic processes, although the exact reasons remained unclear. To overcome this, a genetic screen was established in which wild type *C. reinhardtii* cells were subjected to random mutagenesis, and further screened for colonies that showed increased expression of the transgene. The screen used the ARG<sup>-</sup> selection system to select for transgenic algae, although another level of selection was introduced to screen for clones with increased expression. This was achieved by introducing *CRY1-1*, a ribosomal gene that confers resistance to emetine in a dose-responsive manner. As such, clones with increased *CRY1-1* expression could grow in the presence of high emetine concentrations. Two clones, UVM4 and UVM11, were selected for increased transgene expression and are currently used for in *Chlamydomonas* (Lauersen et al., 2013b).

It is tempting to examine whether a parallel system could be developed for other algae as well.

## Improvement of Transgene Expression by Fusion with a Selection Marker

An expression cassette in which the codon-optimized *GFP* gene was fused to the *ble* selection marker increased GFP expression in *C. reinhardtii* (Fuhrmann et al., 1999). A further improvement was achieved by including the self-cleavable 2A peptide derived from the foot and mouth disease virus (Ryan et al., 1991) between the transgene and the *ble* selection marker. Its presence resulted in processing of the fused polypeptides to yield two independent proteins. This system led to an ~100-fold increase in the expression of several transgenes (Rasala et al., 2012, 2013).

The combination of using novel algal strains that promote increased expression of foreign genes, along with the sophisticated fusion between selection and target genes, opens a new era in transgene expression by the green algae *Chlamydomonas*, although similar developments with other algae are called for as well. The use of *Chlamydomonas* overexpressing strains (i.e., UVM11), combined with the codon optimization of the target gene, was shown to overcome the expression barrier of transgenes in *Chlamydomonas*. This approach led to the efficient expression of an HIV vaccine candidate, P24 (Barahimipour et al., 2016).

## Expression of Transgenes from Episomes

A recent study reported the development of a nuclear episomal vector designed to introduce foreign DNA from *E. coli* into two diatom species, *P. tricornutum* and *T. pseudonana*, via conjugation. The vector contained a yeast-derived sequence that promoted its replication in these diatoms, even after antibiotic selection was eliminated. This episome was maintained as a closed circle at a copy number equivalent to the number of native chromosomes (Karas et al., 2015). This system offers great advantages in that it offers an easy method for introducing large DNA fragments into the host microalga, possibly promoting entry of several genes comprising a metabolic pathway. In addition, it is expected, although has yet to be shown, that expression of foreign genes from an episome would be less interrupted by epigenetic mechanisms or positional effects, since the DNA is not integrated into the chromosomal genome.

## Gene Targeting in Algal Nuclei

Nuclear transformation of microalgae, in most studied cases, exhibited low frequency of homologous recombination (Sodeinde and Kindle, 1993; Nelson and Lefebvre, 1995), as was also observed with most photosynthetic organisms, except for mosses. Although occasional successful homologous recombination in the nuclei of *Chlamydomonas* had been reported in the past (Sodeinde and Kindle, 1993; Nelson and Lefebvre, 1995; Dawson et al., 1997; Hallmann et al., 1997; Minoda et al., 2004; Zorin et al., 2005), its frequency was too low for adaptation as a recommended technology. Efficient homologous recombination suitable for biotechnology applications was recorded in only two eukaryotic algae,



namely *Cyanidioschyzon merolae* (Minoda et al., 2004) and *Nannochloropsis* sp. (Kilian et al., 2011).

Attempts to overcome difficulties that arise from positional effects and/or random integration of transgenes included the development of a Zn-finger nuclease system (Townsend et al., 2009) that can recognize and cleave a relatively long specific sequence, generating site-specific double-strand DNA breaks, thus modifying a gene of interest, as shown for the *COP3* gene encoding a light-activated ion channel (Sizova et al., 2013). Another methodology that is currently being developed for microalgae is based on the CRISPR/Cas9 system, which uses a guide RNA that directs the Cas9 nuclease to restrict a specific sequence of DNA (Jinek et al., 2012). Components of the CRISPR/Cas9 system have recently been shown to function in a transient manner in *C. reinhardtii*, although the high toxicity of Cas9 prevented the successful recovery of stable colonies (Jiang et al., 2014). As such, further improvement of the CRISPR/Cas9 system is required.

Nuclear transgenic expression in microalgae is occasionally inefficient, possibly due to gene silencing (Cerutti et al., 1997a), or other reasons that are not yet fully understood. Still, even if all the elements required for optimal transcription and translation of transgene are provided, expression of exogenous genes can be very low or even non-existent, possibly due to gene silencing (Cerutti et al., 2011). Furthermore, expression of exogenous genes might be eliminated if transgenic algae clones are not maintained under constant selection conditions.

## Gene Silencing in Algae through the RNAi Machinery

The recent sequencing of several algal genomes has provided insight into the great complexity of these species, although algal physiology and metabolomics are still not fully resolved. Nonetheless, key components of the RNA-mediated silencing machinery, such as Dicer and Argonaute that can process double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs; Sontheimer and Carthew, 2005; Ghildiyal and Zamore, 2009; Voinnet, 2009; Fabian et al., 2010), have been found in many algae species, including *C. reinhardtii* (Schroda, 2006; Merchant et al., 2007; Kim and Cerutti, 2009), *V. carteri* (Ebnet et al., 1999; Cheng et al., 2006; Prochnik et al., 2010), *D. salina* (Sun et al., 2008; Jia et al., 2009), *P. tricornutum* (Bowler et al., 2008; De Riso et al., 2009), and *E. gracilis* (Iseki et al., 2002; Ishikawa et al., 2008). RNA-mediated silencing pathways have been studied in the unicellular green alga *C. reinhardtii*, where they are used as a reverse genetics tool for targeted knockdown of a variety of genes (Sineshchekov et al., 2002; Rohr et al., 2004; Soupene et al., 2004; Schroda, 2006). RNAi methodology was also used to examine the function of Aureochrome, a photoreceptor required for photomorphogenesis in stramenopiles. Silencing of the *AUREO2* gene by introduction of dsRNA derived from the target gene induced the formation of sex organ primordia instead of branches, implicating it in the initiation of the development of a branch but not of a sex organ (Takahashi et al., 2007).

RNA-mediated silencing pathways have been studied in the unicellular green alga *C. reinhardtii* and used as a reverse genetics tool for targeted knockdown of a variety of genes (Sineshchekov et al., 2002; Rohr et al., 2004; Soupene et al., 2004; Schroda, 2006). The use of artificial miRNAs (amiRNAs), which mimic the structure of endogenous miRNA precursors was designed to enhance this approach (Molnar et al., 2009; Zhao et al., 2009). AmiRNAs can be designed by the microRNA designer platform (WMD3, <http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>, active on March 14, 2016), which identifies suitable amiRNA candidates, based on optimal and specific hybridization properties, to target mRNA (Ossowski et al., 2008). Stable RNAi gene silencing was demonstrated in different microalgae as well, including *V. carteri* (Ebnet et al., 1999; Cheng et al., 2006), *D. salina* (Jia et al., 2009), *P. tricornutum* (De Riso et al., 2009), and *E. gracilis* (Iseki et al., 2002). Non-integrative dsRNA or siRNA was also used to trigger temporary RNAi in the green alga *D. salina* (Sun et al., 2008), *E. gracilis* (Ishikawa et al., 2008), and *Vaucheria frigida* (Takahashi et al., 2007).

## BIOTECHNOLOGICAL EXPLOITATION OF MICROALGAE

Microalgae offer substantial potential for various biotechnological purposes, including production of animal food, aquaculture and marine agriculture, and biosynthesis of medical products, such as oral vaccines for animals (fish) and humans, high-value nutritional additives (e.g., polyunsaturated fatty acids, carotenoids, etc.), food dyes, and compounds used in the cosmetic industry and various other chemicals, in addition to possible future uses as a sources of energy. Moreover, microalgae-based technologies can be developed into water source treatments. These highly attractive goals justify the great efforts that the scientific community is investing in the development of molecular tools for the generation of transgenic microalgae.

### Using Synthetic Biology for the Production of Commercial Added Value in Algae

In view of the difficulties in obtaining a high level of transgene expression in the cytoplasm of algae, the production of numerous therapeutic proteins was targeted to the chloroplast, including antibodies and proteins for use as oral vaccines. For example, a single-chain antibody was produced against the herpes simplex virus glycoprotein D (Mayfield et al., 2003; Mayfield and Franklin, 2005), and an IgG1 monoclonal antibody was generated against the anthrax protective antigen 83 (Tran et al., 2009). There were also attempts to use the chloroplast for producing oral vaccines against malaria (Gregory et al., 2012, 2013; Jones et al., 2013; Patra et al., 2015), *Staphylococcus aureus* (Dreesen et al., 2010), the white spot syndrome virus protein 28 (Surzycki et al., 2009), the P57 antigen bacterial kidney disease, (Siripornadulsil et al., 2007), and the VP1 antigen of foot and mouth disease virus fused to the Cholera toxin B subunit (Sun et al., 2003). Microalgae were exploited for the production of other immune reactive proteins, such as the human glutamic acid



decarboxylase, a known auto-antigen in type 1 diabetes (Wang et al., 2008).

Heterologous therapeutic proteins were expressed not only in the chloroplast, but also in the nuclei of different algae, such as *D. salina*, *Porphyridium* sp., *Nannochloropsis oculata*, and *C. reinhardtii*, to produce edible vaccines against the malaria parasite *Plasmodium berghei* (Dauvillée et al., 2010), the surface antigen of Hepatitis B virus (Geng et al., 2003) and for the production of food additives such as the xylanase growth hormone (Rasala et al., 2012; Georgianna et al., 2013) and Sep15, a selenium supplement (Hou et al., 2013). Algal-derived oral vaccines were shown to have a long shelf-life and their administration is injection-free. However, as the use of oral vaccines elicits mainly local immunity, their general efficacy must be carefully monitored.

Microalga have been harnessed to express enzymes of interest (Rasala et al., 2010, 2012), as well as for the potential production of biofuels (Georgianna and Mayfield, 2012), although the economical validity of this approach is still not satisfactory. Efforts were also targeted to enhancing the metabolic engineering of algae for enrichment of lipids, although this goal has yet to be reached (Dunahay et al., 1996).

Genetic manipulations could also help in adapting microalgae to different growth conditions. Attempts to convert autotrophic algae into heterotrophs relied on introducing the *HUP1* gene from *C. kessleri*, which encodes for the hexose/H<sup>+</sup> transporter that enables growth in the dark, into *V. carteri* (Hallmann and Sumper, 1996) and *P. tricornutum* (Zaslavskaja et al., 2001). Increased hydrogen production (by 150%) was obtained when the *C. reinhardtii* SMT6 mutant, which is a high H<sub>2</sub>-producing strain, was transformed with the *HUP1* gene. This study established the possibility of improving the production of H<sub>2</sub> from H<sub>2</sub>O and glucose (Doebe et al., 2007). Increased hydrogen production was also obtained by a triple knockdown of the light harvesting complex proteins LHCB1, 2 and 3 by RNAi, which resulted in a 180% increase in hydrogen production (Oey et al., 2013).

Carotenoid production from *D. salina* and *H. pluvialis* is also of great commercial interest, and is based on the natural production of  $\beta$ -carotene and astaxanthin. Manipulation of these species is possible, with increased expression of phytoene desaturase resulting in accelerated biosynthesis of astaxanthin. Production of an L504R mutant of phytoene desaturase resulted in increased production of astaxanthin (Steinbrener and Sandmann, 2006). Furthermore, introducing the *C. zofingiensis* phytoene synthase, an enzyme that participates in the carotenoid biosynthetic pathway, into *C. reinhardtii* resulted in increased levels of violaxanthin and lutein (Cordero et al., 2011). These studies emphasize the great potential in manipulating metabolic pathways of high value products by engineering different algal species. Overall, microalgal systems hold great biotechnological potential that will surely be exploited in the future.

## Biosafety Considerations

In the European Union, all genetically modified organisms (GMOs) or their products must receive approval before they can find their way to market. This practice also applies to genetically

modified (GM) microorganisms. Thus, a guidance protocol for risk assessment of genetically modified microorganisms was prepared by the European Food Safety Authority (EFSA) in 2006 ([http://www.efsa.europa.eu/sites/default/files/scientific\\_output/files/main\\_documents/374.pdf](http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/374.pdf)). The guiding protocols discriminate between the contained use of microalgae and their deliberate release into the environment, as further discussed in an OECD meeting on the Biosafety and Environmental Uses of Micro-Organisms (Wijffels, 2015). Contained use refers to closed conditions in which GM microorganisms are grown, stored, transported, destroyed and disposed, whereas deliberate release refers to GM microorganisms that are released in the environment without restricting their ability to spread. Contained growth can be performed in closed bioreactors (Chaumont, 1993), tubular reactors (Richmond et al., 1993), or polyethylene sleeves (Cohen et al., 1991). These technologies are designed to allow efficient growth and the harvesting of large quantities of microalgal biomass. Examples of deliberate release of GM microorganisms include their use for immunization of fish against various pathogens in open ponds, or when microalgae are employed for cleaning polluted water sources. Risk assessment is usually performed by testing the effects a GM microorganism has on humans, animals, plants and/or the environment, and comparing these effects with those of the conventional non-transformed variant. Risk assessment refers to the toxicity and allergenicity of the GM microorganism or its products, as well as the potential risk of horizontal gene transfer (HGT). To date, there are only a few reports on HGT in microalgae, and these refer mainly to viral-mediated HGT (Monier et al., 2009; Rosenwasser et al., 2014). However, to address this potential risk, even if it is very rare, the use of selection modes based on endogenous metabolic markers that complement a missing activity in a mutant recipient strain would be advantageous over the use of genes that confer resistance to herbicides or antibiotics. Removal of the selection markers is also recommended.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Despite occupying the base of the plant evolutionary tree, algae are distributed worldwide and grow in different climates, under normal and extreme conditions. As such, algae are known to provide a rich repertoire of chemicals and bio-molecules, including those of interest to biotechnological industries. Algae can also be harnessed as an important food source in the frame of aquatic agriculture. Finally, they provide a powerful resource for the production of bio-medical molecules, such as vaccines, antibodies and drugs (Cadoret et al., 2012). Another attractive achievement will enable the introduction of foreign genes that comprise a metabolic pathway that generates a valuable compound. A recent effort to express the complete carotenoid pathway toward enhanced astaxanthin formation was reported for *Xanthophyllomyces dendrorhous* (Gassel et al., 2014). However, these exciting possibilities are fully dependent on efficient expression of transgenes in microalgae. Indeed, this bottleneck must be addressed, since for occasional cases

transgene expression had proven to be rather inefficient, for reasons that were not fully understood. Foreign genes can be targeted for expression in the chloroplast either by integration into the chloroplast genome or by their integration in the nuclear genome as fusion genes equipped with targeting signals that direct the import of the protein product into organelles. With regard to the general feasibility of expressing foreign proteins in microalgae, it appears that despite the considerable body of knowledge accumulated, inducing high expression of transgenes is difficult, possibly due to epigenetic mechanisms. Recent studies have made important improvements for overcoming this problem. Expanding the use of microalga for applied and basic research will benefit from the development of better protocols for gene knock-down by RNAi, as well as for successful deletion of specific genes by gene replacement. The recent revolution in genome editing provided by the CRISPR-Cas9 system should be adapted to microalgae in the coming years. Finally, the

improvement of systems for inducible expression is also of great importance, since this will allow us to control the temporal expression of toxic molecules. Finally, rapid advancements in genome sequencing, now routinely performed for all organisms, will prove instrumental in advancing these goals in the near future.

## AUTHOR CONTRIBUTIONS

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# Specific and Efficient Targeting of Cyanobacterial Bicarbonate Transporters to the Inner Envelope Membrane of Chloroplasts in *Arabidopsis*

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Installation of cyanobacterial bicarbonate transporters to the inner envelope membrane (IEM) of chloroplasts in C<sub>3</sub> plants has been thought to improve photosynthetic performance. However, the method to deliver cyanobacterial bicarbonate transporters to the chloroplast IEM remains to be established. In this study, we provide evidence that the cyanobacterial bicarbonate transporters, BicA and SbtA, can be specifically installed into the chloroplast IEM using the chloroplast IEM targeting signal in conjunction with the transit peptide. We fused the transit peptide and the mature portion of Cor413im1, whose targeting mechanism to the IEM has been characterized in detail, to either BicA or SbtA isolated from *Synechocystis* sp. PCC6803. Among the seven chimeric constructs tested, we confirmed that four chimeric bicarbonate transporters, designated as BicAI, BicAII, SbtAII, and SbtAIII, were expressed in *Arabidopsis*. Furthermore, these chimeric transporters were specifically targeted to the chloroplast IEM. They were also resistant to alkaline extraction but can be solubilized by Triton X-100, indicating that they are integral membrane proteins in the chloroplast IEM. One of the transporters, BicA, could reside in the chloroplast IEM even after removal of the IEM targeting signal. Taken together, our results indicate that the addition of IEM targeting signal, as well as the transit peptide, to bicarbonate transporters allows us to efficiently target nuclear-encoded chimeric bicarbonate transporters to the chloroplast IEM.

**Keywords:** bicarbonate transporter, chloroplast, *Arabidopsis*, protein targeting, cyanobacteria

## INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the enzyme that catalyzes the incorporation of CO<sub>2</sub> into ribulose 1, 5-bisphosphate (RuBP), resulting in the production of two 3-phosphoglycerate (3-PGA) molecules (Whitney et al., 2011). This reaction is known as the first step of inorganic carbon fixation of photosynthesis. In addition to the carboxylation reaction, Rubisco also catalyzes the oxygenation of RuBP. The oxygenation of RuBP produces one molecule of 2-phosphoglycerate (2-PG), as well as one molecule of 3-PGA. However, 2-PG cannot be utilized by the Calvin cycle and must be recycled back into 3-PGA via the photorespiration pathway.

This recycling process results in a partial loss of CO<sub>2</sub>, and energy consumption in C<sub>3</sub> plants, and has been thought to make photosynthesis in C<sub>3</sub> plants inefficient (Price et al., 2013).

Cyanobacteria and algae have evolved unique CO<sub>2</sub>-concentrating mechanisms (CCMs) to overcome this problem (Price et al., 2008, 2013). In cyanobacterial CCM, the key components are CO<sub>2</sub>/bicarbonate transporters, and the microcompartments called carboxysomes, which contain Rubisco. At least five distinct inorganic carbon (Ci) transporters have been identified to date (Price et al., 2008, 2013). BicA and SbtA are single-subunit, Na<sup>+</sup>-dependent bicarbonate transporters at the plasma membrane (Shibata et al., 2002; Price et al., 2004). Both genes are strongly induced by inorganic carbon limitation, and disruption of either gene impairs the photosynthetic capacity in cyanobacteria (Shibata et al., 2002; Price et al., 2004). In contrast, BCT1 is an ATP-binding cassette type bicarbonate transporter, composed of multiple subunits, and thought to utilize ATP as the energy for HCO<sub>3</sub><sup>−</sup> transport (Omata et al., 1999). In addition to the HCO<sub>3</sub><sup>−</sup> transport systems, NDH-I type CO<sub>2</sub> uptake systems are located at the thylakoid membranes and convert CO<sub>2</sub> to HCO<sub>3</sub><sup>−</sup>, thereby preventing the leakage of CO<sub>2</sub> from the cell (Klughammer et al., 1999; Ohkawa et al., 2000).

It has been proposed that the installation of CCMs into chloroplasts is a promising approach to improve photosynthesis in C<sub>3</sub> plants (Price et al., 2013; Price and Howitt, 2014a). According to a theoretical estimation, installation of BicA and SbtA into the chloroplast inner envelope membrane (IEM) improves photosynthetic CO<sub>2</sub> fixation rates (Price et al., 2013). Hence, installation of functional BicA and SbtA into the chloroplast IEM is becoming one of prime targets for the improvement of photosynthesis (Price and Howitt, 2014a). A major technical challenge is how to deliver bicarbonate transporters specifically to the chloroplast IEM. In cyanobacteria, bicarbonate transporter proteins are thought to be inserted into the plasma membrane from the cytosol (Frain et al., 2015). In land plant chloroplasts, at least two chloroplast-encoded proteins, Ycf1/Tic214 and CemaA, have been shown to be inserted into the IEM from the stroma (Sasaki et al., 1993; Kikuchi et al., 2013). This indicates that plastid-encoded membrane proteins can be targeted to the IEM. However, when the cyanobacterial bicarbonate transporter, BicA, was expressed from the plastid genome, the vast majority of the expressed BicA was targeted to the thylakoid membranes instead of the IEM (Pengelly et al., 2014). Because virtually nothing is known about the mechanism by which plastid-encoded membrane proteins are integrated into the chloroplast IEM, it remains a challenge to install plastid-encoded bicarbonate transporters precisely to the IEM.

As an alternative, the installation of nuclear-encoded bicarbonate transporters to the chloroplast IEM can be employed. It has been shown that IEM proteins utilize two distinct pathways for their targeting to the IEM (Inaba and Schnell, 2008; Oh and Hwang, 2015). One is the stop-transfer pathway and the other is the post-import or conservative pathway. To date, aside from Tic40 and Tic110, all the IEM proteins investigated seem to utilize the stop-transfer pathway, suggesting that a stop-transfer mechanism plays a key role in the biogenesis of IEM proteins

(Lubeck et al., 1997; Li and Schnell, 2006; Tripp et al., 2007; Firlej-Kwoka et al., 2008; Viana et al., 2010; Okawa et al., 2014). Both stop-transfer and post-import substrate proteins have bipartite signals, that is, they are composed of the transit peptide and IEM targeting signal (Inaba and Schnell, 2008; Oh and Hwang, 2015). The transit peptide is predictable, usually located at the N-terminus of precursor proteins, and cleaved off after the import into chloroplasts. In contrast, the IEM targeting signal is usually retained within the mature portion (Inaba and Schnell, 2008; Oh and Hwang, 2015). Our previous study demonstrated that the IEM targeting signal is sufficient to deliver the chimeric protein to the chloroplast IEM *in vivo* (Okawa et al., 2014). This suggests that the addition of the IEM targeting signal, as well as the transit peptide to bicarbonate transporters, enables us to install nuclear-encoded bicarbonate transporters into the chloroplast IEM.

In this study, we examined the installation of nuclear-encoded cyanobacterial bicarbonate transporters, BicA and SbtA, to the IEM of chloroplasts in *Arabidopsis*. We successfully expressed chimeric BicA and SbtA proteins in *Arabidopsis* chloroplasts. Furthermore, both chimeric bicarbonate transporters specifically accumulated to the IEM. One of the transporters, BicA, could reside in the chloroplast IEM even after removal of the IEM targeting signal. Based on these results, we propose a new approach to targeting nuclear-encoded cyanobacterial bicarbonate transporters to the chloroplast IEM by using chimeric constructs.

## MATERIALS AND METHODS

### Construction of Vectors and *Arabidopsis* Transformation

The *bicA* and *sbtA* genes were amplified from the genomic DNA of *Synechocystis* sp. PCC6803.

All the plasmids used to amplify each portion are described previously (Okawa et al., 2014). Primers used to amplify each portion are listed in Supplementary Figure S1. For the construction of BicAI and SbtAI, the TP-Cor413im1-protein A portion was amplified using pET/pre-Cor413im1-pA as the template (Okawa et al., 2014). For the construction of BicAII, BicAIII, SbtAII, and SbtAIII, the Cor413im1-Protein A and the K124-Protein A portions were amplified using pET/pre-Cor413im1-pA and pET/TP-K124-pA as the templates, respectively. Both the TP-protein A and Cor413im1 portions of BicAIV was amplified using pET/TP-pA-Cor413im1. The transit peptide portion of BicAII, BicAIII, SbtAII, and SbtAIII, was amplified using pET/TP-pA-Cor413im1.

After amplification of all these fragments, multiple DNA fragments were simultaneously sub-cloned into either *Nco*I-*Xba*I sites of pCambia3300 (for BicA), or *Nco*I-*Nhe*I sites of pCambia1301 (for SbtA) using an In-Fusion HD Cloning Kit (Takara) to obtain the constructs summarized in **Figure 1**.

All pCambia constructs were introduced into *Arabidopsis thaliana* (accession Columbia) via *Agrobacterium tumefaciens*-mediated transformation using the floral dip method (Clough and Bent, 1998).

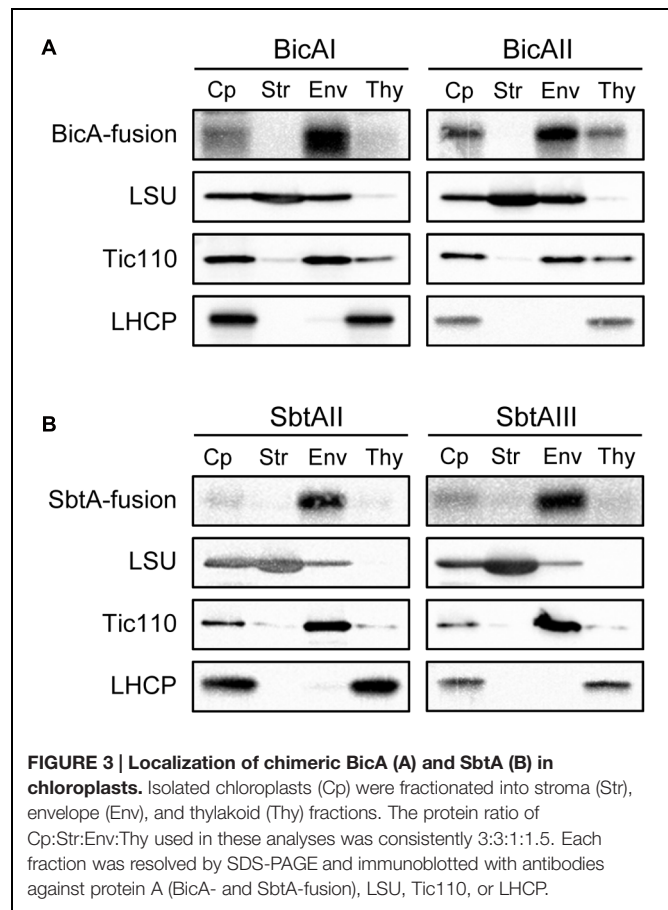
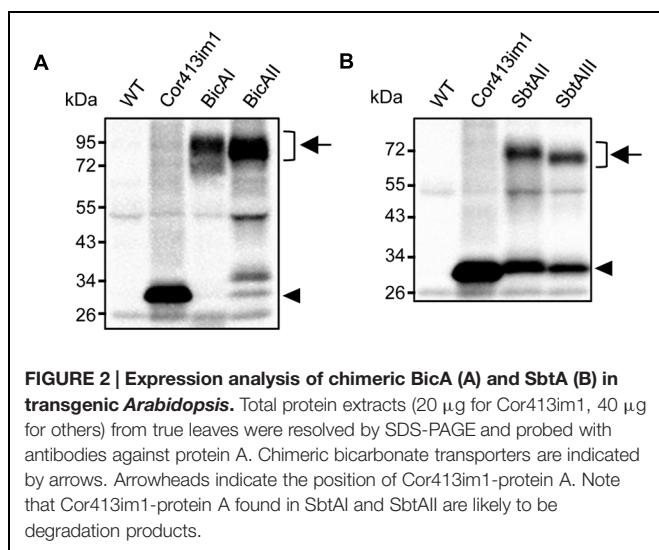




and *Staphylococcus* protein A, were fused to either BicA or SbtA, which are bicarbonate transporters found in cyanobacteria (**Figure 1A**). We also included K124 constructs lacking the sixth transmembrane domain of Cor413im1 as a fusion partner. According to our previous study (Okawa et al., 2014), the topology of K124 has been shown to be flipped at the IEM, and the C-terminus faces toward the stroma (**Figure 1B**). Hence, we assumed that the topology of a bicarbonate transporter fused to K124 is reverted, as compared to those fused to the full-length Cor413im1 (**Figure 1B**). All these constructs were transformed into *Arabidopsis* by *Agrobacterium*-mediated transformation. Among the seven chimeric bicarbonate transporter constructs we tested, we confirmed the expression of four chimeric proteins; BicAI, BicAII, SbtAII, and SbtAIII (**Figures 2A,B**). Although the expression level of each protein was lower than that of Cor413im1-pA (**Figures 2A,B**), it was apparent that the full-length chimeric proteins were indeed expressed in *Arabidopsis*. Based on these data, we concluded that those four chimeric bicarbonate transporters were stably expressed in *Arabidopsis*.

## Localization of Chimeric Proteins Within Chloroplasts

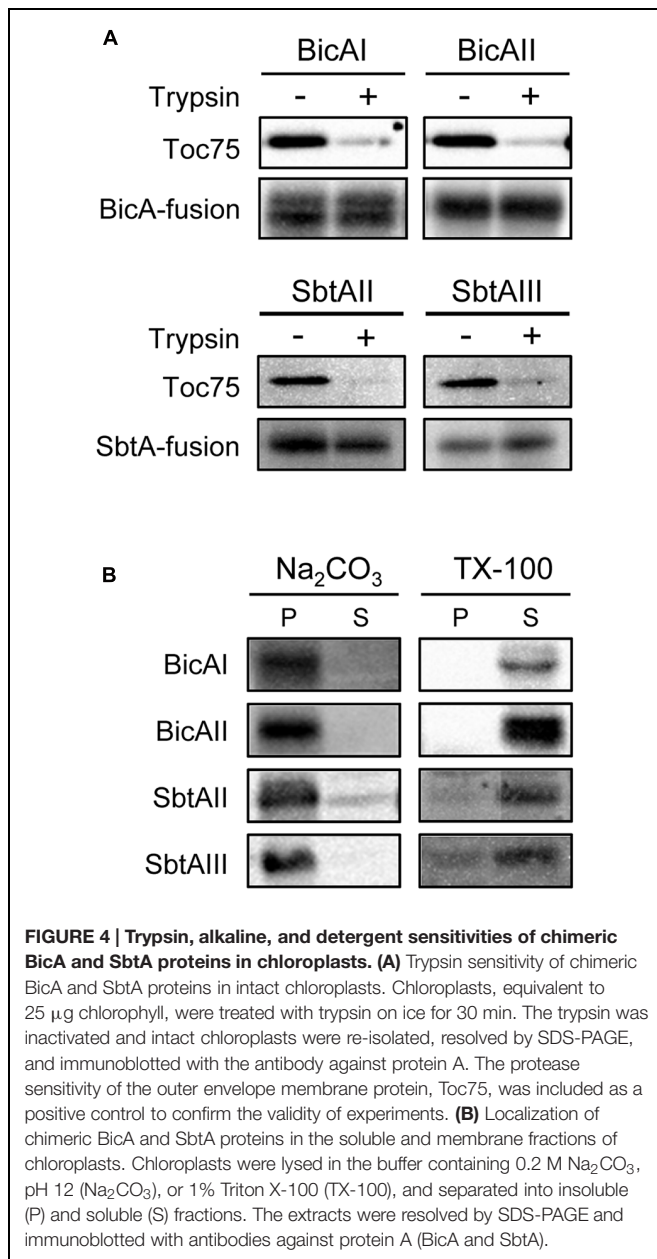
Next, we investigated the localization of these chimeric bicarbonate transporters within the chloroplasts. To this end, we isolated intact chloroplasts from these transgenic plants. Those chloroplasts were further fractionated into stroma, envelope, and thylakoid fractions (**Figures 3A,B**). The purity of each fraction was confirmed using marker proteins such as large subunit (LSU) of Rubisco (stroma), Tic110 (envelope), and light-harvesting complex protein (LHCP; thylakoid). As shown in **Figures 3A,B** (lanes Cp), all chimeric bicarbonate transporters were localized within the chloroplasts. Notably, each chimeric protein was found to be highly enriched in the envelope fraction (**Figures 3A,B**, lanes Env), indicating that the vast majority of these proteins are localized to the envelope membranes of chloroplasts. The level of enrichment of each chimeric protein in the envelope fraction was as high as Tic110, a genuine



chloroplast IEM protein (Supplementary Figure S2). A previous study showed that the vast majority of BicA was targeted to the thylakoid membrane instead of the IEM when it was expressed from the plastid genome (Pengelly et al., 2014). We also observed that a small amount of each chimeric protein was fractionated into the thylakoid fraction (**Figures 3A,B**, lanes Thy). However, those are likely to be contaminants, as a small portion of Tic110 was also observed in the thylakoid fraction (Tic110 in **Figures 3A,B**, lanes Thy). In fact, the thylakoid marker protein, LHCP, was virtually undetectable in the envelope fraction (Supplementary Figure S2). Hence, we concluded that the vast majority of each chimeric bicarbonate transporter was specifically targeted to the envelope membranes of chloroplasts.

## Each Chimeric Bicarbonate Transporter Exists as an Inner Envelope Membrane Protein of Chloroplasts

The fact that each chimeric protein is enriched into the envelope fraction prompted us to further investigate the nature of these proteins in detail. We next investigated whether each chimeric transporter is an outer or inner envelope membrane protein. We isolated intact chloroplasts from transgenic plants expressing chimeric bicarbonate transporters and treated them with trypsin. Trypsin permeates the outer envelope membrane, but not the



IEM, of intact chloroplasts (Jackson et al., 1998). As expected, the outer envelope membrane protein Toc75 was digested by trypsin (Figure 4A, Toc75). In contrast, all the chimeric bicarbonate transporters were resistant to the trypsin treatment (Figure 4A), indicating that those chimeric proteins are localized to the IEM.

Finally, we examined whether these chimeric proteins were integrated into the IEM, or peripherally associated with the IEM. When intact chloroplasts were solubilized with 1% Triton X-100, and fractionated into soluble and insoluble fractions, all chimeric proteins were partitioned into the soluble fraction (Figure 4B, TX-100). In contrast, all these proteins were resistant to alkaline extraction (Figure 4B, Na<sub>2</sub>CO<sub>3</sub>). These data indicate that each chimeric protein is an integral membrane protein at the chloroplast IEM.

Overall, our results indicate that the chimeric bicarbonate transporters fused to the IEM protein, Cor413im1, were specifically and efficiently targeted to the chloroplast IEM.

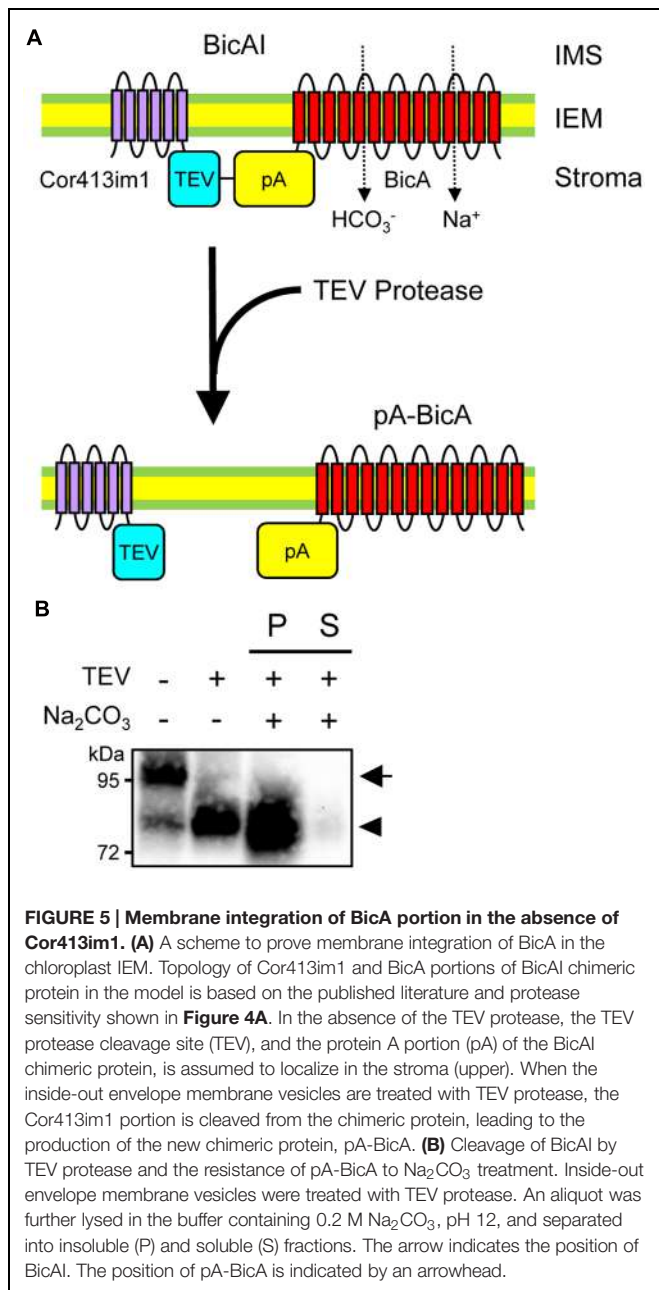
## Bicarbonate Transporters Can Reside in the Chloroplast IEM Even After the Removal of the IEM Targeting Signal

Although chimeric BicA and SbtA were integrated into the IEM, it is still unclear whether the bicarbonate transporters themselves are embedded to the IEM. For instance, one can argue that the Cor413im1 portion of each chimeric protein can serve as a membrane anchor, such that the chimeric proteins can reside in the IEM without the integration of the bicarbonate transporters into the IEM. To address this concern, we investigated if the removal of the Cor413im1 portion from BicAI affects the membrane localization of the BicA portion or not. As shown in Figure 5A, BicAI has a TEV protease cleavage site between Cor413im1 and protein A. Treatment of the inside-out envelope membrane vesicles of BicAI allows the removal of Cor413im1 from the chimeric protein, resulting in the creation of the pA-BicA chimeric protein (Figure 5A). Therefore, we investigated if the chimeric pA-BicA can reside in the IEM of chloroplasts or not. As predicted, the treatment of inside-out envelope membrane vesicles, isolated from BicAI chloroplasts, with TEV protease resulted in the production of a 75–80 kDa protein, which is pA-BicA (Figure 5B). This pA-BicA was resistant to alkaline extraction (Figure 5B). These data indicate that the BicA portion of the chimeric BicAI protein was integrated into the chloroplast IEM. Furthermore, the BicA portion can reside in the chloroplast IEM even after the removal of the IEM targeting signal.

## DISCUSSION

Because of the growing demand on the food supply, increasing crop production by improving photosynthesis is becoming one of the major targets for plant scientists (Whitney et al., 2011; Price and Howitt, 2014a). One such approach is to install bicarbonate transporters into the chloroplast IEM (Price et al., 2011, 2013). However, due to the lack of knowledge concerning the targeting mechanism of chloroplast IEM proteins, it has been challenging to install bicarbonate transporters into the chloroplast IEM. In this study, we took advantage of the IEM targeting signal to specifically target bicarbonate transporters to the IEM. We established a method that allows the efficient and specific targeting of nuclear-encoded cyanobacterial bicarbonate transporters, BicA and SbtA, to the IEM of chloroplasts in *Arabidopsis*. Unlike plastome-expressed BicA, which are primarily targeted to thylakoid membranes (Pengelly et al., 2014), both chimeric bicarbonate transporters were almost exclusively targeted to the IEM within the chloroplasts (Figures 3A,B). Our results strongly suggest that the chloroplast IEM targeting signal, together with the transit peptide, can serve as a potential tool to install CCMs into the chloroplasts of land plants.

A previous study suggested that a certain class of transplastomic IEM proteins can be destined to the chloroplast IEM specifically. When the *TIC40* gene was expressed in the

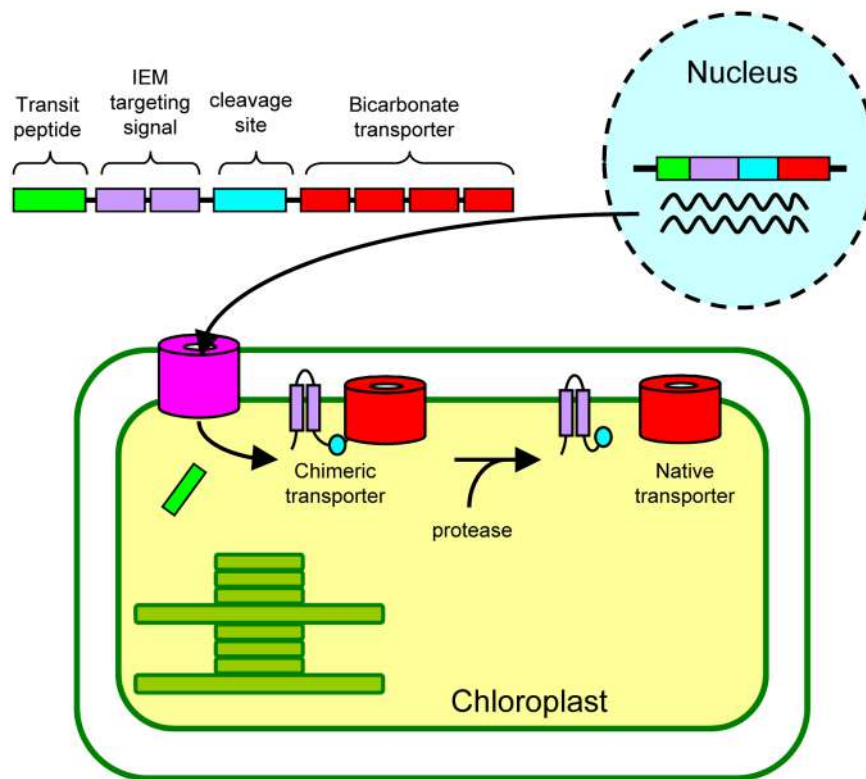


plastid genome, the plastome-expressed Tic40 protein was properly targeted, processed, and inserted into the IEM (Singh et al., 2008). Furthermore, chloroplasts in the transformed plants exhibited massive proliferation of the chloroplast IEM, and accumulated large amounts of plastome-expressed Tic40 (Singh et al., 2008). In contrast, when cyanobacterial BicA was transformed into the plastid genome, chloroplasts in transformed plants failed to accumulate the plastome-expressed BicA at the IEM (Pengelly et al., 2014). Instead, the majority of BicA was targeted to the thylakoid membranes. The reason why plastome-expressed BicA was targeted to the thylakoid membranes can be explained by the fact that a vast majority of IEM proteins are not inserted into the IEM from the stroma

(Oh and Hwang, 2015). Among the nuclear-encoded IEM proteins, Tic40, and Tic110 have been demonstrated to be re-inserted into the IEM from the stroma, and utilize soluble intermediates (Lubeck et al., 1997; Li and Schnell, 2006; Tripp et al., 2007). In contrast, the vast majority of other proteins tested to date appear to be targeted to the IEM by a stop-transfer mechanism, and do not utilize soluble intermediates (Li and Schnell, 2006; Tripp et al., 2007; Firlej-Kwoka et al., 2008; Viana et al., 2010; Froehlich and Keegstra, 2011; Okawa et al., 2014). Furthermore, it appears that hydrophobic proteins lacking the IEM targeting signal seem to be mistargeted to the thylakoid membrane (Okawa et al., 2014). When truncated Cor413im1 lacking IEM targeting signal was expressed in *Arabidopsis*, the majority of Cor413im1 was destined to the thylakoid membrane (Okawa et al., 2014). Hence, we speculate that, without IEM targeting signal, it will be challenging to install plastome-expressed bicarbonate transporters into the chloroplast IEM specifically.

Intriguingly, some of the chimeric proteins were undetectable in transgenic *Arabidopsis*. We speculate that the transmembrane topology of each chimeric protein may be attributable to those observations. According to a topology prediction in the previous study, BicA and SbtA possess 14 and 10 transmembrane segments (Price, 2011; Price and Howitt, 2014b). Both the N- and C-termini of BicA are predicted to face the cytoplasm in cyanobacteria (Price and Howitt, 2014b). In contrast, both the N- and C-termini of SbtA seem to localize in the periplasm (Du et al., 2014). Because the Cor413im1 protein has 6 transmembrane segments and both N- and C-termini faces the stroma (Okawa et al., 2008) (**Figure 1B**), we assume that BicAI and BicAII are likely to possess desirable transmembrane topology for the transport of bicarbonate into chloroplasts. Likewise, the N-terminus of the K124 portion of the SbtAIII construct is likely to be exposed to the intermembrane space because K124 is predicted to have five transmembrane segments (**Figure 1B**). Hence, it is conceivable to speculate that SbtAIII likely exhibits a desirable transmembrane topology for the transport of bicarbonate into chloroplasts. Overall, the chimeric proteins expressed in our study seem to possess the desirable transmembrane topology, with the exception of SbtAII.

Finally, we propose a possible approach by which we can install active cyanobacterial bicarbonate transporters into the chloroplast IEM (**Figure 6**). As demonstrated in our current study, the addition of the IEM targeting signal to the bicarbonate transporters is necessary. Although we have used the full length and truncated Cor413im1, the IEM targeting signals can be further optimized for practical application (e.g., minimizing the length of an IEM targeting signal). These chimeric genes can be transformed into the nucleus. Unlike chloroplast transformation, nuclear transformation can be performed in numerous plant species. Once the chimeric proteins are targeted to the chloroplast IEM, they must be activated, allowing the active incorporation of bicarbonate into chloroplasts. Activity of the chimeric transporter may be sufficient to transport bicarbonate into chloroplasts, but it is entirely possible that addition of an IEM targeting signal could inhibit the activity of the bicarbonate transporter.



**FIGURE 6 | A proposed approach to install cyanobacterial bicarbonate transporters, encoded by the nuclear genome, into the inner envelope membranes (IEM) of chloroplasts in land plants.**

If this is the case, the IEM targeting signal can be removed from the chimeric protein using a protease, resulting in the production of the “native” bicarbonate transporter at the IEM. Recent studies have shown that the  $\beta$ -carboxysome-like structure can be reconstituted within chloroplasts (Lin et al., 2014a,b). Hence, once the activities of the bicarbonate transporters at the chloroplast IEM are evaluated, simultaneous installation of carboxysomes, as well as bicarbonate transporters, would be possible to improve photosynthesis in  $C_3$  plants.

## CONCLUSION

We successfully installed chimeric cyanobacterial bicarbonate transporters into the chloroplast IEM. Although the effects of those chimeric bicarbonate transporters on photosynthesis remain to be characterized, the specific and efficient targeting of cyanobacterial bicarbonate transporters to the chloroplast IEM serve as a milestone toward achieving “turbocharged photosynthesis.”

## AUTHOR CONTRIBUTIONS

TI and YI-I designed and supervised the research. SU, FA, and TI performed research. SU, YI-I, and TI analyzed data. SU and TI wrote the paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00016>



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# The *Arabidopsis minE* mutation causes new plastid and FtsZ1 localization phenotypes in the leaf epidermis

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Plastids in the leaf epidermal cells of plants are regarded as immature chloroplasts that, like mesophyll chloroplasts, undergo binary fission. While mesophyll chloroplasts have generally been used to study plastid division, recent studies have suggested the presence of tissue- or plastid type-dependent regulation of plastid division. Here, we report the detailed morphology of plastids and their stromules, and the intraplastidic localization of the chloroplast division-related protein AtFtsZ1-1, in the leaf epidermis of an *Arabidopsis* mutant that harbors a mutation in the chloroplast division site determinant gene *AtMinE1*. In *atminE1*, the size and shape of epidermal plastids varied widely, which contrasts with the plastid phenotype observed in *atminE1* mesophyll cells. In particular, *atminE1* epidermal plastids occasionally displayed grape-like morphology, a novel phenotype induced by a plastid division mutation. Observation of an *atminE1* transgenic line harboring an *AtMinE1* promoter::AtMinE1-yellow fluorescent protein fusion gene confirmed the expression and plastidic localization of AtMinE1 in the leaf epidermis. Further examination revealed that constriction of plastids and stromules mediated by the FtsZ1 ring contributed to the plastid pleomorphism in the *atminE1* epidermis. These results illustrate that a single plastid division mutation can have dramatic consequences for epidermal plastid morphology, thereby implying that plastid division and morphogenesis are differentially regulated in epidermal and mesophyll plastids.

**Keywords:** *Arabidopsis thaliana*, chloroplast, fluorescent protein, FtsZ, leaf epidermis, MinE, plastid division, stromule

## Introduction

Plastids are ancient prokaryote-derived organelles in plant cells that multiply via division of pre-existing organelles. The photosynthetic plastids, chloroplasts, undergo symmetric division, which produces a homogeneous population of spherical chloroplasts in each mature-leaf cell (Possingham and Lawrence, 1983; López-Juez and Pyke, 2005; **Figures 1A,B**). Chloroplast fissions are driven by

a macromolecular complex located on the double-envelope membrane at the plastid constriction site. In the model plant *Arabidopsis thaliana*, approximately 10 nuclear-encoded proteins are located at distinct subplastidic compartments, which constitute the division apparatus (Yang et al., 2008; Okazaki et al., 2010). Among these proteins, the prokaryotic tubulin-like protein FtsZ, which stands for “Filamenting temperature-sensitive Z”, plays a central role in the initiation and progress of chloroplast division. *A. thaliana* has two phylogenetically distinct, functionally non-redundant FtsZ proteins, FtsZ1 and FtsZ2 (Stokes and Osteryoung, 2003; Miyagishima et al., 2004), both of which have polymerization and filament-bundling activities, enabling them to form a contractile ring structure at the mid-chloroplast site (McAndrew et al., 2001; Vitha et al., 2001; Yoder et al., 2007).

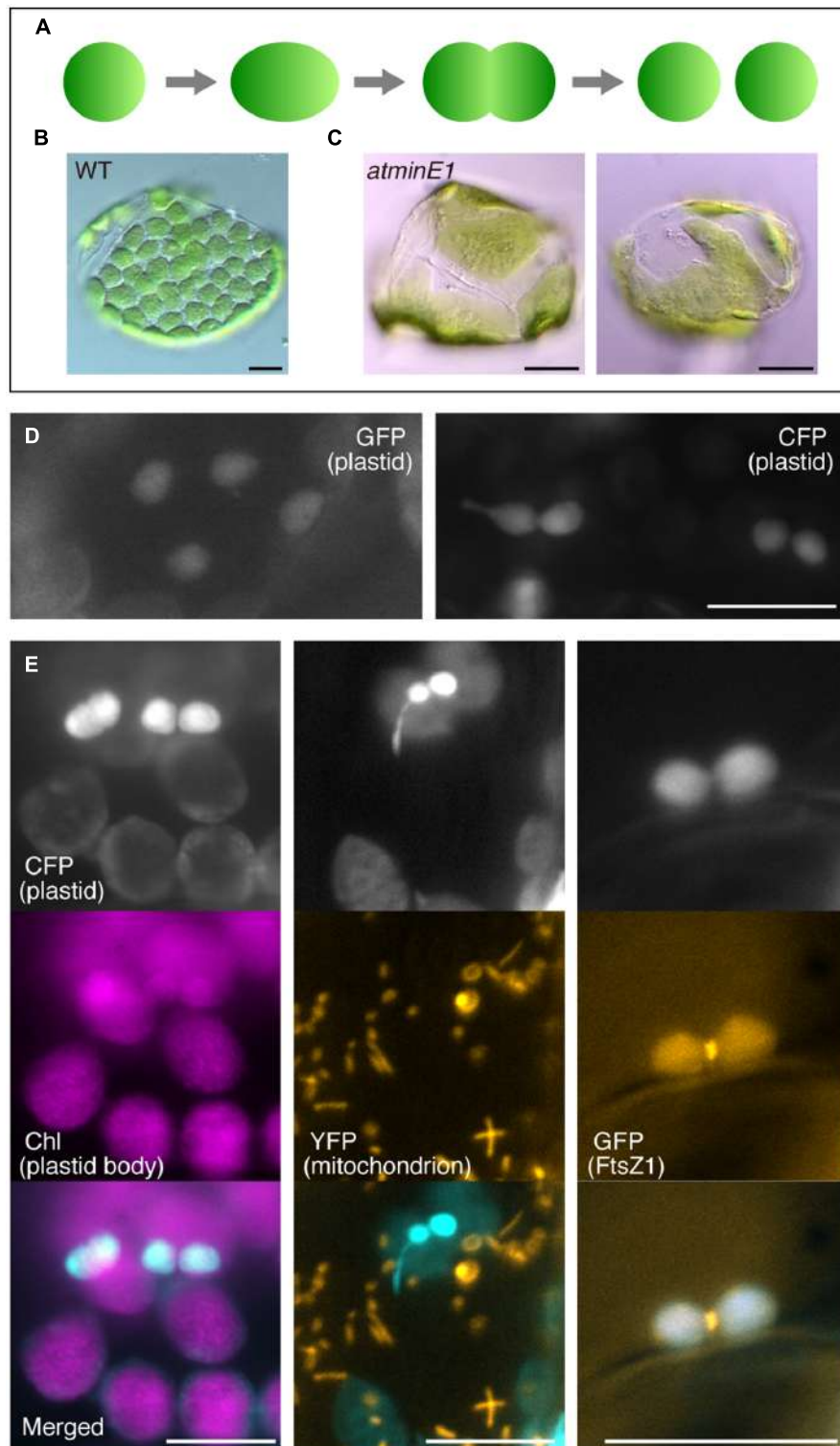
The symmetry and the “one division site (or one FtsZ ring) at a time” manner of normal chloroplast division in leaf mesophyll cells (Figure 1A) rely on at least four chloroplast proteins; MinD (Colletti et al., 2000; Kanamaru et al., 2000), MinE (Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002), ARC3 (Shimada et al., 2004; Maple et al., 2007), and MCD1 (Nakanishi et al., 2009). MinD and MinE are endosymbiont-derived stromal proteins, while ARC3 is a plant-specific stromal protein harboring an FtsZ-like domain. MCD1 is a plant-specific membrane protein that spans the inner envelope and recruits MinD to the division site. In *A. thaliana*, only one homolog of *minE*, designated *AtMinE1*, is encoded by the nuclear genome (Itoh et al., 2001). Overexpression, repression, or insertional mutation of *AtMinE1* results in the generation of enlarged chloroplasts and a reduced number of chloroplasts per mesophyll cell, although the chloroplast morphology differs among these three plant types (Itoh and Yoshida, 2001; Itoh et al., 2001; Fujiwara et al., 2008). The relationship between *minE* expression levels and the chloroplast morphology phenotype in *A. thaliana* is analogous to that in *Escherichia coli* cells: in both cases, *minE* overexpression results in a heterogeneous population of chloroplasts or cells, whereas its repression generates giant chloroplasts or elongated cells due to inhibited division (de Boer et al., 1989).

The best-characterized pathway of plastid division in plants is chloroplast division in leaf mesophyll cells (Pyke, 1997). The discovery and detailed characterization of a series of *A. thaliana* mutants, the *accumulation and replication of chloroplasts* (*arc*) mutants (Pyke, 1997), bearing a numerically and morphologically unusual population of mature-leaf mesophyll chloroplasts, has revealed a good correlation between the state of chloroplast division apparatus and the mutant chloroplast phenotypes. Namely, the degree of division apparatus disorganization is reflected by the degree of reduction in chloroplast number, as well as the extent of organelle enlargement. In *A. thaliana arc6*, the most severe mutant of chloroplast division, only one or a few giant chloroplasts are generated per leaf mesophyll cell (Pyke et al., 1994) because the FtsZ ring assembly is completely blocked in this mutant (Vitha et al., 2003), although the total chloroplast compartment volume per cell remains constant via a compensatory mechanism (Pyke, 1997). This cause-and-effect

relationship fits well with the principle of cell division in organisms. Thus, the chloroplast replication state in terminally differentiated leaf mesophyll cells has been used as a diagnostic aid to determine the significance of target genes in chloroplast division.

In recent years, however, it has become clear that the framework of “division inhibition leading to enlargement of plastids” (Figure 1C, for example) is not necessarily applicable to every cell lineage or plastid type. Forth and Pyke (2006) found that the tomato *suffulta* mutation results in the formation of a few giant chloroplasts per cell, but these convert into a wild-type-like population of chromoplasts by budding and fragmentation of plastids or stromules during fruit ripening. Holzinger et al. (2008) examined the effects of *A. thaliana arc3*, *arc5*, and *arc6* mutations on plastid number and morphology in many plant organs or tissues, showing that stromules are more abundant in several epidermal tissues in these mutants compared to wild type. In addition, Chen et al. (2009) observed plastids in the embryos of *A. thaliana crumpled leaf* (*crl*) and *arc6* mutants, implying that plastid protrusions lacking chlorophylls can serve as precursors of new daughter plastids during cell division. Furthermore, we previously demonstrated that stromule length and frequency increase without a dramatic change in plastid size or number in several tissues of the chloroplast division mutant *atminE1* (Kojo et al., 2009), which harbors a T-DNA insertion in the *AtMinE1* locus and hence produces severely reduced levels of *AtMinE1* transcripts (Fujiwara et al., 2008). These findings prompted us to formulate an alternative framework to explain the relationship between FtsZ-based plastid division and plastid morphogenesis, especially stromule formation, in non-mesophyll cells.

We therefore focused our attention on the leaf epidermis. To date, plastid morphology and division in the leaf epidermis of chloroplast division mutants has largely been overlooked, with the exception of the tomato *suffulta* mutant (Forth and Pyke, 2006), the *A. thaliana msl2 msl3* double mutant (Haswell and Meyerowitz, 2006), and the *atminE1* mutant (Fujiwara et al., 2009). In a previous study (Fujiwara et al., 2009), we observed two distinct types of plastid morphology and an altered configuration of *AtFtsZ1-1* (*A. thaliana* FtsZ1) in the leaf epidermis of *atminE1*. Specifically, we observed giant plastids containing short filaments and multiple dots of FtsZ1 and relatively poor plastids containing multiple FtsZ1 rings or spiral(s). The latter type was observed in the leaf epidermis but not in mesophyll cells, suggesting that the plastid division system operates in a tissue-specific manner. Hence, the *atminE1* mutant is well suited for investigating the tissue-specific characteristics of plastid morphology and division. To effectively visualize epidermal plastids (immature chloroplasts), which are only weakly pigmented, we utilized plastid (stroma)-targeted cyan fluorescent protein (CFP). CFP is an effective fluorophore for labeling organelles in the leaf epidermis, because the background fluorescence signals from chloroplasts in the underlying mesophyll layer are relatively low compared to those obtained using green fluorescent protein (GFP; Kato et al., 2002). In fact, the fluorescent images of CFP-labeled plastids in the leaf epidermis were relatively clear and showed a high signal-to-background ratio compared to those



**FIGURE 1 | Utility of cyan fluorescent protein (CFP) to investigate plastid morphology in *Arabidopsis* leaf epidermis. (A–C)** A framework describing the replication and morphology of leaf mesophyll chloroplasts. Schematic diagram of chloroplast replication by binary fission **(A)** and chloroplast phenotypes in WT **(B)** and *atminE1* **(C)** leaf mesophyll cells are shown. **(D)** Detection of plastid-targeted green fluorescent protein (GFP, left) and CFP (right) in leaf epidermis. **(E)** Dual detection of plastid-targeted CFP and chlorophyll (Chl magenta-colored), mitochondria-targeted YFP (orange-colored) or FtsZ1–GFP (orange-colored) in leaf epidermis. **(D,E)** Leaves from 2-week-old seedlings of WT-background transgenic lines were observed by fluorescence microscopy. In merged images, CFP fluorescence is colored in cyan. Bars = 5  $\mu\text{m}$  (black) and 10  $\mu\text{m}$  (white).



from GFP-labeled plastids (Figure 1D). In the present study, we employed transgenic *atminE1* lines that stably express a transgene encoding plastid-targeted CFP. Extending our earlier observations (Fujiwara et al., 2009), we explored the detailed morphology of leaf epidermal plastids, stromules, and other types of plastid substructures, the localization of FtsZ1 within the plastids, and their possible associations with plastid constriction in the *atminE1* mutant.

## Materials and Methods

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* (L.) Heynh. ecotypes Columbia (Col) and Wassilewskija (Ws) were used as wild-type (WT) plants. A T-DNA insertional mutant of *AtMinE1*, Flag\_056G07 (DLFTV7T3, Ws background), was obtained from Institut National de la Recherche Agronomique (INRA, Versailles, France) (Samson et al., 2002). A transgenic line, Z1g11 (Col background), expressing a full-length AtFtsZ1-1-sGFP(S65T) fusion (FtsZ1-GFP) under an upstream genomic sequence of *AtFtsZ1-1*, and its cross with *atminE1* were previously described (Fujiwara et al., 2008, 2009). Another transgenic line, FL4-4 (Col background), expressing stroma-targeted CFP (TP<sub>FtsZ1-1</sub>-CFP), and matrix-targeted yellow fluorescent protein (YFP; Pre<sub>mHSP60</sub>-YFP) under the control of the CaMV35S promoter, and its cross with *atminE1*, were also generated previously (Itoh et al., 2010). A stable line, ptA5-3 (Col background), expressing stroma-targeted GFP (TP<sub>RBCS3A</sub>-GFP) under the control of the CaMV35S promoter (Niwa et al., 1999), was provided by Dr. Yasuo Niwa. Plants were germinated and grown as previously described (Fujiwara et al., 2009), except for the duration of cold treatment of seeds being 4 days.

### Generation of Transgenic Line Expressing Stroma-targeted CFP

To monitor plastids in living tissues of *A. thaliana*, a gene cassette expressing an N-terminal transit peptide sequence (90 aa) of AtFtsZ1-1 fused to the N-terminus of CFP (provided by Dr. Atsushi Miyawaki) (TP<sub>FtsZ1-1</sub>-CFP) under the control of the CaMV 35S promoter and the NOS terminator was inserted into the *Hind*III and *Eco*RI sites of the binary vector pSMAB704 (Igasaki et al., 2002; provided by Dr. Hiroaki Ichikawa) by simultaneously removing the vector-derived CaMV35S promoter, *uidA*, and NOS terminator cassette (see Supplementary Figure S1). The resulting vector, pSMAB-Z1TP-sC, was employed for *Agrobacterium*-mediated Col transformation by the floral dip method (Clough and Bent, 1998). A total of 429 transformed (T<sub>1</sub>) seedlings were selected on bialaphos (4 µg/l, Meiji Seika, Tokyo, Japan)-containing MS plates. Of 13 lines that showed high and stable CFP fluorescence, one line, FC1-7, was chosen for its fluorescence stability over three generations without occurrence of transgene silencing, with the aid of stereofluorescence microscopy (model FLIII; Leica Microsystems, Heidelberg, Germany). FC1-7 was crossed with Z1g11 and Z1g11 × *atminE1* to efficiently visualize the stroma in these lines. The F<sub>3</sub> progenies were characterized by fluorescence microscopy.

### Complementation Assay of *atminE1* with *AtMinE1-YFP*

A genomic copy of *AtMinE1* was amplified by PCR with oligonucleotide primers E1-9 (5'-GAG TCG ACC CGG GTT ACG AAG AAG CCT TGG TTC-3') and E1-8 (5'-TGT CGA CCT CTG GAA CAT AAA AAT CGA ACC-3') (*Sal*I and *Sma*I restriction sites italicized). The PCR product (2.7 kb), comprising a 1.2 kb upstream genomic region of *AtMinE1* and the *AtMinE1* open reading frame, was introduced into pSMAB704 by simultaneously removing the CaMV35S promoter, *uidA*, and the NOS terminator cassette and co-introducing a 1.0 kb *Sal*I-*Eco*RI fragment of the *YFP::NOS* terminator cassette (the original *YFP* [*Venus*] was provided by Drs. Takeharu Nagai and A. Miyawaki). The resulting plasmid, pSMAB-E1-V, was employed for *Agrobacterium*-mediated transformation of the *A. thaliana atminE1* mutant (Clough and Bent, 1998). A total of 24 T<sub>1</sub> seedlings were selected as described above. The T<sub>2</sub> or T<sub>3</sub> progenies were analyzed by quantitative RT-PCR, immunoblotting, stereofluorescence microscopy, and epifluorescence microscopy.

### Quantitative RT-PCR Analysis

Total RNA from primary leaves of 2-week-old seedlings of the Ws, *atminE1*, and transgenic *atminE1* lines (overexpression line [E1v10], complemented line [E1v24]) was extracted and subjected to quantitative RT-PCR as previously described (Fujiwara et al., 2010). The primers used were as follows: for 18S *rRNA* (used as an internal control), 18S<sub>rRNA</sub>F6: 5'-GAC TAC GTC CCT GCC CTT TGT-3' and 18S<sub>rRNA</sub>R6: 5'-ACT TCA CCG GAT CAT TCA ATC G-3'; for *AtMinE1*, AtMinE1-FOR4: 5'-TCA TTA CCT TCT TCT TCT TCC-3' and AtMinE1-REV4: 5'-TGC AAG AAC CTT CAC CTG ACC-3'; and for *AtMinD1*, MD-FOR3: 5'-AAT GGC GAC AAC TGA GAA ACC-3' and MD-REV: 5'-CGC GTA TCG TCG TTA TCA CCT-3'.

### Western Blotting

Total proteins were extracted from aerial parts (~50 mg) of 17-day-old soil-grown *A. thaliana* plants and subjected to Western blotting as previously described (Fujiwara et al., 2009). The band intensity was quantified with image processing software ImageJ version 1.43j (<http://rsb.info.nih.gov/ij/>).

### Stereofluorescence Microscopy

Whole seedlings or floral organs were observed under a stereofluorescence microscope (FLIII [Leica Microsystems]) equipped with a CCD digital camera (ORCA-ER [Hamamatsu Photonics, Hamamatsu, Japan]). YFP and chlorophyll signals were detected with standard filter sets for EYFP (Leica; excitation: 500–520 nm; emission: 540–580 nm) and Texas Red (excitation: 540–580 nm; emission: >610 nm), respectively. Digital black-and-white images were processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA).

### Epifluorescence Microscopy

Whole plant organs were mounted under glass coverslips and observed under an epifluorescence microscope (IX70 [Olympus, Tokyo, Japan]), equipped with ORCA-ER [Hamamatsu

Photonics]) using 60× (numerical aperture [N.A.] 1.20, water immersion), 60× (N.A. 1.35, oil immersion), and 100× (N.A. 1.40, oil immersion) objective lenses (Olympus). Stroma-targeted GFP was detected with a filter cube U-MWIBA (Olympus; excitation: 460–490 nm; emission: 510–550 nm). FtsZ1–GFP and chlorophyll autofluorescence were detected as described previously (Fujiwara et al., 2009). CFP was detected with CFP-2432A (Semrock, Rochester, NY, USA; excitation 426–450 nm; emission 465–501 nm). To avoid rapid photobleaching of fluorescent proteins and to minimize photoresponses of plant cells, samples were observed at 6–25% excitation strength. No chloroplast photorelocations were observed during microscopy. Digital black-and-white images were imported into RGB channels of Adobe Photoshop CS3 to obtain the final merged images. To obtain line profile data, original images of CFP and GFP were aligned with ImageJ plugin StackReg, available at <http://bigwww.epfl.ch/thevenaz/stackreg/>. Noise reduction was performed by band-pass filtering using KBI plugins.

## Results and Discussion

### Plastid Morphology and Division in the Leaf Epidermis of Wild-type Plants

Leaf epidermal plastids are relatively small and underdeveloped (as suggested by the weak autofluorescence from chlorophyll) compared with mesophyll chloroplasts (**Figure 1E**, left). These plastids are often present in dumbbell- or peanut-shaped form. The number, shape, size, and intracellular distribution of the leaf epidermal plastids in WT observed in the present study were basically in accordance with previous reports (Chen et al., 2009; Schattat and Klösgen, 2011). Dual detection of stroma-targeted CFP and FtsZ1–GFP [AtFtsZ1-1-sGFP(S65T)] (Fujiwara et al., 2008) in a transgenic line (see Materials and Methods for details) revealed that the peanut-shaped plastids in the leaf epidermis were associated with the production of centrally located FtsZ1–GFP signals within the plastids (**Figure 1E**, right). This result indicates that these plastids were in the process of FtsZ1 ring-mediated symmetric division, in a similar manner to mesophyll chloroplast division. Nevertheless, epidermal plastids displayed a greater tendency to form stromules and thus a higher plasticity in envelope morphology than mesophyll chloroplasts.

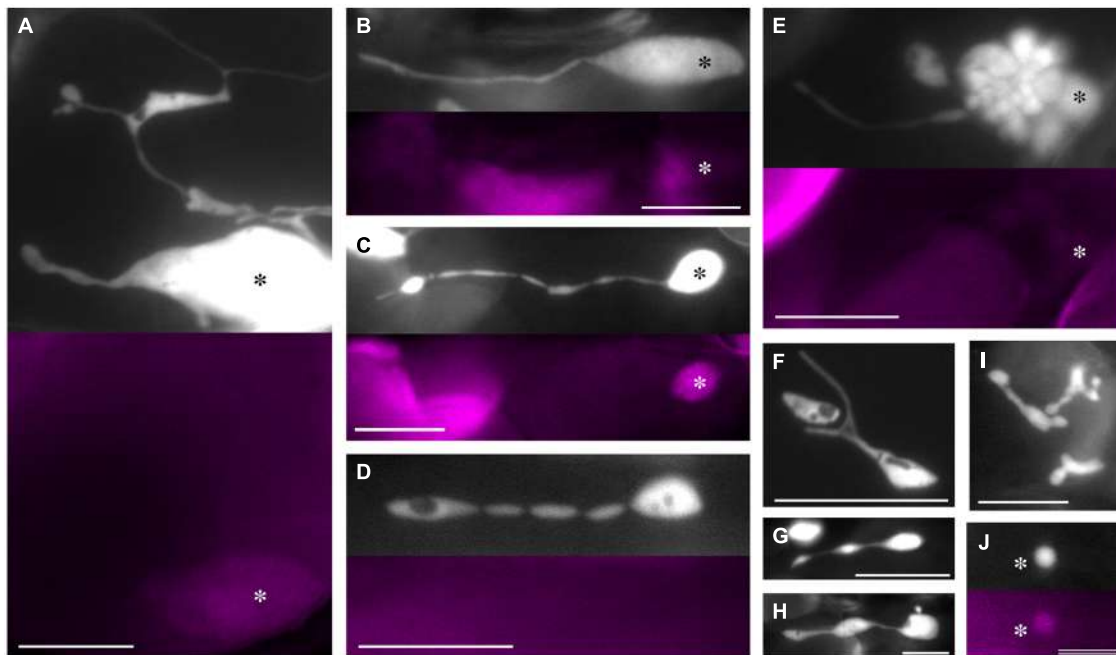
While a detailed model of mesophyll chloroplast division has been proposed (Gao and Gao, 2011; Basak and Möller, 2013; Osteryoung and Pyke, 2014), which is mainly based on thorough observations of mutants and transgenic lines with altered chloroplast size and number, no such model has been established for non-mesophyll plastids, as morphological studies of each type of plastid have not previously been performed. To gain insight into epidermal plastid division, we conducted intensive observations of plastid morphology in the leaf epidermis of the *A. thaliana atminE1* mutant, one of the most severe mutants of chloroplast division (**Figure 1C**; Fujiwara et al., 2008). The *atminE1* mutant harbors a T-DNA insertional mutation at intron 1 of the *AtMinE1* locus, resulting in approximately 2,000-fold reduction in the

*AtMinE1* transcripts as compared to those in WT (Fujiwara et al., 2008).

### Detailed Morphology of Leaf Epidermal Plastids in the *atminE1* Mutant

Taking advantage of the merits of plastid-targeted CFP described above, we investigated the plastid morphology in leaf petiole epidermis of 2- and 3-week-old *atminE1* seedlings using epifluorescence microscopy (**Figure 2**). While epidermal (pavement) cells of the leaf blade are puzzle piece-shaped with interdigitated lobes, those of the petiole assume a flat rectangular shape, making them more suitable for the observation of plastids and other organelles. First, we focused on the morphology of individual plastids. Even in the same cell, plastids were highly polymorphic in terms of their subplastidic structures such as main plastid bodies, stromules, and bulges. Importantly, it was often difficult to distinguish among these three structures (**Figure 2A**), which is often the case for non-green plastids (Schattat et al., 2015). Nonetheless, we typically observed giant plastids with long stromules (**Figure 2B**). As variations of this typical form, we observed shallow, wavy constrictions on stromules (**Figure 2C**) and seemingly fragmenting stromules (**Figure 2D**). These stromules often contained substructures resembling plastid bodies in their interior or terminal regions (**Figures 2C,D**). These characteristic morphologies of stromules were observed regardless of the presence of chlorophyll autofluorescence in their main plastid bodies. The *atminE1* mutant also exhibited a unique morphological feature in the plastid bodies of the leaf epidermis. At low frequency, mini-sized plastid bodies aggregated into a grape-like clump (**Figure 2E**). In some cases, some of the plastid bodies within a clump emitted faint chlorophyll autofluorescence. Also, we noticed the presence of relatively immature, chlorophyll-free plastids, like those observed in the leaf epidermis of the *A. thaliana arc6* mutant (Holzinger et al., 2008). This type of plastid assumed various shapes, appearing round, stretched, or with multiple constrictions (**Figures 2F–I**). We did not detect a significant relationship between plastid size and shape (**Figures 2G,H**), except for the predominance of a round form among small, poorly developed plastids. Another phenotype that is unique to the *atminE1* epidermis among chloroplast division mutants examined thus far is the presence of mini-sized, chlorophyll-containing plastids (**Figure 2J**). Tiny plastids in the epidermis of chloroplast division mutants, which were previously reported, lacked chlorophyll (Holzinger et al., 2008).

In summary, we detected novel, unique phenotypes of leaf epidermal plastids in *atminE1*, which have not been reported in other *A. thaliana* mutants that exhibit severely impaired chloroplast division. These phenotypes include the formation of grape-like plastid clusters (**Figure 2E**) and tiny plastids with positive chlorophyll autofluorescence signals (**Figure 2J**), while *atminE1* epidermal plastids also exhibited enlarged plastid bodies and excessive stromule formation (**Figures 2A–D**), both of which are common phenotypes of non-green plastids in severe chloroplast division mutants. These results imply that the latter “common” phenotypes reflect the fundamental effects of inhibited division whereas the former “unique” phenotypes



**FIGURE 2 | Plastid morphology in leaf epidermis of *atminE1*.** (A–J) Images of CFP-labeled plastids in leaf petiole epidermis of 2- or 3-week-old *atminE1* seedlings. Fluorescence images of chlorophyll (colored in magenta) are also shown. Asterisks indicate plastid bodies with positive chlorophyll signals. Plastids in (D,F–I) lack signals. Bars = 10  $\mu$ m (A–I) and 5  $\mu$ m (J).

represent an additional effect that depends on the specific function of AtMinE1 or the severity of inhibited division.

### Size, Number, and Intracellular Distribution of Leaf Epidermal Plastids in the *atminE1* Mutant

Next, we examined the plastid morphology in the leaf petiole epidermis of *atminE1* in light of their size, number, and distribution within each cell (Figure 3). We found several plastid size, number, and distribution patterns: a single cell could contain only one giant plastid (Figure 3A), one giant plastid coexisting with tiny plastid(s) (Figure 3B), or some plastid bodies connected by stromules, forming a network throughout the entire length of the petiole epidermal cell (Figure 3C; Supplementary Figure S2A). These patterns are basically in agreement with the findings of a previous report on the *arc6* mutant (Holzinger et al., 2008). Moreover, we occasionally detected a cell containing dumbbell-shaped, chlorophyll-bearing plastids (Figure 3D), a novel feature of *atminE1*. In general, only giant plastids, which appeared to be produced via inhibited plastid division, appeared to contain chlorophyll (Figures 2A–C) in the *atminE1* epidermis, although there were some exceptions (Figures 2E,J). The existence of dumbbell-shaped, chlorophyll-bearing plastids implies that some of these chlorophyll-containing plastids maintain the capability of proliferation by division.

While exploring how and why the heterogeneity of plastid shape and size in each individual cell was generated in *atminE1*, we noticed bulge- or vesicle-like subplastidic structures attached to the main plastid bodies or stromules (Figure 3E, magenta

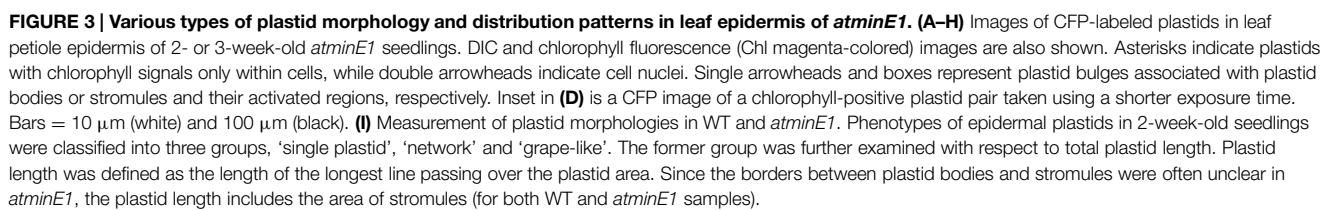
arrowhead, and Supplementary Figure S2B). These structures usually underwent transformation, even during short observation periods, but they remained firmly attached to other parts of the plastids (i.e., bodies and stromules). We frequently observed that the generation of such bulge-like structures was strongly activated in a confined region of plastids (Figures 3F,G, boxes). In all images shown in Figures 3E–G, all of the bulge- or vesicle-like structures and their accompanying plastid bodies or stromules were located in the same focal plane. Notably, those bulges appeared to emerge from a single point, although no discrete structure (such as a “central node”) was identified. These observations suggest that the grape-like plastid clusters (Figures 2E and 3H, Supplementary Figure S2C) represent an extreme state resulting from strong activation of bulge and/or vesicle formation. Figure 3H shows an example of a plastid cluster juxtaposed with the cell nucleus. Indeed, plastid bodies, stromules, and bulges were often located near nuclei in the leaf epidermal cells of *atminE1*.

Taken together, these findings demonstrate that the leaf epidermis of *atminE1* is characterized by a heterogeneous cell population containing various types (Figure 3I) of plastid morphology and distribution patterns.

### Examining the Relationship between Leaf Epidermal Plastids and Mitochondria in the *atminE1* Mutant

The findings described above demonstrate that leaf epidermal plastids in *atminE1* could assume various shapes. A subpopulation of the plastids and their substructures closely







resembled mitochondria. Indeed, the size and shape of stromules and mitochondria, their resemblance to each other, and even the potential relationships between these structures have been discussed previously (Wildman et al., 1962; Kwok and Hanson, 2004). To investigate the possible relationship between subplastidic structures (bulges and stromules) and mitochondria, we employed a transgenic *A. thaliana* line, FL4-4, which stably expresses both mitochondrion-targeted YFP and plastid-targeted CFP, as well as *atminE1* harboring both marker genes (transferred from FL4-4 by crossing; Itoh et al., 2010). Using these lines, we conducted simultaneous detection and morphological comparisons of both organelles.

No colocalization of CFP signals from grape-like plastid clusters, giant plastids, or stromules with YFP signals from mitochondria in *atminE1* was observed (Figures 4A,B), as was the case for plastid bodies and stromules of WT (Figure 1E, middle). Meanwhile, due to the higher surface area of plastids in the mutant, attachment of mitochondria to plastids was more frequently observed in *atminE1* than in WT. Although the bulge structures, which were attached to the plastid bodies or stromules, most closely resembled mitochondria, their CFP signals did not perfectly coincide with mitochondrial YFP signals (Figure 4C), indicating that these subplastidic structures and mitochondria were mutually discrete compartments.

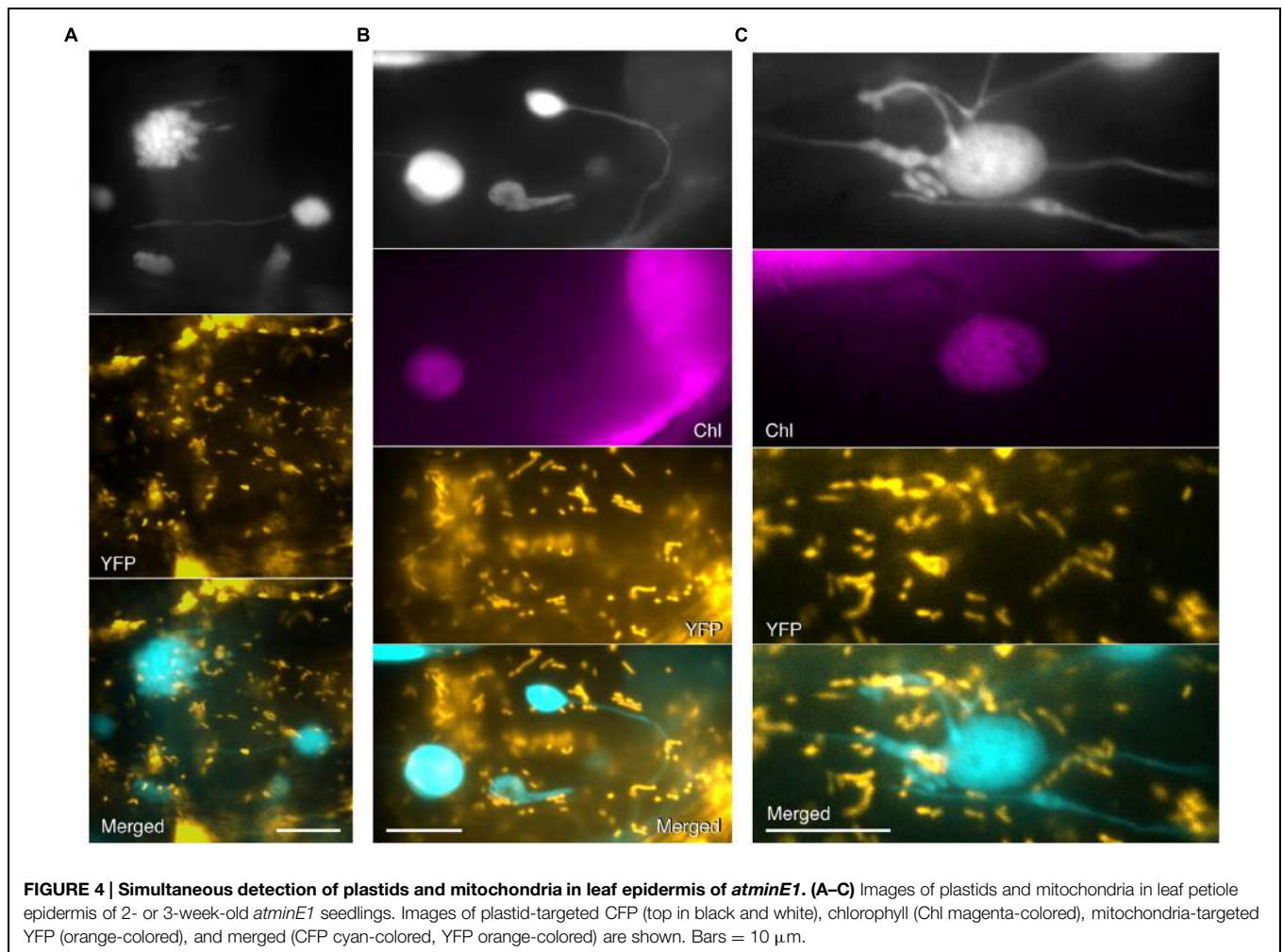
At present, we cannot completely exclude the possibility that the occurrence or dynamics of bulge formation might be regulated by mitochondria through their contact with plastids. In the current study, however, we did not find any significant association between plastid structures and mitochondrial morphology/localization. Since the coincidental behavior of stromules and the endoplasmic reticulum has been reported (Schattat et al., 2011a,b), the interaction of plastids and their derived structures, including stromules, with other organelles still deserves further investigation in future studies.

### Genetic Complementation of *atminE1* with *AtMinE1-YFP*

To elucidate the relationship between the mutant phenotypes and the function of *AtMinE1*, it is important to examine whether *AtMinE1* is expressed in leaf epidermal cells. To investigate the expression profile of *AtMinE1* in planta, we previously performed GUS staining using transgenic *A. thaliana* plants harboring an *AtMinE1*-upstream genomic sequence::*uidA* fusion (Itoh et al., 2001), revealing that GUS activation occurred strongly in the shoot apex and moderately in green tissues and pollen, but not in roots, in most transgenic plants. One exceptional line (Itoh et al., 2001) exhibited GUS staining in whole plants including roots, and we recently considered the possibility that this exceptional transgenic line might represent *AtMinE1* expression in light of a comprehensive *A. thaliana* transcriptome study (Winter et al., 2007) and a study of non-photosynthetic plastids of *atminE1* (Kojo et al., 2009). We constructed an *AtMinE1* promoter::*AtMinE1-YFP* fusion gene (Figure 5A) and introduced it into the nuclear genome of *atminE1* via *Agrobacterium*-mediated transformation. The complemented *atminE1* transgenic plants, if obtained, would produce almost WT levels of *AtMinE1* fused to a visual reporter

subjected to control at the transcriptional, splicing, translational, and post-translational levels.

We obtained 24 T<sub>1</sub> lines with bialaphos resistance conferred by the T-DNA. Of these, two lines showed complemented phenotypes, 11 showed partially complemented phenotypes, nine showed phenotypes that were defective in plastid division site placement, and two showed no effects, based on observations of epidermal plastids and cortex chloroplasts in leaf petioles (details described below). We chose one division site placement-defective line and one complemented line for further study. Quantitative RT-PCR analysis revealed that the former (hereafter referred to as the “overexpression line”) had more than 200-fold higher levels of *AtMinE1* transcripts (including the innate *AtMinE1* and the introduced *AtMinE1-YFP*) compared to WT, while the latter (hereafter referred to as the “complemented line”) had an *AtMinE1* expression level approximately twice that of WT (Figure 5B). No marked differences in *AtMinD1* expression were detected in the plants examined (Figure 5B). We examined the expression of *AtMinE1-YFP* by Western blotting using an anti-GFP antibody (Figure 5C). In both transgenic lines, a major band was specifically detected at 46 kDa, which roughly corresponds to the expected size of *AtMinE1-YFP* (22.4 kDa [predicted mature form of *AtMinE1*] plus 26.8 kDa [YFP] plus 0.6 kDa [linker sequence]). The intensity of the main 46-kDa band in the overexpression line was 1.7-fold higher than that of the complemented line. Furthermore, several high-molecular-weight proteins (>80 kDa) were expressed in the overexpression line, suggesting that overexpression of *AtMinE1-YFP* could result in the formation of stable (SDS-resistant) dimers or multimers of *AtMinE1-YFP* or heteromeric complexes with its interacting proteins. These results indicate that *AtMinE1-YFP* was synthesized in these lines as intended. The overexpression of *AtMinE1-YFP* might have been caused by positional effects of transgene insertion on the chromosomes of transgenic plants. At the macro-morphological level, the inflorescence stems of *atminE1* exhibited reduced gravitropism and an early flowering phenotype, flowering 4 days earlier than WT, under our experimental conditions (Figure 5D), although plant morphology and reproduction in this mutant are normal (Fujiwara et al., 2008). This gravisensitivity of *atminE1* showed consistency with the reported phenotype of *arc12*, another mutant of *AtMinE1* (Pyke, 1999; Yamamoto et al., 2002; Glynn et al., 2007). Restored gravitropism and flowering time were observed in both the complemented (Figure 5D) and overexpression lines (data not shown). We investigated the extent of complementation of the *atminE1* phenotype by observing chlorophyll autofluorescence in leaf petioles (Figure 5E). In WT, epidermal plastids were uniformly sized, with a spherical or dumbbell-shaped (dividing) appearance. By contrast, most epidermal plastids of *atminE1* were relatively large and heterogeneously sized. In both the complemented and overexpression lines, the plastid morphology in the petiole epidermis was similar to that of WT, but the plastid size in the latter was weakly heterogeneous. In the leaf petiole cortex, *atminE1* chloroplasts were enlarged with no detectable constriction sites, as described previously (Fujiwara et al., 2008, 2009), while those of the

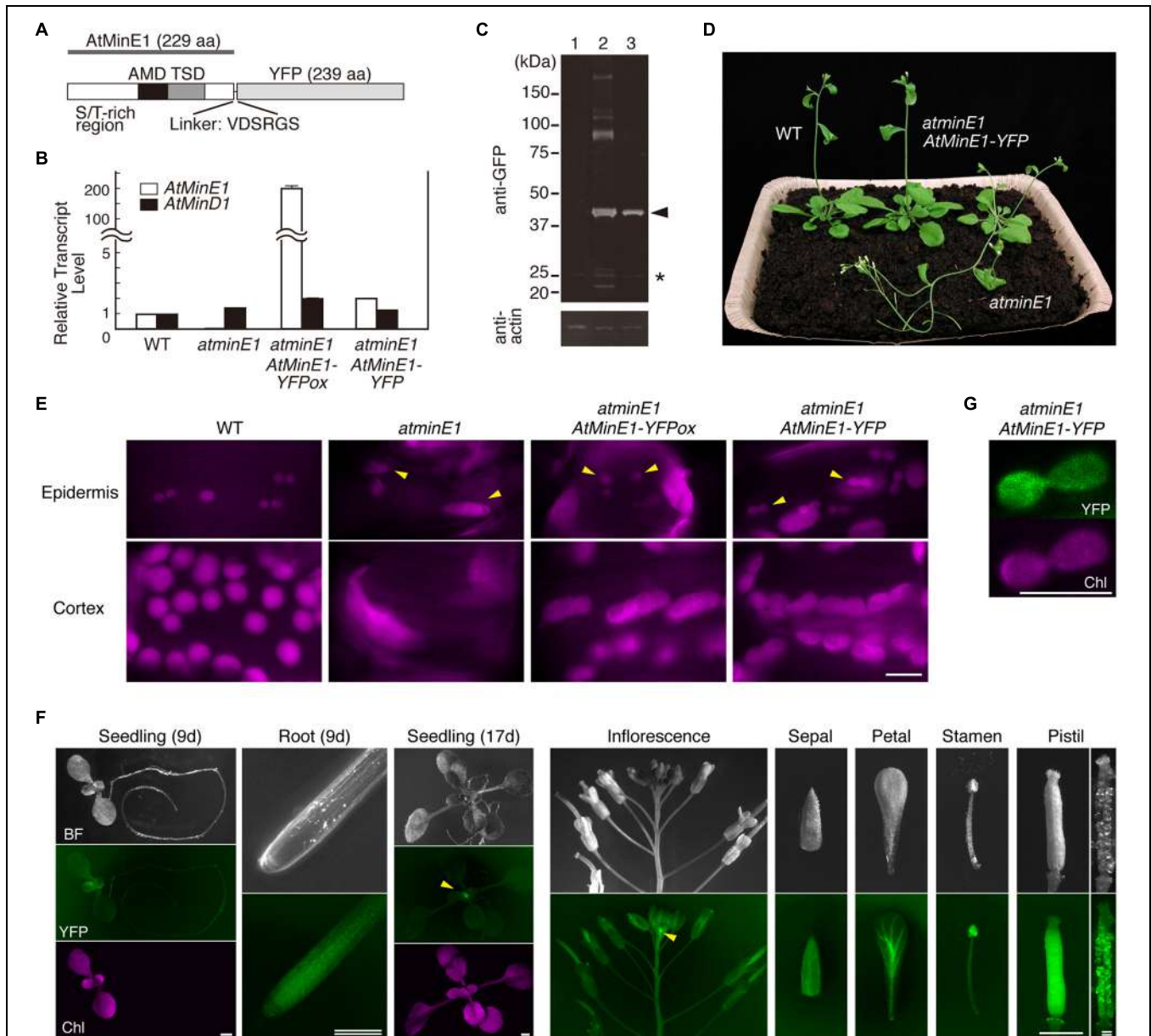


overexpression line were elongated and had multiple constriction sites, indicative of defects in division site placement. The overexpression of AtMinE1-YFP could have disrupted division site placement via the ARC3-mediated mechanism (Zhang et al., 2013). In the petiole cortex of the complemented line, the chloroplast phenotype conferred by the *atminE1* mutation was almost restored to the WT phenotype with respect to their size, morphological uniformity, and the absence of multiple constrictions, although their chloroplasts were only slightly larger than those of WT. Taken together, these results indicate that AtMinE1-YFP plays a role equivalent to that of the native AtMinE1 and exhibits a relationship between gene expression level and chloroplast phenotype as previously demonstrated (Fujiwara et al., 2008). Therefore, the complemented *atminE1* transgenic line is useful for analyzing AtMinE1 expression *in situ*.

We examined this line at the vegetative and reproductive stages by fluorescence stereomicroscopy (Figure 5F). YFP fluorescence was detected in all organs examined: cotyledons, leaves, stems, roots, floral organs (sepals, petals, stamens, pistils), and ovules. AtMinE1-YFP was also expressed in pollen grains (data not shown). Importantly, intense YFP signals were

detected at the apical regions of both shoots and roots, and the fluorescence intensities gradually declined as the organs or tissues matured. These YFP fluorescence patterns in seedlings and floral organs spatially overlapped with the GUS expression data, although newly observed signals were present in roots and were faint and broadly distributed within each organ. While validating the newly detected *AtMinE1* expression patterns in the complemented line, we observed chloroplasts at the basal regions of the petal epidermis (Supplementary Figure S3). The morphological phenotype of chloroplasts was restored to that of WT with concomitant AtMinE1-YFP expression in the cells. These results indicate that *AtMinE1* is globally expressed in vegetative and reproductive plant organs and is expressed at high levels in young tissues, with maximum expression occurring in the shoot apex.

We further examined the subcellular localization of AtMinE1-YFP in leaf epidermal cells of the complemented plants (Figure 5G). YFP signals were exclusively localized to plastids, with diffuse signals present throughout an area of deeply constricted organelles. The morphological phenotype of the plastids was restored to that of WT, with AtMinE1-YFP fluorescence detected within the plastids.



**FIGURE 5 | Expression and function of AtMinE1-YFP in *atminE1*.** (A) Domain structure of AtMinE1-YFP. Three regions of AtMinE1, an S/T-rich N-terminal region, an *E. coli* AMD (anti-MinCD domain)-like region, and an *E. coli* TSD (topological specificity domain)-like region, as well as a linker sequence between AtMinE1 and YFP, are indicated. (B) Quantitative RT-PCR analysis. RNAs from leaves of WT, *atminE1*, and transgenic *atminE1* with AtMinE1p::AtMinE1-YFP (an overexpression line and a complemented line) were analyzed. Relative amounts of AtMinE1 (white bars) and AtMinD1 (black bars) transcripts compared to 18S rRNA (WT = 1) are shown. (C) Immunoblotting. Proteins from seedlings of WT (lane 1) and transgenic *atminE1* plants (an overexpression line [lane 2] and a complemented line [lane 3]) were analyzed using mouse anti-GFP and anti-actin antibodies. Chemiluminescent signals of AtMinE1-YFP (arrowhead) and non-specific, extra signals (\*) are indicated at the right. (D) Complementation of plant phenotype of *atminE1* by AtMinE1-YFP. One-month-old WT, *atminE1*, and a complemented plant are shown. (E) Complementation of plastid morphology of *atminE1* by AtMinE1-YFP. Images of chlorophyll autofluorescence from epidermal (top; from 3-week-old seedlings) and cortex (bottom; from 2-week-old seedlings) plastids of WT, *atminE1*, and transgenic *atminE1* plants are shown. Arrowheads represent epidermal plastids. Bar = 10  $\mu$ m. (F) Fluorescence stereomicroscopy. A complemented *atminE1* line at both the vegetative and reproductive stages was observed. Images of bright field (BF), YFP fluorescence (green), and chlorophyll autofluorescence (Chl, magenta) are shown. Arrowheads indicate accumulation of YFP signals at the shoot apices. Bars = 1 mm (single) and 200  $\mu$ m (double). (G) Localization of AtMinE1-YFP signals in leaf epidermal plastids. Fluorescence images of YFP (green) and chlorophyll (magenta) in a leaf epidermal cell of the complemented *atminE1* line are shown. Bar = 5  $\mu$ m.

Altogether, these results demonstrate that the role of AtMinE1 is intimately associated with plastid division and morphogenesis in the leaf epidermis, and its deficiency causes abnormal

plastid phenotypes, likely as a direct consequence rather than a secondary effect. Moreover, this new information about AtMinE1 expression in *A. thaliana* also suggests that the plastid phenotypes



observed in diverse tissues of *atminE1* (Kojo et al., 2009) may be closely associated with the role of AtMinE1 in these tissues.

### Subplastidic Localization of AtFtsZ1-1 and Plastid Constriction in the Leaf Epidermis of the *atminE1* Mutant

In mesophyll chloroplasts of WT *A. thaliana* plants, AtMinE1 is involved in the formation of the mid-chloroplast FtsZ ring, an early event in chloroplast division (Fujiwara et al., 2008). In fact, in enlarged mesophyll chloroplasts of the *atminE1* mutant, AtFtsZ1-1 (hereafter referred to as FtsZ1) fails to assemble into a mid-chloroplast ring but instead forms various structures such as multiple dots, short filaments, and small, isolated rings (Fujiwara et al., 2009). In this study, we examined the localization of FtsZ1 in the *atminE1* leaf epidermis using a transgenic line in which the FtsZ1-GFP fusion was expressed under its own promoter (Fujiwara et al., 2008). Previously, we observed multiple FtsZ1 rings in mini-sized plastid bodies in the *atminE1* leaf epidermis (Fujiwara et al., 2009). Here, by simultaneously utilizing FtsZ1-GFP and stroma-targeted CFP, we further monitored FtsZ1 localization (with great precision) within stromules and bulges in addition to plastid bodies (Figure 6).

In many cases, the signal intensity of FtsZ1-GFP was weak. Like stroma-targeted CFP, FtsZ1-GFP appeared to accumulate much more strongly in the plastid bodies than in stromules, probably reflecting differences in stroma volume (Figure 6A). In those plastids, we did not find any characteristic structures of FtsZ1 in their bodies and stromules, except for unfixed dots, which were occasionally observed. While we detected apparent disconnection and constriction of the stromal CFP signal within a single stromule, no particular FtsZ structure was associated with these disconnected or constricted parts of stromules (Figure 6A). In certain cases, however, FtsZ1 rings were observed in stromules (Figure 6B). As chloroplast division progresses, the appearance of chloroplast FtsZ rings under a fluorescence microscope changes from two dots (at both sides of the isthmus) or a filament (traversing the isthmus) to a single patch (a putative equivalent of the compacted ring; Vitha et al., 2001; Kuroiwa et al., 2002). Since stromules are very thin, FtsZ rings in stromules are considered to be equivalent to those at a late or final stage of normal chloroplast division. Therefore, it was not easy to discriminate between FtsZ1 rings and non-ring structures in stromules, and thus we relied on the following criteria for discrimination: when in focus in the top focal plane of the FtsZ1 ring, the diffusion signal of FtsZ1-GFP dropped at the flanking regions, while the stromal CFP signal dropped at the site of FtsZ1 ring formation, both due to the presence of a ring-associated isthmus. For reference, the FtsZ1 ring in wild-type, dividing plastids (Figure 1E, right) obviously met these criteria. In light of these criteria, in the plastid shown in Figure 6B, at least four FtsZ1 rings were recognized (magenta arrowheads) in the two stromules emanating from the single body. As shown in Figures 6A,B, FtsZ1 rings were not necessarily associated with the constricted/disconnected parts of stromules. However, in some cases, unfixed dots of FtsZ1-GFP were observed within the diffusion signal. Most of these FtsZ1 dots could be distinguished from FtsZ1 rings (Figure 6B) based on the above criteria. In the *atminE1* leaf epidermis, we

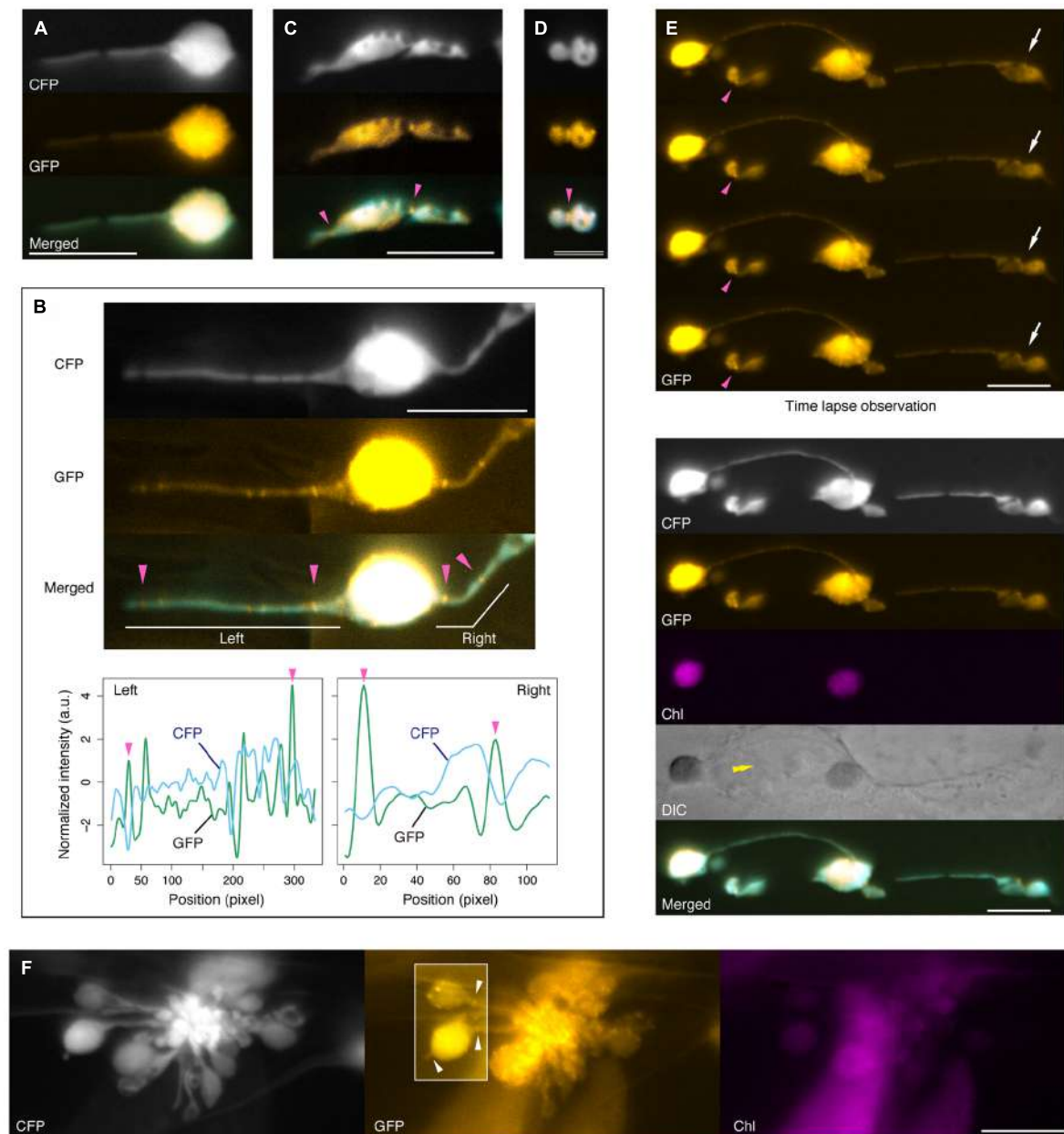
also observed a putative FtsZ1 ring at one or more constriction site(s) of the plastid main bodies (Figures 6C,D). In such plastids, FtsZ1-GFP signals were concentrated either in a short filament traversing the shallow constriction or in a spot at the deep constriction (Figures 6C,D, magenta arrowheads) in addition to the stromal diffusion signal. Since epidermal plastids in *atminE1* are highly irregular and pleomorphic, it was previously difficult to judge whether such a plastid was undergoing fission merely based on its shape. It is noteworthy that the use of FtsZ1-GFP in combination with stromal CFP enabled us to identify epidermal plastids undergoing fission (Figure 6C, for example).

Based on the above observations, together with our previous findings (Fujiwara et al., 2009), we postulate that, even without AtMinE1, (i) FtsZ1 could assemble into a ring in non-swollen plastid bodies, stromules and bulges, and (ii) the formation of the FtsZ1 ring could lead to constriction and, ultimately, membrane fission of those plastid compartments. While we have provided evidence for postulate (i), postulate (ii) has yet to be verified experimentally. To address this issue, we performed time-lapse fluorescence imaging of FtsZ1-GFP according to the method of Fujiwara et al. (2009), demonstrating that plastid bodies were capable of isthmus formation and constriction without AtMinE1 (Figure 6E). Intriguingly, as exemplified by the plastid shown in Figure 6E, the constricting isthmus of plastid bodies often appeared to lack a clear FtsZ1 ring. In leaf epidermal plastids, FtsZ1 ring formation may not be a prerequisite for the constriction of plastid bodies.

Within grape-like plastid aggregations, putative FtsZ1 rings were observed in the narrow, stromule-like regions of bulges, whereas the vast majority of FtsZ1-GFP fluorescence was detected as a diffuse signal within the plastid stroma (Figure 6F; see also Supplementary Figure S4). The apparent aggregations of plastids that occasionally occurred in the *atminE1* leaf epidermis (Figures 2E, 3H, 4A and 6F, Supplementary Figure S2C) may have resulted from the failure of the stroma-containing bulges (initially in the form of tubes, lobes, or vesicles) to separate; the FtsZ1 rings in the plastid aggregations (Figure 6F) might represent suspended plastid fission. In other words, the “plastid aggregate” might, in fact, represent a single or a few plastid(s), inside which a number of bulges formed and grew, rather than a product of massive connection or aggregation of independent plastid bodies and bulges.

Based on our detection of plastid FtsZ1 rings (Figures 6B–D,F) and time-lapse imaging of plastids (Figure 6E), we conclude the following: (1) In the *atminE1* leaf epidermis, the formation of FtsZ1 rings can occur both within non-giant plastid bodies and within plastid bulges and stromules. (2) Deep plastid constrictions with FtsZ1 rings can be generated in two ways, either by constriction of FtsZ1 rings in the plastid bodies or by sustained presence of FtsZ1 rings in the bulges/stromules in conjunction with the growth of both sides of the ring. (3) Because of depletion of AtMinE1, the FtsZ1 rings at deep constrictions can inefficiently (or scarcely) mediate the scission of plastid envelope membranes and, as a result, may help stabilize the constrictions by counteracting the swelling and deformation of plastid bodies, bulges, or stromules.





**FIGURE 6 | FtsZ1 localization in stromules and plastid bulges in leaf epidermis of *atminE1*.** (A–F) Dual detection of FtsZ1–GFP and stroma-targeted CFP in epidermal plastids of *atminE1* leaf petioles. Fluorescence images of CFP, GFP (orange-colored), chlorophyll (magenta-colored), and merged (CFP cyan-colored, GFP orange-colored) are shown. In (B), line profiles of normalized CFP and GFP signal intensity in left and right stromules in the image are also presented. Single arrowheads indicate position of FtsZ1 ring placement, while box in (F) highlights FtsZ1 ring placement at stromules or between plastid body and plastid vesicle within a grape-like plastid association. Arrows and a double arrowhead in (E) indicate a plastid constriction event by time-lapse microscopy and the position of cell nucleus, respectively. Bars = 5 μm (D), and 10 μm (others).

## MinE-independent FtsZ1 Ring Formation and Plastid Constriction: Hidden Diversity of Plastid Division/Constriction Patterns in Leaf Tissues

Epidermal plastids replicate by FtsZ1 ring-associated binary fission, in the same manner as mesophyll chloroplasts (Figure 1E, right). Despite this, the effects of a mutation in *AtMinE1* on

plastid division and morphology differed markedly, even in the same leaf, depending on tissue type. Our present data provide insight into the process by which epidermal plastids become pleomorphic. First, our observations indicate that FtsZ1 ring-mediated membrane constriction occurs in stromules, bulges, and small plastid bodies, but not in giant plastids, in the absence of *AtMinE1*, although it remains unknown whether the FtsZ1

ring is actually capable of stromule fission. The above data support our previously proposed hypothesis (Fujiwara et al., 2008, 2009) that excessive expansion of plastids may inhibit FtsZ-based constriction, while plastids with a small diameter and stromules may be capable of efficient constriction. Second, this study suggests that FtsZ1 can assemble into a ring and produce a membrane constriction, even without AtMinE1 (**Figure 6B**), but it appears unable, or scarcely able, to complete membrane fission in the (almost) complete absence of AtMinE1. This finding implies that there is a novel, MinE-independent mode of plastid division/constriction, which might be specific to the epidermal or non-green tissue. Such FtsZ1 rings at the plastid constrictions may play an envelope-tightening or holding role in the AtMinE1-deficient epidermis. It is possible that the MinE-independent mode is also present in stromules in the epidermis of WT plants but is hidden, perhaps due to the relatively low number and short length of the stromules in WT or to the completion of “stromule fission” by the functional FtsZ1 ring, which would produce difficult-to-identify plastid-derived structures. In this context, it is interesting that detachment of a vesicle-like plastid from the tip of the stromule, a process known as “tip-shedding”, was previously observed in trichome cells of tomato (*Solanum lycopersicum*) using video microscopy with differential interference contrast optics (Gunning, 2005).

Assuming the above hypotheses are correct, we can explain the terminal phenotype of plastid morphology in the *atminE1* epidermis as the following “stromule-derived subcompartments” model: (Step 1) stromules and bulges emanating from giant plastids undergo FtsZ-based constriction to produce even smaller subcompartments, while the giant plastid bodies themselves cannot constrict due to their large diameter; (Step 2) these smaller subcompartments undergo further rounds of constriction, producing still more subcompartments; (Step 3) these subcompartments may continuously grow wider or longer whereas the pre-existing constrictions largely remain unchanged due to the presence of static FtsZ1 rings; and (Step 4) the above steps are repeated in epidermal cells, giving rise to a population of plastids with rapidly increasing shape heterogeneity. This model would also explain how the “grape-like” plastid clusters (**Figures 2E, 3H, 4A and 6F**, Supplementary Figure S2C) could be generated from a single or a few plastids, while such images are usually interpreted as the aggregation and connection of several independent plastids (the “connected plastids” model). As another example, the plastid image in **Figure 2A** may have been interpreted as multiple plastids connected by stromules according to the conventional viewpoint (the “connected plastids” model). However, based on the new viewpoint, this image can be interpreted as a single giant plastid whose stromules have grown into subplastid bodies or bulges (the “stromule-derived subcompartments” model). Localization of FtsZ1 rings at the constriction sites of plastid bulges and stromules (**Figure 6**) also supports the present “stromule-derived subcompartments”

model rather than the conventional “connected plastids” model.

## Conclusion

In previous studies, terminal phenotypes of various chloroplast division mutants were interpreted based on the established framework of mesophyll chloroplast division. Specifically, plastid enlargement has been regarded as an indicator that plastids cannot divide, and heterogeneity of plastid size was thought to indicate that plastid division (and associated formation of the FtsZ ring) had occurred at abnormal (i.e., non-central and random) site(s). However, the terminal phenotype of plastids in the *atminE1* epidermis could not be fully explained by this model. Our results suggest that the well-studied control of mesophyll chloroplast division is not necessarily true for plastid replication in other leaf tissues. Our data also emphasize the need to establish a framework of non-mesophyll plastid division, which would undoubtedly be important for elucidating the development and differentiation of plastids.

## Author Contributions

MF, RI designed the study, interpreted the data, and wrote the paper. MF, YK, SS, RI performed the experiments. MF, KK conducted the image analysis. TA, MF, RI contributed reagents/materials/analysis tools.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00823>

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# Fluorescent Protein Aided Insights on Plastids and their Extensions: A Critical Appraisal

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Multi-colored fluorescent proteins targeted to plastids have provided new insights on the dynamic behavior of these organelles and their interactions with other cytoplasmic components and compartments. Sub-plastidic components such as thylakoids, stroma, the inner and outer membranes of the plastid envelope, nucleoids, plastoglobuli, and starch grains have been efficiently highlighted in living plant cells. In addition, stroma filled membrane extensions called stromules have drawn attention to the dynamic nature of the plastid and its interactions with the rest of the cell. Use of dual and triple fluorescent protein combinations has begun to reveal plastid interactions with mitochondria, the nucleus, the endoplasmic reticulum and F-actin and suggests integral roles of plastids in retrograde signaling, cell to cell communication as well as plant-pathogen interactions. While the rapid advances and insights achieved through fluorescent protein based research on plastids are commendable it is necessary to endorse meaningful observations but subject others to closer scrutiny. Here, in order to develop a better and more comprehensive understanding of plastids and their extensions we provide a critical appraisal of recent information that has been acquired using targeted fluorescent protein probes.

**Keywords:** plastids, fluorescent proteins, photoconvertible fluorescent protein, stromules, stroma, retrograde signaling

## INTRODUCTION

Plastids are organelles of purported endosymbiogenic origin characterized by the presence of multi-layered bounding membranes (Margulis, 1970; Hooper, 2007; Sato, 2007). Plastids with an inner and an outer bounding membrane are accepted as a defining feature of plants and green algae (Wise, 2007; Pyke, 2009). Publications on plastids and the fundamentals of our present knowledge on these organelles are traceable to the late seventeenth century (Leeuwenhoek, 1674; reviewed by Gunning et al., 2007). A paradigm shift in plastid biology came with the realization that irrespective of their wide diversity of form and function, all plastid types are inter-convertible and are derived from colorless pro-plastids (Schmidt, 1870; Schimper, 1882). A classification based upon internal pigmentation was suggested (Schimper, 1882, 1883; Senn, 1908) and is followed even today; Accordingly plastids containing green pigment (chlorophyll) are called chloroplasts, plastids with other colored pigments are considered chromoplasts and colorless plastids are called leucoplasts.

Light microscopy observations and transmission electron microscopy (TEM) further established the presence of internal membranes in all plastids. The flattened membrane sacs were named thylakoids (Menke, 1962) and their stacking into prominent grana accounts for the characteristic lens shaped plastid body of chloroplasts. Relatively less organized pro-lamellar bodies and scattered thylakoids account for the lack of a well-defined plastid body and the elongated and pleomorphic leucoplasts and etioplasts (Gunning, 2001; Wise, 2007). Thylakoids in all plastid types are surrounded by a fluid stroma. Plastids also possess their own DNA condensed within nucleoids as well as protein translation machinery. Since different plastids synthesize and accumulate starches, lipids, oils and proteins they are further sub-classified on the basis of their major content and function (Wise, 2007). Despite the diversity of form and function the plastid unit is circumscribed by the double membrane-envelope.

Although transmission electron micrographs form the basis for our understanding of plastid ultrastructure, an appreciation of the dynamic nature of these fundamental organelles developed has with the advent of time-lapse imaging and cinephotomicrographic techniques (Wildman et al., 1962; Green, 1964; Menzel, 1994; Gunning, 2005). Whereas chloroplasts display strong auto-fluorescence (**Figure 1A**) and can therefore be easily identified under ultra-violet and blue light excitation, many more insights on plastids have come through the discovery of GFP and its potential as a fluorescent probe for living cells (Chalfie et al., 1994). Now, after more than 20 years of fluorescent protein (FP) aided research a large number of protein fusions have highlighted plastids and sub-plastidic structures as well as transient metabolites such as starches and lipids (**Table 1; Figure 1**). The use of double and triple transgenic plants has also facilitated observations on plastid interactions with other cellular components (Kwok and Hanson, 2003, 2004a,b; Schattat et al., 2011a). However, in comparison to conventional botanical micro-techniques and TEM where chemical fixation ensure that the cells and tissue do not change during observations living plant cells continue responding even as they are being observed. While every new publication underscores the tremendous potential of the FP-based approach for increasing insights on plastids it is also equally apparent that many artifacts are being reported and perpetuated. The situation becomes quite problematic when multiple reviews and follow-up publications strengthen a particular viewpoint and require considerably more effort for reevaluation of the original observations. This critical appraisal applauds the considerable insights on plastids obtained to date through the use of plastid-targeted FPs. It also points to the pitfalls and in some cases suggests alternative explanations that might be useful in furthering our knowledge on these essential organelles of the plant cell.

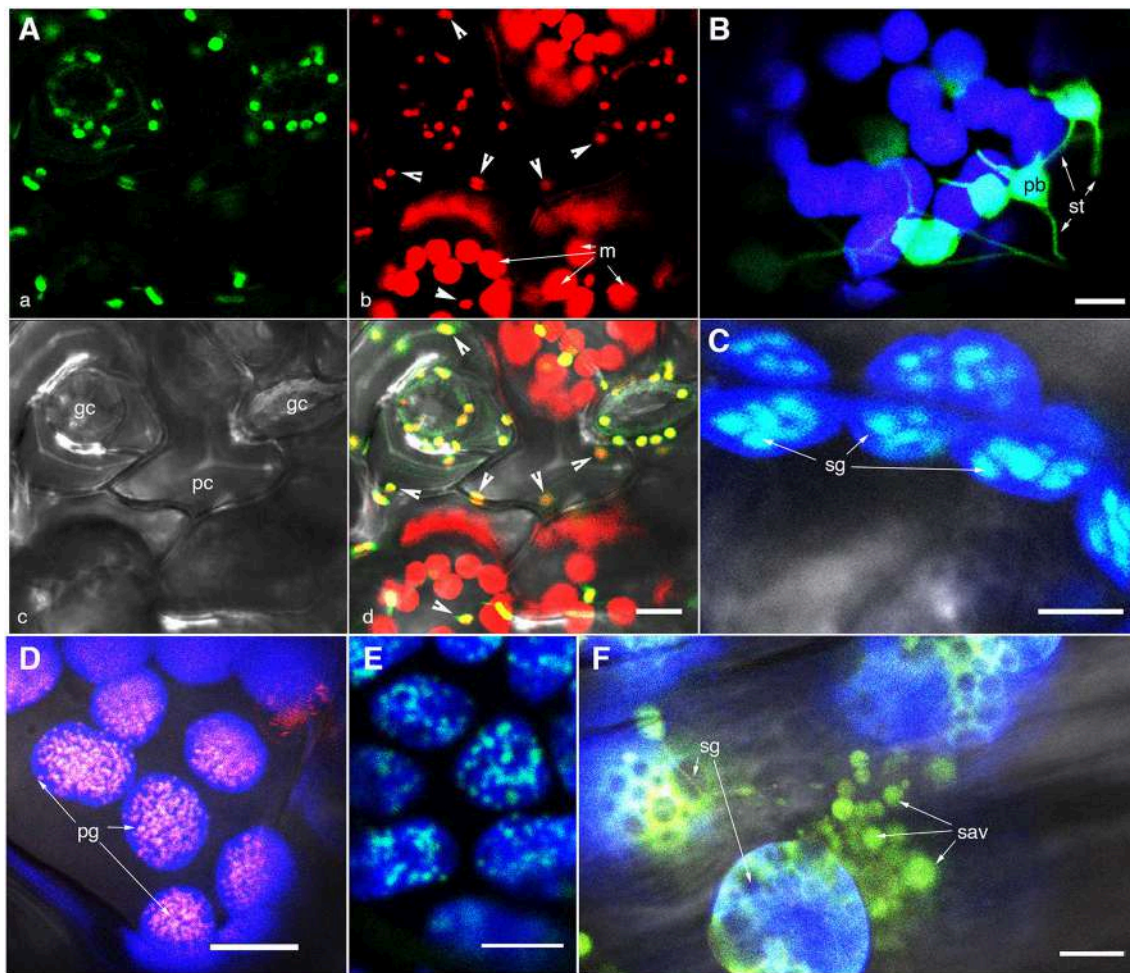
## STROMA-TARGETED FPs HAVE LED TO MAJOR INSIGHTS CONCERNING THE DYNAMIC NATURE OF PLASTIDS

The targeting of a GFP to the stroma (Köhler et al., 1997) was one of the earliest successful demonstrations of

the use of FP-technology for understanding plastids. Earlier light microscopy based investigations had already established the dynamic behavior of plastids in response to light and other environmental factors, now considered as text-book information (Pyke, 2009; Buchanan et al., 2015; Taiz et al., 2015). Differential interference contrast (DIC) cinephotomicrography of chloroplasts suggested an undulating envelope that was likened to a mobile jacket surrounding the plastid body (Wildman et al., 1962). Stroma-targeted GFP confirmed the earlier observations and highlighted thin stroma filled tubules, subsequently named stromules, that extended and retracted in relation to the main chloroplast body (Köhler et al., 1997; Köhler and Hanson, 2000; **Figure 1B**). The excitement generated by this seminal discovery led several groups to start generating fusing proteins (Tirlapur et al., 1999; Arimura et al., 2001; reviewed by Natesan et al., 2005) that could highlight stromules and allow investigations on the conditions that promote or repress stromule formation and can provide insights into their function. In general in plants stably expressing stroma-targeted FPs the epidermal plastids appear more fluorescent as compared to mesophyll chloroplasts. This has led to an erroneous impression in the mind of the non-specialist that mesophyll chloroplasts do not exhibit stromules while the most extensive and numerous stromules are observed in non-green plastids (Köhler and Hanson, 2000). Stromule formation has been observed in response to alteration in plastid redox status (Itoh et al., 2010; Brunkard et al., 2015), elevated temperatures (Holzinger et al., 2007a), symbiotic interactions (Fester et al., 2001; Hans et al., 2004; Lohse et al., 2005), virus and bacterial infection (Caplan et al., 2008; Krenz et al., 2012, 2014; Erickson et al., 2014) and growth regulator and mineral nutrient stress (Gray et al., 2012; Glińska et al., 2015). Stromule formation is also attributed to changes in plastid size and density within a cell (Pyke and Howells, 2002; Waters et al., 2004). As observations on stromules and changes in plastid morphology increase the fresh insights and opinions resulting from them are discussed in more detail.

## DIURNAL CHANGES IN PLASTID MORPHOLOGY

Stroma-targeted FPs made it easier to follow plastid behavior in real time under different physiological states of the plant cell. It was found that the morphology of plastids changed considerably during the day-night cycle. The frequency of stromule formation from plastids increased during daytime and reverted to a low, basal frequency at night (Schattat et al., 2011a; Brunkard et al., 2015). A clear link to photosynthesis and sucrose production was suggested by this diurnal phenomenon (Schattat and Klösgen, 2011). This was confirmed through exogenous sucrose feeding which also increased the frequency of stromule formation (Schattat and Klösgen, 2011; Schattat et al., 2012a). Notably, other conditions such as pathogen infection (Fester et al., 2001; Lohse et al., 2005; Krenz et al., 2010, 2012; Erickson et al., 2014; Caplan et al., 2015) and senescence (Ishida et al., 2008), that affect the sugar status of a plant cell also increase stromule frequency. Whereas sugar appears to be a universal signal for changes in plastid morphology a recent report (Brunkard et al., 2015)



**FIGURE 1 | Representative images of fluorescently highlighted plastids and some sub-plastidic features. (A)** A top-down view of epidermal and mesophyll chloroplasts in the upper epidermis of a green house grown *Arabidopsis* plant expressing the stroma-targeted tpFNR:GFP. Panel “a” shows the green fluorescent stroma (488 nm excitation; emission collected—509–520 nm). Panel “b” shows chlorophyll fluorescence in red (emission band 650–750 nm) in guard cells (gc), pavement cells (pc; arrowheads in b,d), and mesophyll cell (m) chloroplasts. Note the difference in size and the GFP signal intensity between the epidermal and mesophyll chloroplasts. **(B)** A view of thin stroma-filled tubules (stromules; st) and the bulky, grana-containing plastid body (pb) in epidermal chloroplasts of tobacco. **(C)** Starch grains (sg) in mesophyll chloroplasts highlighted in an *Arabidopsis* plant expressing a granule bound starch synthase (GBSS) fused to GFP. **(D)** Clusters of plastoglobuli (pg) observed in senescent leaves of *Arabidopsis* expressing a Fibrillin4:mEosFP fusion. **(E)** The highlighting of nucleoids in chloroplasts is indicated in a transgenic *Arabidopsis* plant expressing a plastid envelope DNA-binding (PEND) GFP fusion. **(F)** View of gerontoplasts in senescent leaves in an *Arabidopsis* plant expressing stroma-targeted tpFNR:GFP shows their swollen appearance suggesting compromised envelope membranes, degrading chlorophyll, the presence of starch grains (sg) visible as dark non-fluorescent regions and clusters of senescence associated vesicles (sav) containing fluorescently GFP-labeled stroma content. Chlorophyll auto-fluorescence in **(B–F)** is false colored blue. Size bars = 5  $\mu$ m in **(B,C)**; 10  $\mu$ m in **(A,D,E,F)**.

suggests that changes in the internal redox status of chloroplasts, which precede the production of photosynthates, are responsible for stromule formation.

The conclusion that light-sensitive redox signals triggered within chloroplasts play a major role in stromule formation are based on the use of DCMU and DBMIB, two chemical inhibitors of the photosynthetic electron transport chain (pETC) (Brunkard et al., 2015). It was observed that treatment of 14-day old excised cotyledons of *Nicotiana benthamiana* and *Arabidopsis thaliana* for 2 h with these inhibitors resulted in a significant increase in stromule frequency of chloroplasts. The presence of chloroplasts was demonstrated in pavement and guard cells in the

tobacco epidermis (Dupree et al., 1991) and the researchers found increased stromule frequency in both cell types (Brunkard et al., 2015). However, the increase in stromules was limited to only guard cells and not observed in the pavement cells of *Arabidopsis*. In order to explain the absence of stromules in *Arabidopsis* cotyledon pavement cells an unreferenced statement—“unlike *N. benthamiana*, the epidermis of *A. thaliana* has two distinct types of plastids: chloroplasts in the guard cells and leucoplasts in the pavement cells,” was presented (Brunkard et al., 2015). A diagrammatic depiction of this statement was used to present a model where reactive oxygen species (ROS) generated from the pETC triggers stromule formation in chloroplasts but sucrose

**TABLE 1 | A non-comprehensive list of fluorescent proteins targeted to plastids.**

Localization	Gene	FP	Organism of expression	T/P	References
Stroma	TP-RecA	G	<i>Petunia</i> and <i>N. tabacum</i>	P	Köhler et al., 1997
	TP- <i>ent</i> -kaurene synthase (TP-AtKS1)	G	<i>N. tabacum</i>	T	Helliwell et al., 2001
	TP- <i>ent</i> -kaurene oxidase (TP-AtKO1)	G	<i>N. tabacum</i>	T	Helliwell et al., 2001
	TP-copalyl diphosphate synthase (TP-AtCPS1)	G	<i>N. tabacum</i>	T	Helliwell et al., 2001
	TP-small subunit of ribulose 1,5 biphosphate carboxylase (TP-RbcS)	G	<i>N. tabacum</i>	T	Helliwell et al., 2001
	Acyl-carrier protein (ACP)	R	<i>A. cepa</i>	T	Schnurr et al., 2002
	TP-ferredoxin NADP(H) oxidoreductase (TP-FNR)	G	<i>A. thaliana</i>	P	Marques et al., 2004
	TP-Plastocyanin (TP-PC)	G	<i>A. thaliana</i>	P	Marques et al., 2004
	TP-33kDa subunit of the oxygen evolving system of photosystem II (TP-PSII-O)	G	<i>A. thaliana</i>	P	Marques et al., 2004
	TP-ferredoxin NADP(H) oxidoreductase (TP-FNR)	E	<i>N. benthamiana</i> , <i>A. thaliana</i>	T/P	Schattat et al., 2012a
	Small subunit of ribulose 1,5 biphosphate carboxylase (SSU)	G	<i>A. thaliana</i>	P	Kim and Apel, 2004
	NADPH-dependent protochlorophyllide oxidoreductase A (PORA)	G	<i>A. thaliana</i>	P	Kim and Apel, 2004
	Thylakoid formation 1 (THF1)	G	<i>A. thaliana</i>	P	Wang et al., 2004
	Aspartate aminotransferase 5 (ASP5)	G	<i>N. tabacum</i>	P	Kwok and Hanson, 2004a
	Small subunit 3A of ribulose 1,5 biphosphate carboxylase (RbcS-3A)	C	<i>N. tabacum</i>	P	Kwok and Hanson, 2004a
	TP- Small subunit 3A of ribulose 1,5 biphosphate carboxylase (TP-RbcS-3A)	C	<i>N. tabacum</i>	P	Kwok and Hanson, 2004a
	$\alpha$ -carbonic anhydrase (CAH1)	G	<i>A. thaliana</i>	T	Villarejo et al., 2005
	Snowy cotyledon 1 (SCO1)	G	<i>A. thaliana</i>	P	Albrecht et al., 2006
	Allene oxide cyclase (AOC)	G	<i>A. thaliana</i> , <i>S. tuberosum</i> cv. Desiree	T/P	Farmaki et al., 2007
	Mesophyll-cell RNAi library line 7 -like (MRL7-L)	G	<i>N. tabacum</i>	T	Qiao et al., 2011
	Accumulation and Replication of Chloroplasts 3 (ARC3)	Y	<i>N. tabacum</i>	T	Maple et al., 2007
	Chloroplast sensor kinase (CSK)	G	<i>N. tabacum</i>	T	Putthiyaveetil et al., 2008
	ADP-sugar pyrophosphatase (StASPP)	G	<i>A. thaliana</i> , <i>S. tuberosum</i>	P	Muñoz et al., 2008
	TP-Spo0B GTP-binding protein like (TP-AtOBGL)	G	<i>N. tabacum</i>	T	Chigri et al., 2009
	TP-Granule bound starch synthase I (TP-GBSSI)	Y	<i>T. aestivum</i> L	P	Shaw and Gray, 2011
	Starch synthase 1 (SS1)	G	<i>N. benthamiana</i>	T	Gámez-Arjona et al., 2014a
	3-ketoacyl-ACP reductase (KAR)	G	<i>P. patens</i>	T	Mueller et al., 2014
	Peroxisredoxin Q A (PrxQA)	G	<i>P. patens</i>	T	Mueller et al., 2014
Outer envelope	Outer envelope membrane protein 7 (AtOEP7)	G	<i>A. thaliana</i>	T/P	Lee et al., 2001
	GTP-Binding domain of AtToc159 (AtToc159G)	G	<i>A. thaliana</i>	T	Bauer et al., 2002
	Long-chain acyl-CoA synthetase 9 (LACS9)	G	<i>A. cepa</i>	T	Schnurr et al., 2002
	Crumpled leaf (CRL)	G	<i>A. thaliana</i>	P	Asano et al., 2004
	Chloroplast unusual positioning 1 (CHUP1)	G	<i>A. thaliana</i>	P	Oikawa et al., 2008
	Sensitive to freezing 2 (SFR2)	C	<i>A. thaliana</i>	P	Ferro et al., 2010
	Translocon at the outer membrane of chloroplasts 64 (AtTOC64)	G	<i>N. benthamiana</i>	T	Breuers et al., 2012
Inner envelope	Monogalactosyldiacylglycerol synthase 1 (MGD1)	G	<i>A. thaliana</i>	T	Awai et al., 2001
	Inner envelope protein 60 (IEP60)	G	<i>A. thaliana</i>	T	Ferro et al., 2002
	Chloroplast envelope quinone oxidoreductase homolog (ceQORH)	G	<i>A. thaliana</i> , <i>N. Tabacum</i>	T	Miras et al., 2002
	Triose phosphate translocator (AtTPT)	G	<i>N. benthamiana</i>	T	Breuers et al., 2012
	Albino or pale green mutant 1 (AtAPG1)	G	<i>N. benthamiana</i>	T	Breuers et al., 2012
	Giant Chloroplast 1 (GC1)	Y	<i>A. thaliana</i>	P	Maple et al., 2004

(Continued)



TABLE 1 | Continued

Localization	Gene	FP	Organism of expression	T/P	References
	Chloroplast import apparatus 5 TP and first 2 transmembrane domains (prCIA5TP-TM2)	R	<i>A. thaliana</i>	T	Teng et al., 2006
	Translocon at the inner envelope membrane of chloroplasts 40 (Tic 40)	Y	<i>A. thaliana</i>	T	Bédard et al., 2007
	Translocon at the inner envelope membrane of chloroplasts 110 (Tic110)	Y	<i>A. thaliana</i>	T	Bédard et al., 2007
	Translocon at the inner envelope membrane of chloroplasts 20 I (TIC20-I)	Y	<i>A. thaliana</i>	T	Kasmati et al., 2011
	Translocon at the inner envelope membrane of chloroplasts 20 II (TIC20-II)	Y	<i>A. thaliana</i>	T	Kasmati et al., 2011
	Translocon at the inner envelope membrane of chloroplasts Tic20 IV (TIC20-IV)	Y	<i>A. thaliana</i>	T	Kasmati et al., 2011
	Translocon at the inner envelope membrane of chloroplasts Tic20 V (TIC20-V)	Y	<i>A. thaliana</i>	T	Kasmati et al., 2011
	Translocon at the inner membrane of chloroplasts 21 (TIC21)	Y	<i>A. thaliana</i>	T	Yang et al., 2012
	AtLrgB	G	<i>A. thaliana</i>	P	Yang et al., 2012
Thylakoid	Sulfurtransferase 15 (AtSTR15)	G	<i>A. thaliana</i>	T	Bauer et al., 2004
	NADPH-dependent protochlorophyllide oxidoreductase B (PORB)	G	<i>A. thaliana</i>	P	Kim and Apel, 2004
	N-terminal region of P-type ATPase of Arabidopsis 2 (PAA2)	G	<i>A. thaliana</i>	T	Abdel-Ghany et al., 2005
	Allene oxide synthase 1 (AOS1)	G	<i>A. thaliana</i>	T	Farmaki et al., 2007
	Allene oxide synthase 2 (AOS2)	G	<i>A. thaliana</i>	T	Farmaki et al., 2007
	Hydroperoxide lyase (HPL)	G	<i>A. thaliana</i>	T	Farmaki et al., 2007
	Chlorophyll A/B binding protein 180 (CAB180)	G	<i>A. thaliana</i>	T	Farmaki et al., 2007
	FE superoxide dismutase 2 (FSD2)	G	<i>N. tabacum</i>	T	Myounga et al., 2008
	High chlorophyll fluorescence 106 (Hcf106)	G	<i>N. tabacum</i>	T	Vladimirov et al., 2009
	Thylakoid soluble phosphoprotein (AtTSP9)	C	<i>A. thaliana</i>	P	Ferro et al., 2010
	Curvature thylakoid 1A (CURT1A)	R	<i>A. thaliana</i>	T	Armbruster et al., 2013
	Curvature thylakoid 1B (CURT1B)	R	<i>A. thaliana</i>	T	Armbruster et al., 2013
	Curvature thylakoid 1D (CURT1D)	R	<i>A. thaliana</i>	T	Armbruster et al., 2013
	Starch synthase 4 (SS4)	G	<i>N. benthamiana</i>	T	Gámez-Arjona et al., 2014a
	TP-16kDa subunit of the oxygen evolving system of photosystem II (TP-PSII-Q)	G	<i>A. thaliana</i>	P	Marques et al., 2004
	TP-23kDa subunit of the oxygen evolving system of photosystem II (TP-PSII-P)	G	<i>A. thaliana</i>	P	Marques et al., 2004
Starch granule	Granule bound starch synthase (GBSS)	G	<i>A. thaliana</i>	P	Szydlowski et al., 2009; Bahaji et al., 2011
	Dual-specificity protein phosphatase 4 (DSP4)	G	<i>A. thaliana</i>	P	Sokolov et al., 2006
	Isoamylase 3 (ISA3)	G	<i>A. thaliana</i>	T	Delatte et al., 2006
	Starch binding domain of Glucan, water dikinase 3 (GWD3-SBD)	Y	<i>N. benthamiana</i>	T	Christiansen et al., 2009
	Like SEX4 1 (LSF1)	G	<i>N. benthamiana</i>	T	Comparot-Moss et al., 2010
Plastoglobules	Plastoglobulin 30.4 (AtPGL30.4)	G	<i>A. thaliana</i>	T	Vidi et al., 2006
	Plastoglobulin 34 (AtPGL34)	G	<i>A. thaliana</i>	T	Vidi et al., 2006
	Plastoglobulin (AtPGL35)	G	<i>A. thaliana</i>	T	Vidi et al., 2006
	Fructose-1,6-bisphosphate aldolase 1 (AtFBA1)	G	<i>A. thaliana</i>	T	Vidi et al., 2006
	Fructose-1,6-bisphosphate aldolase 2 (AtFBA2)	G	<i>A. thaliana</i>	T	Vidi et al., 2006
	Tocopherol cyclase 1 (AtVTE1)	Y	<i>A. thaliana</i>	T	Vidi et al., 2006
	NAD(P)H dehydrogenase C1 (NDC1)	Y	<i>N. benthamiana</i>	T	Piller et al., 2011
	Phytoene synthase (AtPSY)	R	<i>V. unguiculata</i> subsp. <i>unguiculata</i>	T	Shumskaya et al., 2012

(Continued)

TABLE 1 | Continued

Localization	Gene	FP	Organism of expression	T/P	References
	Phytoene synthase 1 (OsPSY1)	G	<i>Z. Mays</i>	T	Shumskaya et al., 2012
	Phytoene synthase 2 (OsPSY2)	G	<i>Z. Mays</i>	T	Shumskaya et al., 2012
	Phytoene synthase 3 (OsPSY3)	G	<i>Z. Mays</i>	T	Shumskaya et al., 2012
	Phytoene synthase 2 (ZmPSY2)	G	<i>Z. Mays</i>	T	Shumskaya et al., 2012
	Phytoene synthase 3 (ZmPSY3)	G	<i>Z. Mays</i>	T	Shumskaya et al., 2012
	Plastoglobulin 2 (ZmPG2)	R	<i>Z. Mays</i>	T	Shumskaya et al., 2012
	Fibrillin 1b (FBN1b)	G	<i>N. benthamiana</i>	T	Gámez-Arjona et al., 2014b
Nucleoids	N-terminus of Plastid envelope DNA binding (PEND)	G	<i>A. thaliana</i>	P	Terasawa and Sato, 2005
	Apurinic endonuclease-redox protein (ARP)	G	<i>A. thaliana</i>	T	Gutman and Niyogi, 2009
	Endonuclease three homolog 1 (AtNTH1)	G	<i>A. thaliana</i>	T	Gutman and Niyogi, 2009
	Endonuclease three homolog 2 (AtNTH2)	G	<i>A. thaliana</i>	T	Gutman and Niyogi, 2009
	Fructokinase-like (FLN1)	Y	<i>N. tabacum</i>	T	Arsova et al., 2010
	Fructokinase-like (FLN2)	Y	<i>N. tabacum</i>	T	Arsova et al., 2010
	Mesophyll-cell RNAi library line 7 (MRL7)	G	<i>N. tabacum</i>	T	Qiao et al., 2011
	Plastid transcriptionally active chromosome 3 (pTAC3)	G	<i>A. thaliana</i>	T	Yagi et al., 2012
	Lac repressor (LacI)	G	<i>N. tabacum</i>	P	Newell et al., 2012
	SWIB domain containing protein 2 (SWIB-2)	G	<i>N. tabacum</i>	T	Melonek et al., 2012
	SWIB domain containing protein 3 (SWIB-3)	G	<i>N. tabacum</i>	T	Melonek et al., 2012
	SWIB domain containing protein 4 (SWIB-4)	G/R	<i>N. tabacum</i>	T	Melonek et al., 2012
	SWIB domain containing protein 6 (SWIB-6)	G/R	<i>N. tabacum</i>	T	Melonek et al., 2012

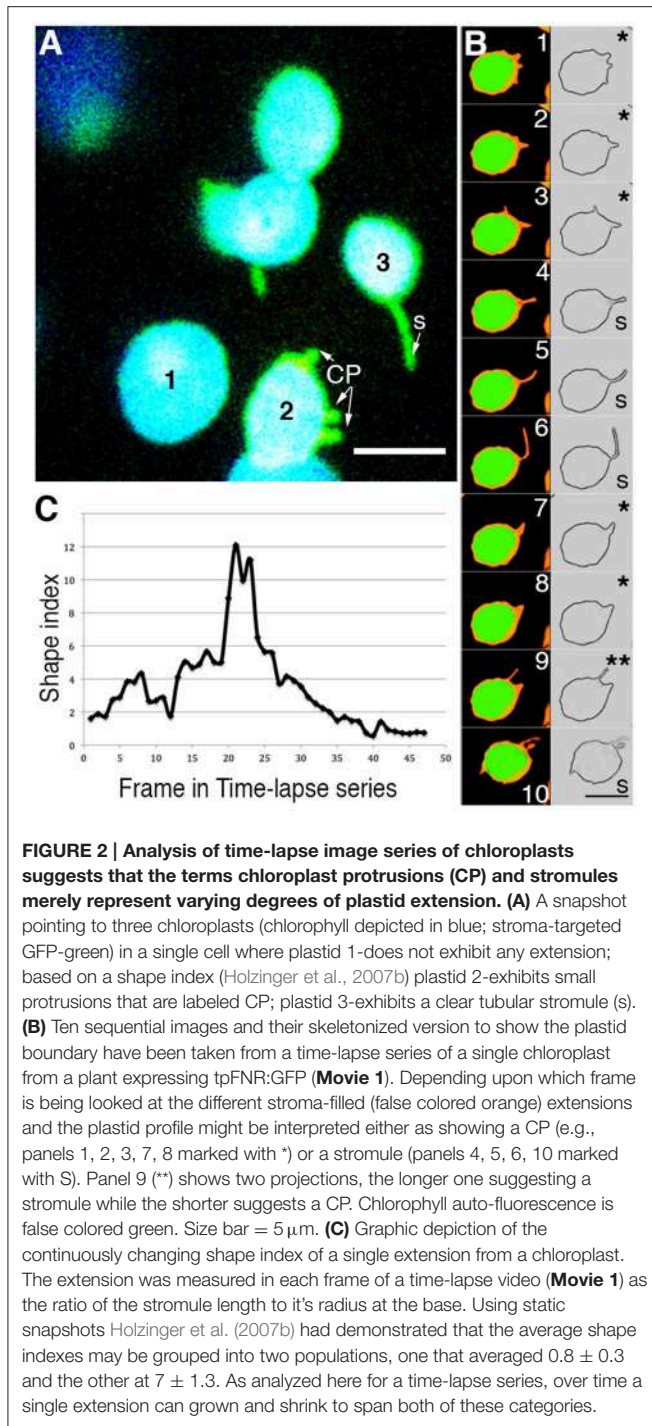
Plant species: *Triticum aestivum* L.; *Arabidopsis thaliana*; *Nicotiana benthamiana*/*tabacum*; *Solanum tuberosum*; *Zea mays*; *Allium cepa*; *Physcomitrella patens*. FP, Fluorescent Protein; E, mEosFP; G, GFP; R, RFP; Y, YFP; P, Transgenic Plant; T, Transient expression; TP, Transit Peptide/presequence. With the exception of the TP-GBSS driven under the Rice Act1 promoter and the LacI plastid nucleoid probe driven by a tobacco psbA gene all other probes reported here used the Cauliflower Mosaic Virus 35S promoter.

produced by chloroplasts in the mesophyll layer is responsible for stromules in the so-called pavement cell leucoplasts (Brunkard et al., 2015). Interestingly a number of publications actually document the presence of chloroplasts in epidermal pavement cells in *Arabidopsis* (Robertson et al., 1996; Vitha et al., 2001; Joo et al., 2005). An authoritative book on plastid biology (Pyke, 2009) provides the unambiguous statement—“in many texts, it is stated that epidermal cells lack chloroplasts, which is untrue.” It is also noteworthy that the major conclusions of Brunkard et al. (2015) are based on observations of excised cotyledons and not true, photosynthesizing leaves. Plastids in wounded as well as senescent tissue are known to show increased stromule frequency (Krupinska, 2007; Ishida et al., 2008). We conclude that the model presented by Brunkard et al. (2015) suggesting change in internal chloroplast redox as a trigger for stromule formation, even though based on an assumption of leucoplasts in *Arabidopsis* pavement cells, is very interesting and requires further critical evaluation.

CHLOROPLAST PROTRUSIONS AND STROMULES: AN ARTIFICIAL DISTINCTION?

During recent years FP-highlighted plastids and stromules have garnered a fair bit of attention but another contemporary

undercurrent of contextual publications based on TEM studies has also existed and requires discussion. Several publications that predate the discovery and naming of stromules, presented double membrane bound stroma-filled protrusions that were simply called chloroplast protrusions (CP) (Bonzi and Fabbri, 1975; Lütz and Moser, 1977; Lütz, 1987; Bourett et al., 1999). Serial TEM sections of leaves in *Ranunculus glacialis* and *O. digyna* (Lütz and Moser, 1977; Lütz, 1987; Larcher et al., 1997; Lütz and Engel, 2007) showed that CP appear as broad or long, grana-free extensions and occasionally form pocket-like structures with mitochondria and microbody aggregates (Lütz and Engel, 2007). While the underlying basis for the statement is unclear researchers on CP appear to have distanced themselves from observations of stromules by declaring that CP and stromules are different (Buchner et al., 2007a,b, 2013, 2014; Holzinger et al., 2007b; Lütz and Engel, 2007; Lütz et al., 2012; Moser et al., 2015). An appraisal of the publications suggests that the only difference is that as compared to CP observed in electron micrographs the stromules are very thin, with diameters less than 800 nm and up to 50 μm long (Köhler and Hanson, 2000). However, emphasis on the thinness of the stromule was made in order to differentiate them from the generally flexible non-photosynthetic plastids that appear irregularly shaped, amoeboid, round to oblong to elongated and form lobes, knobs and loops (Köhler et al., 1997; Köhler and Hanson, 2000; Kwok and Hanson, 2004d). While discussing the early studies in relation to the paucity



of electron micrographs of stromules it was pointed out that studies on CP focused on the leaf tissue, in which stromules are not common, and that stromules are not well preserved by standard fixation methods for electron microscopy (Köhler and Hanson, 2000). Today both statements cannot be upheld since numerous observations on stromules in leaf tissue have been published at both the light microscopy and TEM level (Holzinger et al., 2008; Sage and Sage, 2009; Schattat et al., 2012a). A major effort was made to figure out clear differences between the two

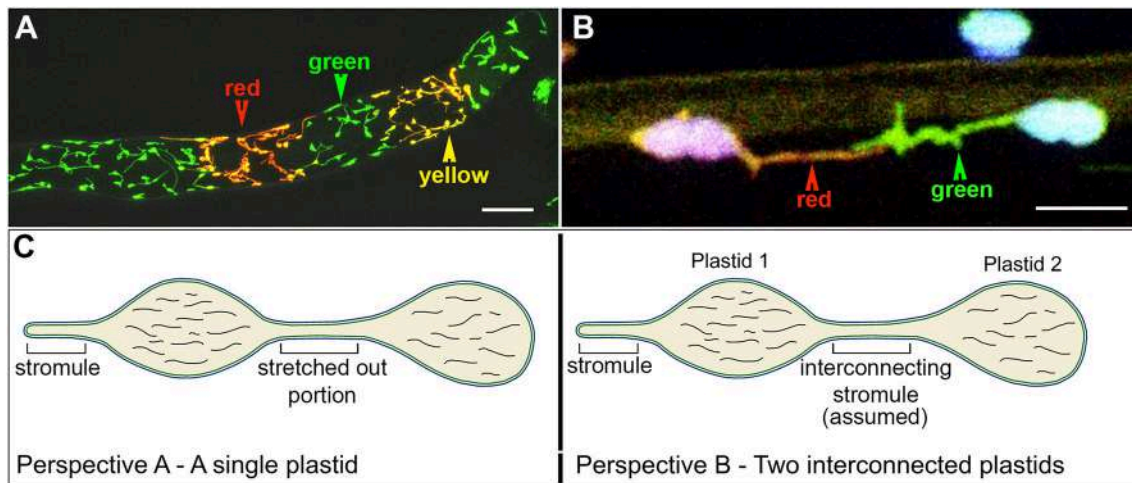
sets of observations by Holzinger et al. (2007b) by creating a “shape index” to compare the different sizes and volumes of stromules with those of temperature-induced protrusions in *A.thaliana*. Interestingly this study concedes that “an interchange between these groups might still be possible,” and whether a protrusion goes on to become a stromule of more typical length and diameter might depend on the sub-cellular space available and the unknown factors that cause stromule growth (Holzinger et al., 2007b). Equally interesting is a contextual comprehensive review that cites the Holzinger et al. (2007b) publication as strong evidence of differences between CP and stromules but also presents a table that lists *Arabidopsis* as a plant that does not produce CP (Lütz, 2010).

The publications on CP have largely been based on TEM snapshots while the FP-aided observations on stromules elegantly reveal the dynamic nature of the plastid. Nevertheless, the distinction appears quite artificial and a report of chloroplast extensions in bundle sheath cells in rice leaves used the terms CP and stromules interchangeably after realizing that the plastid extensions observed might be placed into either category (Sage and Sage, 2009). In addition the excellent transmission electron micrographs of plastids in *Arisarum proboscideum* (Bonzi and Fabbri, 1975) depicted protrusions that today might just as easily be labeled stromules. On the other hand reports published well after the term stromule was introduced (Köhler and Hanson, 2000) persisted in presenting narrow tubules as CP (Figures 2E, 4A in Holzinger et al., 2007a; Figures 5.2D,E, 5.4C,E in Lütz et al., 2012).

As part of our critical appraisal we investigated the behavior of numerous plastids expressing stroma-targeted tp-FNR:GFP. We found that in a snapshot of any leaf expressing stroma-targeted FP might suggest some chloroplasts to be exhibiting CP and others stromules (**Figure 2A**). Time-lapse images (**Figures 2B,C**) show that all stromules, irrespective of whether they are from chloroplasts or any other plastid type, develop from small protrusions that might stretch into tubules of varying lengths and thickness and retract to produce beaked plastids (**Movie 1**).

## THE NOTION OF PROTEIN EXCHANGE BETWEEN INDEPENDENT PLASTIDS

While the use of stroma-targeted GFP allowed plastid stromules to be visualized in living plant cells another FP-based technique involving fluorescence recovery after photo-bleaching (FRAP) was presented alongside to suggest a very important finding (Köhler et al., 1997). The finding was that stromules could interconnect plastids and GFP could flow between them (Köhler et al., 1997). This conclusion was reached by carrying out FRAP on elongated leucoplasts from tobacco roots expressing stroma-targeted GFP. Although the interconnection of plastids was not observed it was assumed that it must have taken place and would have involved stromules. Köhler et al. (1997) were able to demonstrate flow of GFP within a single plastid compartment. Presentation of the FRAP-based view on leucoplasts in reviews and textbooks established a general idea that all plastids are able to connect and exchange proteins with each other



**FIGURE 3 | The use of a stroma targeted green to red photo-convertible mEosFP for differential coloring of plastids allowed the long-standing idea of plastid-interconnectivity through stromules to be reassessed. (A)** A row of single cells showing leucoplasts in a tobacco BY2 cell line expressing the tpFNR:mEosFP shows the three colors (green, red, yellow) that are typically achieved using the probe. Non-photoconverted plastids and stromules appear green; after a 5–7 s exposure to  $490 \pm 30$  nm light fully photoconverted leucoplasts appear red while yellow plastids are obtained after a short 2–5 s photoconversion period. **(B)** Chloroplasts in a pavement cell of a stably transformed Arabidopsis line expressing stroma-targeted tpFNR:mEosFP and chlorophyll (false colored blue) with extended stromules that appear to be interacting. Prolonged observations of hundreds of similar, differentially colored, dynamic plastids and stromules failed to show protein exchange between the chloroplasts. **(C)** Two perspectives of the plastid are presented. Perspective A interprets it as a single, elongated plastid with a narrow intervening tubular region such as that observed during normal pleomorphy of dynamic etioplasts, chromoplasts, and leucoplasts. This perspective is favored by Schattat et al. (2012a,b, 2015). Perspective B underlies the assumption of “interconnected plastids” and considers the narrow intervening region to be a stromule that connects two bulged domains considered as two independent plastid bodies. Leucoplasts with a very similar morphology were used in FRAP experiments to establish the idea of FP flow between plastids (Köhler et al., 1997). Whereas independent plastids actually becoming interconnected have not been observed the flow of a fluorescent protein from one point to another within a single, continuous, membrane bound compartment as depicted here can hardly be disputed. Size bar: **A** = 25  $\mu$ m; **B** = 5  $\mu$ m.

(Hanson and Köhler, 2006; Hanson and Sattarzadeh, 2008, 2011). This view challenges the unitary nature of a plastid but the precise mechanism of plastid fusion implied in this idea has still not been elucidated.

Meanwhile advances in FP technology resulted in the discovery and availability of monomeric Eos, a green to red photoconvertible fluorescent protein (Wiedenmann et al., 2004; Mathur et al., 2010) and allowed a stroma-targeted tpFNR:mEosFP probe to be created (Schattat et al., 2012a). This probe was originally designed to investigate the mechanism leading to protein exchange between plastids whose stromules exhibit prolonged interactions. The probe allows all plastids expressing it to be differentially colored in hues ranging from green to red (Figures 3A,B). Schattat et al. (2012a) reasoned that true fusion of stromules to inter-connect two independent plastids (e.g., Figure 3B) would result in a mixing of stromal color and provide an unequivocal demonstration of protein flow between two plastids. Alternatively maintenance of separate green and red plastid stroma colors despite apparent interaction between their stromules would suggest an inability to exchange fluorescent proteins. To demonstrate that the differential coloring technique and mixing of colors between two fusing organelles actually works they used mitochondria, which like plastids are also double membrane envelope bound organelles. Observations by Schattat et al. (2012a,b) and Mathur et al. (2013) did not support plastid fusion at all and thus strongly contradicted the FRAP-based work on root leucoplasts reported

by Köhler et al. (1997). Despite the evidence that the plastid unit is maintained and no inter-plastid exchange of proteins is observed (Schattat et al., 2012a,b) Hanson and Sattarzadeh (2013) continue to support the original leucoplast-based findings of Köhler et al. (1997). The matter is therefore presently considered as a controversy.

An additional viewpoint propagated through literature based on stroma-targeted FPs suggested the occurrence of interconnected plastids (Köhler et al., 1997; Hanson and Sattarzadeh, 2013). This idea has also been challenged (Schattat et al., 2015), and it is noteworthy that with the exception of artificially initiated chloroplast fusion and in observations of senescent or diseased plant tissue, no one has actually observed two normal and independent plastid units fuse with each other. Further, etiolated plants often display etioplasts with two or more bulged regions connected by a thin tubule (Gunning, 1965; Schattat et al., 2015). Following exposure to light these regions, that appear very similar to plastid bodies, exhibit fluorescence as the protochlorophyllide changes into chlorophyll. As part of our critical appraisal the two views of an elongated plastid are summarized in Figure 3C.

## INSIGHTS FROM FPs TARGETED TO PLASTID MEMBRANES

A number of probes localize to the three types of plastid membranes; the internal, thylakoid membranes; and the inner



and outer membranes of the envelope (**Table 1**; Breuers et al., 2011). Many of the membrane-targeted probes have been expressed transiently under the constitutively active Cauliflower Mosaic Virus 35S promoter with a view of confirming their subcellular localization and are supported by biochemical evidence (Seo et al., 2009; Tan et al., 2011; Mueller et al., 2014). In several cases the overexpression of such fusion proteins has resulted in observations of protein patches on the plastid envelope (Seo et al., 2009; Tan et al., 2011). Alternatively it has led to the ectopic proliferation of membranes (Oikawa et al., 2008; Breuers et al., 2012). Specific patterns of extra membrane formation observed upon transient overexpression show that when proteins of the inner membrane such as AtTIC40:GFP are over-expressed multiple membrane layers are formed on the interior of the plastid envelope while outer membrane proteins such as AtTOC64:GFP form ectopic membrane extensions into the cytoplasm (Breuers et al., 2012; also see **Figure 5** for protein over-expression induced artifacts). Using electron microscopy the authors found that ectopic outer membrane formation was accompanied by a proliferation of the inner membrane and thus concluded that the membrane protrusions represented stromules. However, an electron microscopy based investigation generally does not provide as many chances of observing a phenomenon as provided by fluorescence microscopy of living cells. Thus, at present it is unclear whether all the protrusions formed due to overexpression of an outer membrane protein are actually stromules. Nevertheless, the observations of Breuers et al. (2012) provide an important and testable idea that membrane envelope remodeling such as that suggested during stromule formation might occur through changes in the protein: lipid ratio.

## FPs TARGETED TO STARCH GRAINS, PLASTOGLOBULI, AND NUCLEOIDS

Two distinct types of storage products: starch and plastoglobuli are found in plastids. Starch is composed of long, branched polymers of glucose molecules and either takes a long term storage form, typically found in specialized leucoplasts called amyloplasts, or can transiently accumulate in photosynthesizing chloroplasts and be degraded subsequently during the dark period (Zeeman et al., 2010). Although several probes that target starch grains have been developed (**Table 1**; **Figures 1C,D**) and significant advances have been made in targeting FPs into economically important cereal (Primavesi et al., 2008; Wu et al., 2013; Krishnakumar et al., 2015) and tuberous crops (Sidorov et al., 1999;) their use in understanding the dynamic process of starch grain development is still rather limited. Similarly while biochemical and molecular analysis has identified mutants with different starch composition and properties the effect of different mutations on starch-accumulating plastids is just beginning to be assessed (Matsushima et al., 2014; Sun et al., 2014; Hara et al., 2015; Zhang et al., 2015). It is also notable that although many FP probes highlight plastids in roots the diurnal behavior of leucoplasts, their rapid response to stimuli such as gravity, physical barriers, water and nutrient stress and to soil

microorganisms remain relatively unexplored areas of FP-based research.

In this context one particular starch probe that has remained underexploited is GBSS-GFP (Bahaji et al., 2011; **Figure 1C**). This probe exhibits dual localization; it highlights large starch grains, but when the grains are small or non-existent GBSS:GFP expressed under a CaMV35S promoter predominantly localizes to the stroma. This localization masks small starch grains in some plastid types and makes it a challenging probe for studying the early steps of starch formation. Since GBSS is found exclusively bound to the starch grain when chloroplast fractions are studied (Smith et al., 2004) the stromal localization might result from the 35S promoter induced overexpression or from altered fusion protein turnover due to the presence of GFP.

In contrast to starch plastoglobuli are found in nearly all plastids and their biochemical composition varies between plastid types. They can be formed from a wide variety of molecules including plastoquinone-9, plastoquinol-9,  $\alpha$ -tocopherol, galactolipids, tri-acylglycerols, and carotenoids (Lichtenthaler, 2013). Since plastoglobules can be readily purified biochemically and are being subjected to proteomics (Ytterberg et al., 2006; Nacir and Bréhélin, 2013) the FP-probes for plastoglobuli (**Table 1**) are presently rather under utilized. However, the formation of plastoglobules, their spatio-temporal relation to thylakoids, their characteristic accumulation in different plastid types during development and their function in senescent tissues are all interesting questions that are beginning to be explored using live-imaging approaches (Nacir and Bréhélin, 2013; Shanmugabalaji et al., 2013). A very similar situation exists for plastid nucleoids that have been visualized (**Figure 1E**) but whose localization details during plastid development, differentiation and division await further exploration.

## FP-AIDED INSIGHTS ON PLASTID INTERACTIONS

The endosymbiont theory for the origin of plastids also points to their interactions with all other components and compartments of the plant cell (Margulis, 1970). Plastid interactions have been suggested through organelle/membrane proximity in electron micrographs and concluded from biochemical investigations that have tracked plastid products such as sugars and lipids (Block and Jouhet, 2015; Kölling et al., 2015) as well as signaling components (Sandalio and Foyer, 2015 and cited publications) to other cytoplasmic structures. Several proteins exhibit dual or multiple localization patterns (e.g., **Table 2**), and whereas some of the localizations in transient expression studies might turn out to be artifacts others suggest biochemical relationships shared between different organelles. Some localization patterns might reflect a condition specific status. In addition recent years have seen widespread availability of various FP-probes for plastids and other organelles (Mathur, 2007; The Illuminated Plant Cell, <<http://www.illuminatedcell.com>>; Mano et al., 2011; The maize GFP data base <<http://maize.jcvi.org/cellgenomics/index.php>>) and these have been very useful in establishing views

**TABLE 2 | Some proteins that show multiple localizations.**

Localization		Organism/cell type	Protein	FP	Key features	References
Dual	Chl ER	<i>C. reinhardtii</i>	RB60	G	Protein disulfide isomerase; part of redox regulatory protein complex involved in translation in chloroplasts; exists as soluble form in stroma or tightly bound to thylakoid membrane; also retained in the ER	Levitan et al., 2005
	Chl ER	<i>N. benthamiana</i>	BnCLIP1	G	Lipase; MCS between plastids and ER. Putative plastid inner membrane of envelope localized	Tan et al., 2011
	Chl P	<i>A. thaliana</i>	DRP5B (ARC5)	G	Chloroplast and peroxisome fission; cytosolic, recruited to a discontinuous ring around membrane fission sites	Zhang and Hu, 2010
	Chl Cyt	<i>P. patens</i>	FtsZ	G	Part of division ring; cytosolic assembles into a ring in chloroplasts	Kiessling et al., 2004
	Chl M	<i>A. thaliana</i>	AtDEF1	G	Peptide deformylase; catalyzes <i>N</i> -formyl group removal from methionine residues of nascent polypeptides; AtDEF1.2 and AtDEF2 found in stroma and thylakoid; AtDEF1.1 localizes to mitochondria	Dinkins et al., 2003
	Chl M	<i>A. thaliana</i>	MST1	G	Mercaptopyruvate sulfurtransferase	Nakamura et al., 2000
	Chl M	<i>N. tabacum</i>	AtHRS1	G	Histidyl-tRNA synthetase	Akashi et al., 1998
	Chl M	<i>O. sativa</i>	Virescent2 (V2)	G	Plastid and mitochondrial guanylate kinase (pt/mtGK)	Sugimoto et al., 2007
	Chl M	<i>Z. mays</i>	ZmSig2B	G	Nucleus-encoded sigma factor; accumulates in chloroplasts and mitochondria	Beardslee et al., 2002
	Pl M	<i>G. max</i>	glutathione reductase	G	Component of ascorbate-glutathione cycle	Chew et al., 2003
	Pl M	<i>Z. mays</i>	Myosin XI	Ab*	Myosin motor protein	Wang and Pesacreta, 2004
	Pl M	<i>Oryza</i> spp.	OsNIN1 (M)	G	Alkaline/neutral invertase; transported into both mitochondria and plastids	Murayama and Handa, 2007
			OsNIN3 (Pl)	G		
	Pl Pm	<i>A. thaliana</i> <i>N. tabacum</i>	AtGLR3.4	Y	Glutamate receptor	Teardo et al., 2011
	Pl Vac	<i>A. thaliana</i>	ATG8	G	ATG-dependent autophagy; co-localizes with stroma-targeted DsRed in RCBs in vacuoles	Ishida et al., 2008
Triple	Chl Cyt M	<i>A. thaliaa</i>	tRNA nucleotidyl transferase	G	Adds 3'-terminal cytidine-cytidine-adenosine to tRNAs	von Braun et al., 2007
	Chl/Pl	<i>A. thaliana</i>	FIS1A	Y	Tail anchored membrane protein; implicated in mitochondrial and peroxisomal fission	YFP: Ruberti et al., 2014
	P M			E		mEosFP: Jaipargas, 2015
	Chl/Pl ER- Go	<i>A. cea</i>	Amyl-1	G	$\alpha$ -amylase isoform; localized in amyloplasts degrades starch	Kitajima et al., 2009

Chl, chloroplasts; Pl, plastids; Pm, plasma membrane; P, peroxisomes; M, mitochondria; ER, endoplasmic reticulum; N, nucleus; Go, Golgi bodies; Vac, vacuole; Ly, Lysosomes; E, mEosFP; G, GFP; Y, YFP; \*Ab, antibodies were used, not FP.

regarding plastid interactions with other organelles. Some of the resultant insights are presented.

## PLASTIDS AND THE CYTOSKELETON

Plants need light in order to undergo photosynthesis. Photosynthesis takes place in the chloroplasts of plants but too much or too little light can have negative effects on plant health. Plants have developed two chloroplast responses to combat the lack of or excess of light, the chloroplast accumulation and avoidance responses (Sakai et al., 2001; Kagawa et al., 2004; Wada, 2013). Chloroplasts have been shown to accumulate on the irradiated side of the cell under low intensity blue light, or move away from the light source under high light intensity (Sakai et al., 2001; Kagawa et al., 2004). Two photoreceptors phototropin 1 and phototropin 2 (PHOT1, PHOT2) are implicated in mediating this response (Briggs et al., 2001; Sakai et al., 2001). The light avoidance response possibly minimizes chloroplast damage, thus saving photosystem II (Kasahara et al., 2002, 2004; Takahashi and Badger, 2011) and is mediated by F-actin that surrounds a chloroplast (cp-actin; Kandasamy and Meagher, 1999; Kadota et al., 2009). The cp-actin appears to facilitate chloroplast movement in both the accumulation and avoidance responses through the formation and disassociation of cp-actin on the leading edge and the trailing end of the chloroplast, respectively (Kadota et al., 2009). Major insights have come from analyses of the CHLOROPLAST UNUSUAL POSITIONING gene (CHUP1) and different FP-fusions of its domains and the *chup1* mutant (Oikawa et al., 2003; Schmidt von Braun and Schleiff, 2008; Lehmann et al., 2011). The involvement of myosin motor proteins in plastid movement has been strongly indicated (Paves and Truve, 2007; Kong and Wada, 2011; Wada, 2013).

The involvement of cytoskeletal elements and motor proteins in stromule extension was also investigated (Kwok and Hanson, 2003). The use of different cytoskeleton inhibitors suggested that the formation of stromules and their behavior relies to different degrees upon both microfilaments and microtubules (Kwok and Hanson, 2003). The myosin ATPase inhibitor 2,3-butanedione 2-monoxime (BDM) also resulted in decreased stromule dynamics and suggested the involvement of myosin motors (Gray et al., 2001). Subsequently using transient RNA interference of myosin XI and by localizing a GFP fused to the tail domain of this motor protein to the chloroplast envelope, again in transient expression Natesan et al. (2009) concluded that myosins are essential for stromule formation. Notably, their transient expression based observations using the cargo domain of myosin XI fused to GFP suggest a rather non-specific localization as it includes several other organelles (Natesan et al., 2009). Another transient expression based study using a truncated version of myosin XI reached a similar conclusion (Sattarzadeh et al., 2009).

## PLASTIDS AND THE ENDOPLASMIC RETICULUM

Electron microscopy based investigations have indicated intimate connections between the plastid and the endoplasmic reticulum

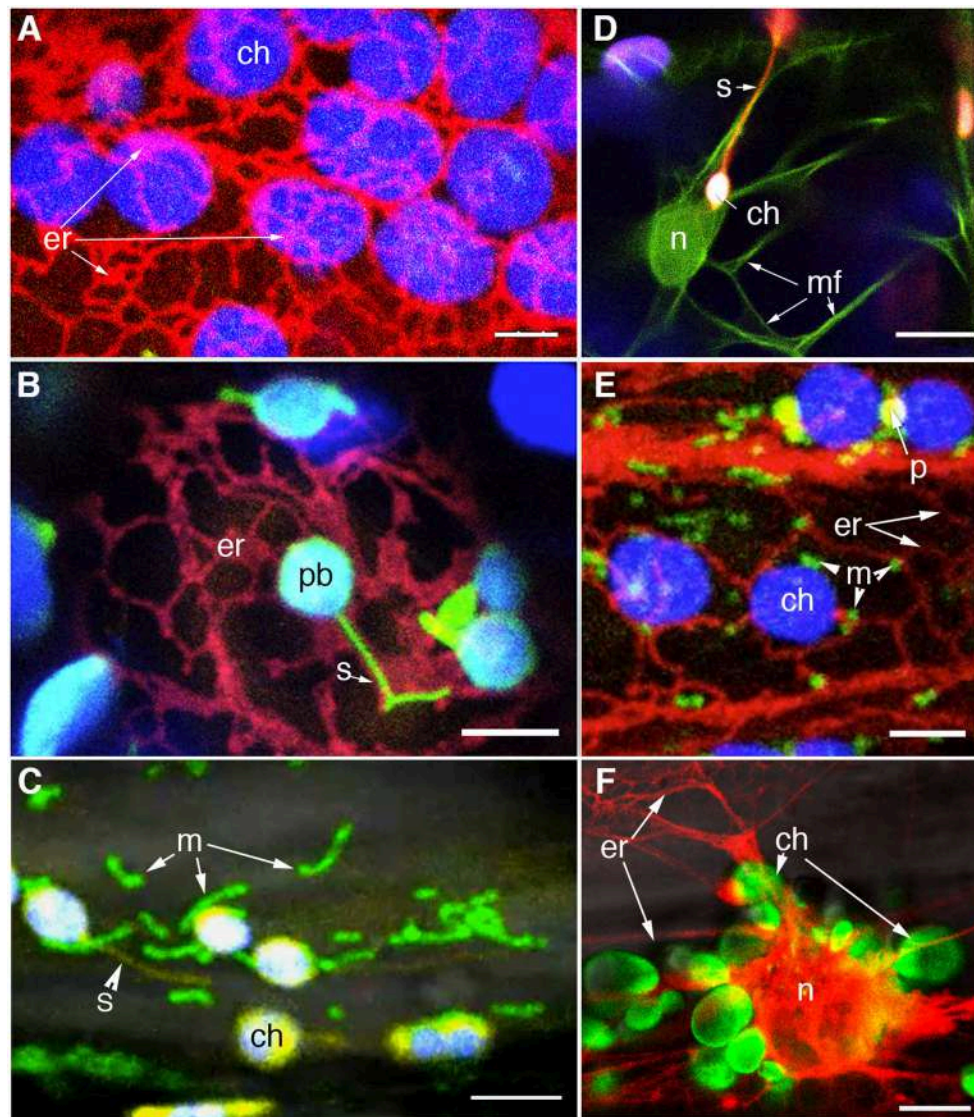
(ER) membranes (Wooding and Northcot, 1965; McLean et al., 1988; Whatley et al., 1991). However, a clear demonstration of plastid and ER interactivity was achieved through simultaneous imaging of different colored FPs targeted to the two organelles (Schattat et al., 2011a,b; **Figure 4**). A loose ER cage around the plastid body (**Figure 4A**), and stromules co-aligned with ER tubules (**Figure 4B**) were observed. The organelle interactivity suggested by these observations was attributed to the presence of membrane contact sites (MCS) between the plastid envelope and the ER (Schattat et al., 2011a,b). The presence of MCS and their strong interconnectivity has been suggested through laser optical tweezers assisted pulling of GFP-labeled ER strands attached to chloroplasts (Andersson et al., 2007). In addition a chloroplast localized lipase from *Brassica napus* fused to GFP (BnCLIP1:GFP) that shows co-localization with ER tubules has been interpreted as indicative of MCS (Tan et al., 2011). While the precise nature of plastid-ER interactions remains to be characterized the identification of the trigalactosyldiacylglycerol (TGD) transporter complex and its association with the ER during lipid biosynthesis are promising leads that are being actively pursued (Xu et al., 2008, 2010; Block and Jouhet, 2015).

## THE PLASTID-NUCLEUS RELATIONSHIP AND VIEWS ON RETROGRADE SIGNALING DURING RESPONSE TO PATHOGENS

As purported descendants of prokaryotic endosymbionts and possessing their own genetic and protein machinery chloroplast gene expression must be highly coordinated with nuclear encoded genes in order to maintain optimal functionality within the cell. Indeed observations of plastids clustered around the nucleus in different epidermal cells with stromules ramifying the grooves and infoldings of the nuclear envelope (Kwok and Hanson, 2004b; **Figure 4F**) favor the idea of signaling between the two organelles. Retrograde signaling from chloroplasts to the nucleus is known to depend upon exposure to light and the redox state of the plastid, might be mediated through metabolite sensing as well as reactive oxygen species (ROS), and involve plastid membrane bound transcription factors (Fernández and Strand, 2008; Stael et al., 2014; Chi et al., 2015). Fluorescent proteins have proved useful in understanding this aspect of plastid integration within the cell.

An elegant approach to understand retrograde signaling from the plastid during pathogen response was taken to follow the movement of N-Receptor Interacting Protein 1 (NRIP1) from chloroplasts to nuclei using NRIP1 fused to the Cerulean fluorescent protein with an N-terminal nuclear export signal (NES) (Caplan et al., 2015). NES-NRIP1-Cerulean can only accumulate within the nucleus after it has been imported and processed within the chloroplast, where the chloroplast transit peptide of NRIP1 is cleaved off along with the NES. Movement of NRIP1, which accumulates within the chloroplast, to the nucleus is triggered in response to Tobacco Mosaic Virus (TMV) infection or expression of the TMV effector protein p50. When NES-NRIP1-Cerulean was co-expressed with p50, processed NES-NRIP1-Cerulean accumulated within the nucleus while no





**FIGURE 4 | Visualization of different colored FP to specific organelles facilitates investigations on plastid interactions. (A)** Confocal image of chloroplasts (chlorophyll autofluorescence false colored blue) and RFP-highlighted ER shows the ER-cage around plastids in a stable transgenic Arabidopsis line. **(B)** An Arabidopsis line co-expressing stroma-targeted tpFNR:GFP [green; plastid body (pb) with chlorophyll false colored blue] and RFP targeted to the ER allowed the stromule (s) -ER correlation to be investigated (Schattat et al., 2011a,b). **(C)** A stable transgenic line coexpressing stroma targeted tpFNR:YFP and mito:GFP (Logan and Leaver, 2000) is allowing an investigation on the mitochondria (m) relationship to chloroplasts (ch) and stromules (s). **(D)** Investigations on F-actin (mf) relationship to chloroplasts (ch) and stromules (s) are being facilitated through a double transgenic line expressing GFP:mTalin (Kost et al., 1998; green) and tpFNR:mEosFP (red). F-actin around the nucleus (n) is apparent. **(E)** A small region from a hypocotyl cell of a triple transgenic expressing RFP targeted to the ER (er), GFP targeted to mitochondria (m) and a YFP targeted to peroxisomes (p). Chloroplasts (ch) are discernable due to their autofluorescence. The line is being used for investigating the relationship between the four organelles. **(F)** A double transgenic line co-expressing tpFNR:GFP and RFP-ER shows the peri-nuclear ER cage and the cluster of chloroplasts (ch) surrounding the nucleus (n) in a hypocotyl cell from a dark grown seedling. The probes might provide several interesting observations and insights into retrograde signaling between plastids and the nucleus. Size bars: **A–C,E,F** = 5  $\mu$ m; **D** = 10  $\mu$ m.

such accumulation was observed when NES-NRIP1-Cerulean was expressed alone. The observation that during the response to p50 expression stromules and plastid bodies can be found in close proximity to the nucleus has been used to suggest that stromules are involved in the direct movement of NRIP1 to the nucleus during the innate plant immune response (Caplan et al., 2008, 2015).

Stromules have also been implicated in facilitating plastid-to-nucleus trafficking during infection of *N. benthamiana* with *Abutilon* mosaic virus (AbMV; Krenz et al., 2010, 2012). Using BiFC (Bimolecular Fluorescence Complementation) as interaction between the AbMV movement protein (MP) and the plastid localized heat shock cognate 70 kDa protein (cpHSC70-1) was observed regardless of infection; however, when challenged



with AbMV infection the number of plastids producing stromules as well as the length of the stromules was increased (Krenz et al., 2010, 2012). Similar observations made using the outer envelope protein-7 (OEP7) during AbMV infection have led to the proposal that stromules are involved in the trafficking of AbMV MP from the cell periphery to the nucleus, or vice-versa, during the infection process (Krenz et al., 2010, 2012, 2014).

These observations are interesting and the conclusions derived from them seem very well thought out. However, it is difficult to reconcile the direct involvement of stromules in the retrograde signaling since none of the studies appear to consider the diurnal fluctuations that lead to stromule extension and retraction. The diurnal cycle of stromules either as a response to a change in chloroplast redox status or a change in cellular sugar levels is quite clear (Schattat and Klösigen, 2011; Schattat et al., 2012a; Brunkard et al., 2015). What happens to the postulated retrograde signaling at night when stromules are not extended? Perhaps the observations are the result of a physiological perturbation of the cell during infection and not indicative of a function of stromules (Krenz et al., 2012). Indeed previous work has interpreted Geminivirus-induced plastid alterations to perturbed carbon metabolism that is likely caused by the disruption of sugar translocation through phloem during infection (Jeske and Werz, 1978). The use of *Agrobacterium* mediated overexpression of proteins under consideration again suggests caution in the interpretations since *Agrobacterium* infiltration itself has been shown to increase stromule frequency (Schattat et al., 2012b; Erickson et al., 2014). Furthermore, as the development of AbMV is known to be affected by light intensity as well as diurnal and seasonal conditions (Krenz et al., 2012), observations linking AbMV infection and stromule formation should be reconsidered to account for the diurnal rhythm of stromule formation (Schattat et al., 2012a; Brunkard et al., 2015) and how the plant's response to a pathogen might affect this cycle. Similarly, given the importance of a plant's developmental stage in relation to stromule formation (Waters et al., 2004) it would be interesting to extend these observations over the course of development in both challenged and unchallenged plants instead of assessing a single time point following infection. Although Caplan et al. (2015) conclude that stromules are involved in the direct transfer of processed NES-NRIP1-Cerulean to the nucleus, it is equally possible that after cleavage of the NES signal NRIP1-Cerulean leaks to the cytosol and then accumulates in the nucleus. Accumulation of an untargeted FP in the nucleus is one of the major caveats associated with their use (Haseloff et al., 1997; Mathur et al., 2010). Interestingly many of the observations involving pathogens span several days without really describing or characterizing the state of the cells or the plastids during those days. Furthermore, clustering of plastids and stromules around the nucleus is not restricted to pathogen response and can be observed throughout the normal development of plants (Kwok and Hanson, 2004b; Figure 4F).

We conclude that the coincidental observations of stromules in virus or other pathogen infected tissue and the suggestion that stromules facilitate retrograde signaling between the plastid and nucleus is a possibility but at present it does not fit in into

the well-documented diurnal phenomenon of stromule extension and retraction.

## TARGETED FPs HAVE PROVIDED A COMPREHENSIVE VIEW OF THE PLASTID DIVISION PROCESS

In higher plants plastid division by binary fission involves a coordinated assembly of four concentric division rings that together constrict both the inner and outer membranes of the plastid envelope (Osteryoung and Pyke, 2014). Whereas some of the proteins such as the internal ring localized FtsZ appear to be of prokaryotic origins others such as the ARC5/DRP5B indicate a eukaryotic derivation. Fluorescent proteins have been used to confirm the localization of several division related proteins at the mid-plastid division site as well as provide convincing proof for their sequential activity through complementation of the pertinent mutant (Vitha et al., 2001; Gao et al., 2003; Miyagishima et al., 2006; Fujiwara et al., 2008; Glynn et al., 2008, 2009; Nobusawa and Umeda, 2012). Using FP-probes it was determined that FtsZ proteins are the first to align on the mid-plastid (Vitha et al., 2001). In subsequent experiments the expression of ARC5-GFP in *pdv1 pdv2* mutants showed impaired localization of ARC5 and led to the conclusion that PDV proteins are necessary for ARC5 localization (Miyagishima et al., 2006). Glynn et al. (2008) performed similar experiments to determine that ARC6 is required to recruit PDV2 to the division ring. FP-based observations have thus provided a comprehensive understanding of the construction of the plastid division ring (Nakanishi et al., 2009; Osteryoung and Pyke, 2014). Additional information on the phenomenon was obtained by using a GFP fused to a bacteria-derived FtsZ1 to assess the effects of higher or lower FtsZ1 expression on division efficiency (Vitha et al., 2001). In other experiments, the use of FtsZ2-GFP probes to observe division ring formation in the presence or absence of cafenstrole, an inhibitor of very-long-chain fatty acids (VLCFA) synthesis, provided an insight on the involvement of VLCFAs in plastid division (Nobusawa and Umeda, 2012).

## INSIGHTS INTO PLASTID BREAKDOWN USING FPs

Senescence is an integral part of the plant's life cycle and involves orchestration of physiological changes designed to recapture and recycle cellular resources. Chloroplasts are amongst the more robust cellular elements and in many tissues are the last to disappear. Senescent chloroplasts, also called gerontoplasts (Figure 1E), appear swollen and often display an amoeboid behavior. They also acquire very different behavioral and biochemical characteristics as compared to healthy chloroplasts (Wise, 2007). At the ultra-structural level gerontoplasts exhibit a progressive un-stacking of grana, a loss of thylakoid membranes and a massive increase in the number of plastoglobuli (Harris and Arnott, 1973; Krupinska, 2007). The controlled disassembly of the photosynthetic apparatus often resembles autophagy (Ishida et al., 2014; Izumi et al., 2015) and results in the formation of

vesicles containing stromal and thylakoid material (Krupinska, 2007; **Figure 1E**). Amongst the degradation-vesicles are the Rubisco-containing bodies (Chiba et al., 2003) that have been observed using stroma-targeted FPs (Ishida et al., 2008; Yamane et al., 2012).

## FPs ARE USEFUL IN LEARNING ABOUT PLASTID ASSOCIATED REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS), such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), are commonly thought of as toxic molecules leading to cellular damage; primarily through lipid peroxidation and membrane degradation. There is an obvious association between the increase of different ROS within the cell during senescence as well as during abiotic and biotic stresses (Zentgraf, 2007; Foyer and Noctor, 2009). Several recent studies have employed fluorescent proteins, such as the redox sensitive GFP (roGFP) (Jiang et al., 2006; Meyer et al., 2007; Schwarzländer et al., 2008) to measure subcellular redox states within living plant cells. The roGFP is sensitive to reduced glutathione pools within the cell which, with the help of endogenous glutaredoxin, reduces roGFP and produces a disulfide bridge between two cysteines that have been engineered into roGFP (Sugiura et al., 2015). Formation of the disulfide bridge causes a conformational change that shifts the excitation maxima and allows ratiometric quantitation of the reduced glutathione pool within a living cell (Hanson et al., 2004; Jiang et al., 2006; Meyer et al., 2007; Schwarzländer et al., 2008; Sugiura et al., 2015). Another FP used to directly estimate the relative concentrations of  $\text{H}_2\text{O}_2$  is the modified YFP known as HyPer (Costa et al., 2010). This probe comprises of YFP fused to a regulatory domain of the *Escherichia coli*  $\text{H}_2\text{O}_2$  sensor OxyR. When HyPer is exposed to  $\text{H}_2\text{O}_2$ , two cysteine bonds form within the OxyR and produce a conformation-induced shift in the excitation maxima from 420 to 500 nm, while the emission maximum of 516 nm remains constant, to allow a ratiometric measurement of  $\text{H}_2\text{O}_2$  (Belousov et al., 2006).

HyPer was first characterized in plants using the guard cells of stable transgenic *Arabidopsis* as well as in suspension cell cultures obtained from these plants where a dosage dependent increase in cytosolic HyPer fluorescence was observed following treatments with exogenous  $\text{H}_2\text{O}_2$  (Costa et al., 2010). HyPer has since been used to assess the response of plastids to  $\text{H}_2\text{O}_2$  produced during pathogen response and to investigate potential plastid-to-nucleus signaling via plastid produced  $\text{H}_2\text{O}_2$  (Caplan et al., 2015). Using a chloroplast targeted HyPer, Caplan et al. (2015), demonstrated that following expression of p50 in *N. benthamiana*, which is known to elicit ROS bursts and augment  $\text{H}_2\text{O}_2$  levels the stromule frequency also increased. Furthermore, when chloroplasts clustered closely around a nucleus were scanned with a 405 nm laser to generate light-induced ROS in chloroplasts, the fluorescence intensity of nuclear localized NLS-HyPer increased; indicating that chloroplast generated  $\text{H}_2\text{O}_2$  accumulated in the nucleus and could be involved in chloroplast to nucleus signaling (Caplan et al., 2015). These studies clearly demonstrated the utility of HyPer in assessing  $\text{H}_2\text{O}_2$  levels within

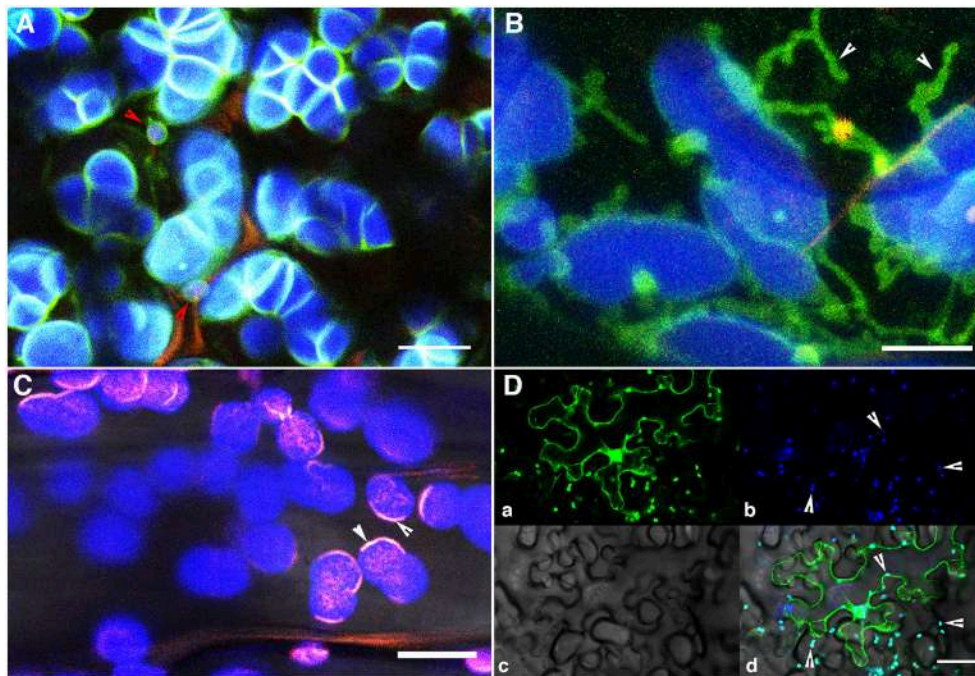
different compartments of the plant cell. It will be interesting to see whether these probes can be applied to investigate changes in cellular redox states during other stresses.

In addition to senescence associated plastid degradation the breakdown of chloroplasts is also linked to a programmed cell death phenomenon that occurs under oxidative stress produced by exposure to high light or physical injury (Apel and Hirt, 2004). The plastid-associated PCD involves the release of singlet oxygen ( $^1\text{O}_2$ ) and leads to the formation of micro-lesions without impairing the general viability of the plant. In green tissue one of the first signs of this localized phenomenon is the loss of chloroplast integrity. An elegant FP-based assay estimated the damage to chloroplasts by observing the leakage of stroma-targeted GFP into the cytoplasm following the  $^1\text{O}_2$  stress (Kim et al., 2012).

## TARGETED FPs AND IDENTIFYING THE POTENTIAL FOR ARTIFACTS

As reviewed here the use of FPs has resulted in several commendable insights on plastids. However, it is important to remember that any fusion protein, despite its expression under the control of the cellular machinery in a living plant cell, is still an artificially created chimera that is quite different from the tag-free protein under investigation. In general, the addition of a 20–30 kDa FP changes the properties of a protein, including its stability and turnover characteristics. In addition the expression of many FP-fusions is augmented through the use of the strong CaMV-35S, or even a double 35S promoter, and thus does not represent the actual protein levels that would be achieved under the native promoter. FP-fusions, specifically those targeted to the plastid membranes are prone to zippering and clumping (**Figure 5A**), can produce abnormal aggregates and large patches, lead to ectopic protrusions (**Figures 5B,C**) and sometimes even provide wrong localizations due to overexpression. Whereas transient expression of fusion proteins is quite efficient and relatively easy to perform it results in a wide range of protein expression levels that vary with time. Such heterogeneity of gene expression promotes a “pick and choose” approach that may bias the observations and resultant conclusions. The creation of multiple stable transgenic lines expressing a specific construct allows for more convincing observations that can be revisited, be subjected to more critical assessments, be studied under different growth and development conditions, and most importantly, can be verified by other investigators. However, transgenic plant creation does require much more time and labor.

A common practice for most plant labs involves the transient agroinfiltration technique where fusions for *Arabidopsis* genes might be carried out in *N. benthamiana* or other tobacco species. While non-matching observations are not usually reported it is worth noting that transient expression patterns obtained using tobacco plants or single cell cultures are not always replicated in stable transgenic *Arabidopsis* plants. Again, consistency between materials chosen for agroinfiltration remains an important factor since young leaves are physiologically quite different from older, fully expanded leaves, which in turn are very different from



**FIGURE 5 | Some of the artifacts resulting from overexpression of a fusion protein. (A)** Overexpression of a N-CHUP:GFP fusion results in sticky plastid envelopes and their massive clumping. **(B)** N-CHUP:GFP overexpression may also result in ectopic protrusions resembling stromules. Whether all such protrusions are actually stromules remains to be determined. **(C)** OE of FIB4:mEosFP that normally localizes to plastoglobuli (**Figure 1D**) can also produce localized artifacts such as extra lining of the inner membrane of the envelope. Observation made using transient expression in tobacco cells. **(D)** Leakage of stroma-targeted FP due to pressure/touch—induced damage to the cell makes the cytoplasm fluoresce due to mis-localization. Note the presence of chloroplasts in pavement cells. Size Bar = 5  $\mu\text{m}$  in **(A,B)**; 10  $\mu\text{m}$  in **(C)**; 50  $\mu\text{m}$  in **(D)**.

senescent ones. For plastids specially, this is an important criterion as the plastid types between green tissue and yellow-green (senescent/stressed) tissue are different. A technique being used quite often involves virus induced gene silencing (VIGS). Studies aimed at understanding stromules must consider the developmental stage of the plant being used as it can have a major effect on the overall conclusions.

During our critical appraisal of published literature on stromules we have become aware of a major discrepancy. Whereas several researchers report and emphasize the diurnal nature of changes in plastid morphology involving the extension and retraction of stromules (Schattat et al., 2011a,b, 2012a; Brunkard et al., 2015) others completely overlook this important fact and draw conclusions from observations that stretch into several days and even weeks. At this stage we can only wonder if conclusions obtained after prolonged periods on a subcellular phenomenon that is observable within 2–5 h should still be worthy of consideration. A very similar reasoning requiring attention concerns inferences on ROS mediated effects on plastid behavior. The term ROS encompasses many different types of oxygen species, each with a different lifetime that affects its ability to penetrate, interact and alter the behavior of cellular membranes (Foyer and Noctor, 2009). Different ROS also trigger chain reactions that can involve several other ROS as well as reactive nitrogen species (RNS). The commonly available point-scanning laser microscopes are not usually calibrated to

deal with the small time scales involved in ROS induced changes and provide real-time data. Whereas the emission of ROS as a general stress induced occurrence in living cells cannot be challenged the estimation of a single ROS through fluorescence decay of a specific FP cannot be indicative of the true ROS levels and the perturbations caused by them in a living cell.

## FUTURE PROSPECTS FOR FP BASED INVESTIGATIONS ON PLASTIDS

Despite the considerable advances in knowledge about plastids where the use of FPs has played an important role some very important questions about these essential organelles of plant cells remain unanswered. The recognition that plastids are independent functional units still requires unequivocal proof. If this idea has to hold true then it should be possible through the use of FPs to distinguish between plastids that look similar but might be metabolically dissimilar. Further, much of our information on plastids comes from the study of chloroplasts. FPs targeted to other plastid types might allow us to fully comprehend the versatile and inter-convertible nature of these organelles. The availability of probes that are already targeted to plastid inclusions such as starch and plastoglobuli suggests that we could start now start combing these probes with an aim to investigate carbon partitioning within plastids. Although some



investigations have been carried out on the interactions between plastids, mitochondria and peroxisomes (Kwok and Hanson, 2003, 2004b,c; Jouhet et al., 2004; Mathur et al., 2012) more details are expected to emerge from double and triple transgenic plants (Figure 4). It will be interesting to actually observe inter-organelle co-operation during photorespiration and high-stress conditions to perhaps add more information to that built up on from seminal TEM and biochemical studies.

## AUTHOR CONTRIBUTIONS

All authors provided input in writing this manuscript.

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Many more publications that use FPs to understand plastids and plastid targeted proteins exist and we apologize to colleagues whose work has not been cited here. We gratefully acknowledge

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01253>

**Movie 1 | Time-lapse imaging of a single chloroplast to show that the difference between a chloroplast protrusion (CP) and a stromule is completely artificial since the two terms represent snapshots of a dynamic phenomenon.** Whereas this chloroplast is quite dynamic other plastids might not show pronounced extensions specially if the cytoplasm is also in a relatively low-dynamic mode. False colored chlorophyll autofluorescence is in green and the stroma in orange (See Figure 2).

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# Chloroplast DNA Copy Number Changes during Plant Development in Organelle DNA Polymerase Mutants

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Chloroplast genome copy number is very high in leaf tissue, with upwards of 10,000 or more copies of the chloroplast DNA (ctDNA) per leaf cell. This is often promoted as a major advantage for engineering the plastid genome, as it provides high gene copy number and thus is expected to result in high expression of foreign proteins from integrated genes. However, it is also known that ctDNA copy number and ctDNA integrity decrease as cells age. Quantitative PCR (qPCR) allows measurement of organelle DNA levels relative to a nuclear gene target. We have used this approach to determine changes in copy number of ctDNA relative to the nuclear genome at different ages of Arabidopsis plant growth and in organellar DNA polymerase mutants. The mutant plant lines have T-DNA insertions in genes encoding the two organelle localized DNA polymerases (PolIA and PolIB). Each of these mutant lines exhibits some delay in plant growth and development as compared to wild-type plants, with the PolIB plants having a more pronounced delay. Both mutant lines develop to maturity and produce viable seeds. Mutants for both proteins were observed to have a reduction in ctDNA and mtDNA copy number relative to wild type plants at all time points as measured by qPCR. Both DNA polymerase mutants had a fairly similar decrease in ctDNA copy number, while the PolIB mutant had a greater effect of reduction in mtDNA levels. However, despite similar decreases in genome copy number, RT-PCR analysis of PolIA mutants show that PolIB expression remains unchanged, suggesting that PolIA may not be essential to plant survival. Furthermore, genotypic analysis of plants from heterozygous parents display a strong pressure to maintain two functioning copies of PolIB. These results indicate that the two DNA polymerases are both important in ctDNA replication, and they are not fully redundant to each other, suggesting each has a specific function in plant organelles.

**Keywords:** chloroplast DNA, DNA polymerase mutants, genome copy number, photosynthesis, quantitative PCR

## INTRODUCTION

Through the process of endosymbiosis, ancient bacteria were engulfed by precursors of eukaryotic cells, and over time most of the genes required for organelle function from these ancestral bacteria have been moved into the nucleus. This raises the question, if most genes have migrated to the nucleus, why not all of them? How do chloroplasts benefit from maintaining their genomes? Most

evidence suggests that the unique physiological environment of chloroplasts is required for proper regulation of chloroplast-specific genes. In a recent paper, John Allen (2015) proposes, supported by significant evidence from the literature, that redox regulation of gene expression is required within the membrane-bound compartment. A chloroplast sensor kinase may detect disruptions in the photosynthetic electron transport chain, which responds to changes in redox conditions to activate or repress chloroplast gene expression, allowing response and regulation of photosynthesis to changing environmental conditions (Allen, 2015). Light has been shown to affect the amount of chloroplast DNA (ctDNA) during plant development (Shaver et al., 2008). Evidence for regulation of chloroplast DNA (ctDNA) by the redox state of cells has been reported in *Chlamydomonas reinhardtii* (Kabeya and Miyagishima, 2013), and similarly for yeast mitochondrial DNA (mtDNA; Hori et al., 2009).

Despite the importance of these organelles, chloroplast and mitochondrial genomes possess relatively few of the genes required for their functions in photosynthesis and respiration. In *Arabidopsis thaliana* chloroplasts there are 87 protein-coding genes and 41 rRNA and tRNA genes (Sato et al., 1999). These numbers are very similar in chloroplast genomes from other higher plant species (Palmer, 1985). The organelle genomes require fully functional transcriptional and translational machinery for expression of the genes. However, plant organelles do not use nuclear DNA replication proteins. Instead, they utilize their own unique set of nuclear-encoded organellar localized DNA replication proteins to maintain their genomes. Many of these are dual-localized to chloroplasts and mitochondria (Christensen et al., 2005; Gualberto et al., 2013; Cupp and Nielsen, 2014; Moriyama and Sato, 2014).

In this paper we focus on chloroplast genome replication and maintenance. CtDNA in higher plants has been shown to replicate by a double-displacement loop mechanism from two specific replication origins (Kolodner and Tewari, 1975; Kunnimalaiyaan and Nielsen, 1997a,b) but may also replicate by a recombination-dependent (RDR) mechanism (Oldenburg and Bendich, 2004; Rowan et al., 2010; Nielsen et al., 2010). The use of two distinct replication mechanisms has been observed for many bacterial virus genomes (Kreuzer and Brister, 2010), where one mechanism is used during the initial stage of infection and another [RDR or rolling circle (RC) replication] for rapid replication of the phage genome for incorporation into new phage particles. The use of two or more mechanisms has been discussed as a possibility for ctDNA replication in plants (Nielsen et al., 2010). Replication via a double-displacement mechanism from specific origins may be involved in maintaining low levels of the chloroplast genome in mature or quiescent cells, while recombination-dependent replication may drive rapid replication to generate high copy numbers of the genome during early stages of plant development.

Tobacco (Ono et al., 2007) and *Arabidopsis* (Christensen et al., 2005; Parent et al., 2011) have been found to encode two closely related bacterial-like DNA polymerases, which have been designated PolIA and PolIB. Both are dual-localized to chloroplasts and mitochondria in these species (Christensen et al., 2005). PolIB has been shown to play a role in ctDNA repair

(Mori et al., 2005; Parent et al., 2011) and mtDNA maintenance, photosynthesis, and respiration (Cupp and Nielsen, 2013). However, in rice (Kimura et al., 2002) and maize (Udy et al., 2012) a single chloroplast-localized DNA polymerase has been identified. By analysis of mutants the maize enzyme, encoded by the *w2* gene, appears to be the only DNA polymerase that functions in chloroplasts and may also function in mitochondria (Udy et al., 2012). There is a paralog of this gene in maize, but the protein has not been detected in chloroplasts. Both maize proteins appear to be involved in mtDNA replication (Udy et al., 2012).

Although the identification and biochemical analysis of plant organelle-localized DNA polymerases has been progressing, limited research has been reported on the role and degree of redundancy of the two DNA polymerases that are found in *Arabidopsis* and some other species. We have examined the effects of mutations in the *A. thaliana* organellar DNA polymerases on ctDNA replication by quantitative PCR (qPCR) analysis of organelle DNA levels. We provide an analysis of the effects of T-DNA insertion mutations in either of the DNA polymerase genes on plant growth and development and chloroplast genome copy numbers.

## MATERIALS AND METHODS

### Planting and Growing Conditions

We obtained the following T-DNA insertion lines from the *Arabidopsis* Biological Resource Center (Figure 1; ABRC; www.arabidopsis.org): Salk\_022624 for PolIA (At1g50840); Salk\_134274 (this is the same line designated polIb-1 in Cupp and Nielsen, 2013) for PolIB (At3g20540). Pots with the approximate dimensions 3 × 3 × 4 (width × length × height) inches were firmly packed with potting soil and placed in a tray. The soil was then saturated with nutrient water prepared with water-soluble fertilizer (Peter's Houseplant Food). *Arabidopsis* seeds were planted directly onto the surface of the soil and placed in a 4°C cold room in the dark for up to 3 days. Plants were then moved to a growth room maintained at 22°C with an average surface-light exposure of 80–100 μmol m<sup>-2</sup> s<sup>-1</sup>. During the first 5 days of germination trays were covered with transparent plastic covers to maintain humidity and prevent drying, after which the covers were removed.

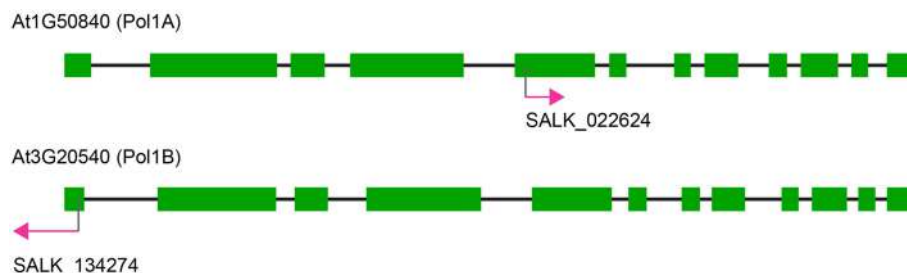
### Tissue Harvesting and DNA Extraction

Leaf tissue was harvested from plants at 7, 10, 14, and 21 dpi (days post-imbibition). Genomic DNA from these plants was then isolated following a cetyltrimethylammonium bromide (CTAB) method for isolating high quality DNA (Minas et al., 2011).

### Screening of T-DNA Insertion Lines

To determine if the T-DNA insertion was present, T-DNA specific primers were used in conjunction with native gene primers. Primers were designed so that native gene primers produced a PCR product about 1 kb in length, and that the T-DNA insertion primer paired with the native gene primer produced a PCR product ~500 b in length. Details of the primers used in zygosity screening are shown in **Supplementary Table 1**.





**FIGURE 1 | Map of the DNA polymerase genes and T-DNA insertions.** Note the overall similarity between both genes for PolIA and PolIB. Both genes possess 12 exons although SALK\_022624 inserts in the fifth exon of PolIA whereas SALK\_134274 inserts in the first exon of PolIB.

In order to obtain plants that were heterozygous for PolIA and PolIB genes, homozygous PolIA and PolIB plants were emasculated and then pollinated from either homozygous PolIA or PolIB flowers. This cross generated offspring that were heterozygous for both PolIA and PolIB, confirmed via PCR. Seeds from the first generation of heterozygous plants were collected to screen for all possible combinations of PolIA and PolIB using PCR as described above.

### Genome Copy Number Analysis

Mitochondrial and chloroplast genome copy number was analyzed using an Applied Biosystems StepOne Plus qPCR machine and PowerUp SYBR green reagents. To analyze genome copy number, sequences unique to either ctDNA or mtDNA were identified. For ctDNA analysis, the targets *psbK*, *petD*, and *ndhH* were used. For mtDNA analysis, these targets included *nad9*, *orf25*, and *cox1*. The housekeeping gene *AtRpoTp* was used as a positive nuclear control and a reference for  $\Delta\Delta C_t$  calculations. A summary of these targets and their specific genes are listed in **Supplementary Table 2**. Technical and biological replicates were compiled and analyzed using the  $\Delta\Delta C_t$  method (Schmittgen and Livak, 2008; Cupp and Nielsen, 2013).

### Analysis of Gene Expression Analysis in PolIA Insertion Line

mRNA was isolated from 7 dpi plants using PureLink Plant RNA Reagent (Life Technologies). RNase free DNaseI was added to remove residual DNA. Purity of mRNA was confirmed by running a small amount on a gel and checking for the absence of large DNA bands. cDNA for RT-PCR was generated from the purified mRNA using SuperScript III reverse transcriptase (Thermo Fisher). Primers for RT-PCR were designed to amplify a portion of the gene near the 3' end of the mRNA. Primers for RT-PCR are described in **Supplementary Table 3**.

### Photosynthesis Assays

Seeds from each mutant were germinated in plastic scintillation vials and grown under the same conditions as described above. At 14 dpi the vials were placed in a Licor 6400-22 Lighted Conifer Chamber Package connected to a Licor Li-6400XT analyzer. This system has the ability to measure photosynthetic rates and can automatically generate  $CO_2$  and light response curves. For this

study, net photosynthetic rates of PolIA and PolIB mutants were calculated by measuring total leaf surface area. Total leaf area was calculated by scanning each plant and using ImageJ to trace and calculate surface area.

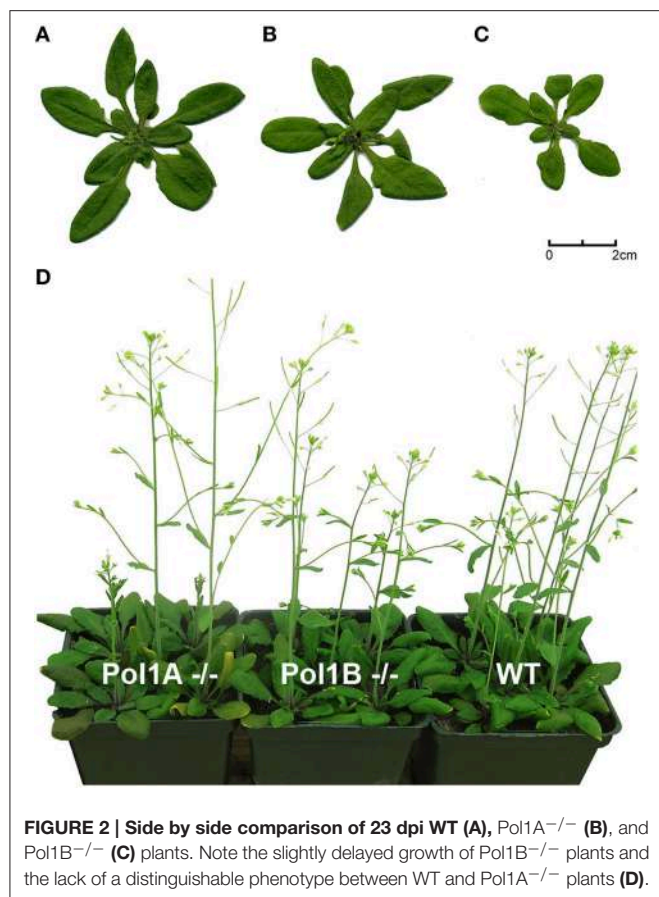
## RESULTS

### Phenotype and Expression Analysis of Organelle DNA Polymerase Mutants

The T-DNA insertion in PolIA is in the fifth exon of the gene, while the insertion in PolIB is in the first exon (**Figure 1**). The homozygous single mutant plants exhibited slight growth delays but both grow to maturity and produce seeds. Mutants in PolIB mutant plants exhibit a slower growth rate than the PolIA mutants. This pattern is consistent over time and reproducible (**Figure 2**; **Supplementary Movie 1**). This indicates that neither DNA polymerase is completely essential for development.

We previously showed that both DNA polymerases are expressed in most plant tissues during development, but there is a difference when comparing expression levels of the two genes. DNA PolIA is most highly expressed (relative to DNA PolIB) in rosette leaves, while DNA PolIB is expressed more abundantly in non-photosynthetic tissue (Cupp and Nielsen, 2013). We previously reported that in PolIB mutant plants, when expression of PolIB is knocked down a substantial increase (60–70%) in PolIA expression was observed by qRT-PCR analysis (Cupp and Nielsen, 2013). We were interested to determine if a similar compensatory effect occurs for the PolIA mutant. However, relative expression of PolIB in PolIA mutant plants was not significantly different from wild-type levels (**Figure 3**). This suggests an important role for DNA PolIA in chloroplasts and ctDNA maintenance, while PolIB may play a more significant role in mtDNA replication and maintenance.

Our findings are consistent with expression of the Arabidopsis DNA PolIA gene compiled from microarray analysis in the Arabidopsis eFP browser (<http://bar.utoronto.ca/~dev/eplant/>). PolIA expression is highest in rosette leaves of wild-type plants, especially the youngest leaves, but is also high in imbibed seeds and developing flowers, and remains relatively high in cauline and older leaves. Expression of PolIA is low in embryos and siliques and in pollen (**Figure 4**), and is stimulated by drought and greatly repressed by osmotic stress (Nakabayashi et al., 2005;



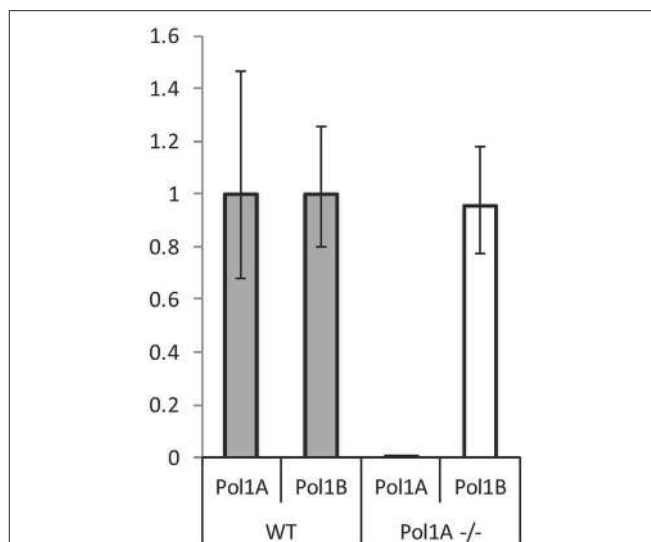
**FIGURE 2 | Side by side comparison of 23 dpi WT (A), *Pol1A*<sup>-/-</sup> (B), and *Pol1B*<sup>-/-</sup> (C) plants. Note the slightly delayed growth of *Pol1B*<sup>-/-</sup> plants and the lack of a distinguishable phenotype between WT and *Pol1A*<sup>-/-</sup> plants (D).**

Schmid et al., 2005). Coexpression data (ATTED-II) indicates that the *PolIA* gene is coexpressed along with chloroplast-localized RecA, OSB2 (a single-stranded DNA binding protein, Gualberto et al., 2013) and some helicase genes. These proteins may all be involved in ctDNA replication, which would be compatible with the involvement of DNA recombination in chloroplast genome replication (RDR) and/or repair. There is very little information available for DNA PolIB in these databases.

Field-inversion gel electrophoresis (FIGE) and restriction pattern analysis of ctDNA from the mutants showed no discernable differences in the mutants compared to wild-type plants (data not shown). We used a PCR assay to detect any differences in rearrangement frequency in the mitochondrial genome, as has been observed for mutants affected in mtDNA recombination (Xu et al., 2011). However, the *PolIA* and *PolIB* mutants showed no differences in rearrangement frequency, indicating that there is no major disruption or change in the mechanism for DNA replication/recombination in the individual gene mutants for ctDNA or mtDNA (not shown).

## CtDNA and mtDNA Copy Number Determination

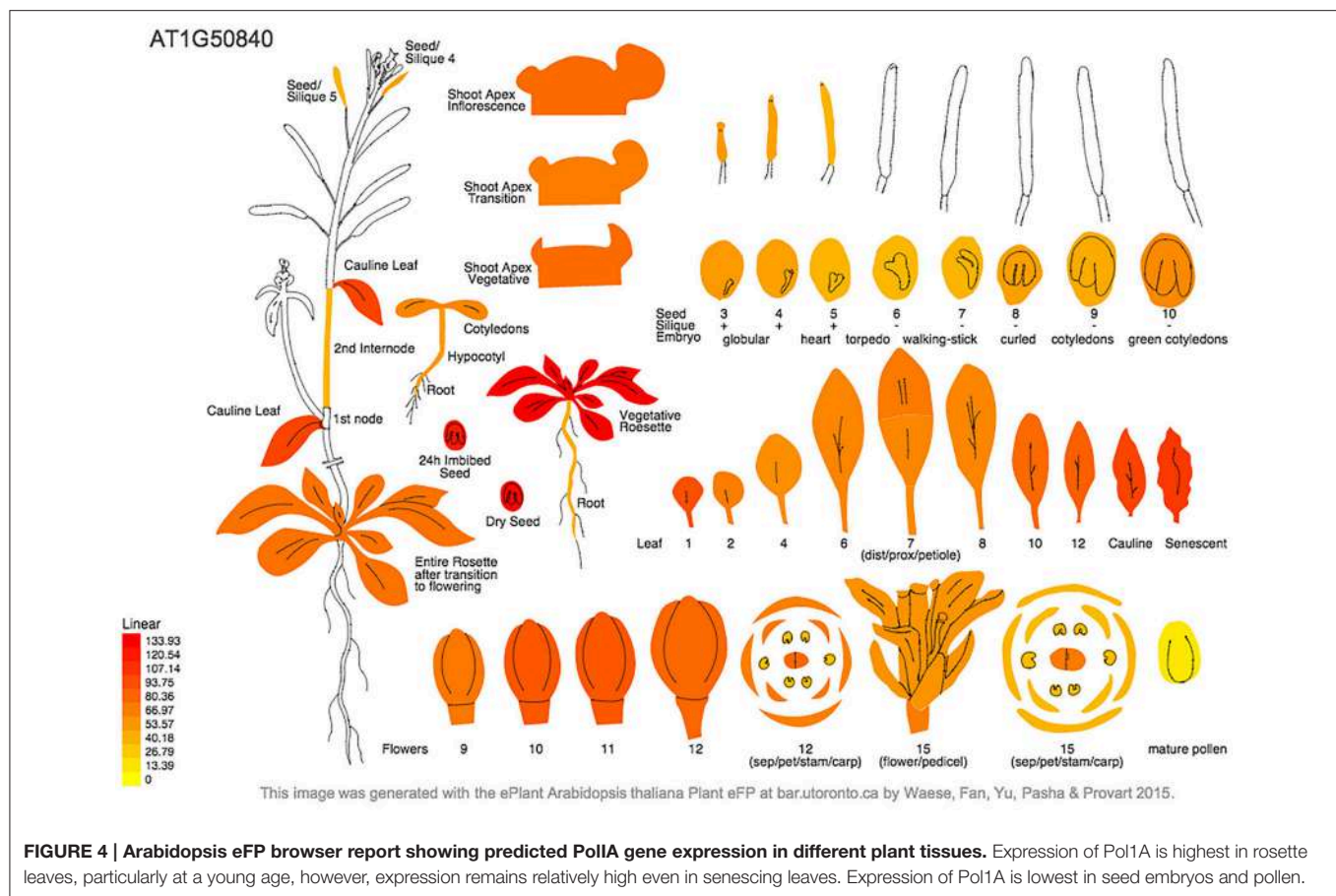
qPCR analysis of ctDNA and mtDNA levels in each of the DNA polymerase mutant lines compared to wild-type showed that



**FIGURE 3 | RT-PCR of *Pol1A* and *Pol1B* expression in *Pol1A* mutant plants.** Although previous work has suggested that mutation in *Pol1B* causes an increase in *Pol1A* expression, mutation of *Pol1A* does not affect expression of *Pol1B*. This experiment shows relative levels of each polymerase transcript normalized against Actin mRNA. Although mutation in *Pol1A* knocks down its expression, no significant change in *Pol1B* expression can be observed.

relative ctDNA levels and mtDNA levels, compared to the nuclear genome, are reduced in both *PolIA* and *PolIB* mutants, similar to what has been reported before for single time points (Parent et al., 2011; Cupp and Nielsen, 2013). To determine DNA levels at additional stages of growth, we analyzed samples at different time points. We examined DNA levels at 7, 10, 14, and 21 days of growth. At all time points there is a decrease in organelle DNA copy number in both mutants compared to wild-type plants of the same age for all 3 separate targets for each organelle genome at each age (Figure 5). Both *PolIA* and *PolIB* mutants showed a ~30% reduction in ctDNA at 7 days, a ~40% reduction at 10 and 14 days, and a 50% reduction at 21 days. At 21 days, there is a slightly greater reduction in the *PolIB* mutant (~60% decrease) compared to the *PolIA* mutant (~50% decrease). These results indicate that both DNA polymerases affect ctDNA copy number, in contrast with the finding in maize that a single DNA polymerase is responsible for ctDNA replication (Udy et al., 2012).

Similar but slightly different results were observed with the two mutant lines when mtDNA targets were analyzed. At 7 days the *PolIA* mutant showed only a slight drop in mtDNA copy number, while *PolIB* showed nearly a 40% drop (Figure 5), similar to what we previously reported (Cupp and Nielsen, 2013). At 10 and 14 days the *PolIA* mutant had a 20–40% drop in mtDNA copy number, while in *PolIB* the decrease was about 50%. At 21 days, the *PolIA* mutant had a 40% decrease in mtDNA, while the *PolIB* mutant showed a decrease of more than 60%. These results suggest that while both DNA polymerases contribute to mtDNA copy numbers, *PolIB* appears to play a greater role in maintenance of the mitochondrial genome. While qPCR analysis does not directly address quality of the DNA,



it does show trends over time for the mutants compared to wild-type plants, indicating changes in organelle DNA levels during development in the mutants compared to wild-type plants.

## Analysis of Photosynthesis in DNA Polymerase Mutants

The decreases in organelle DNA copy number in the mutants raises a question as to whether these changes affect photosynthesis. In previous work with PolIB mutants increases in photosynthesis and related parameters were observed (Cupp and Nielsen, 2013). Current measurements showed an increase in net photosynthesis was observed in 14 dpi PolIA<sup>-/-</sup> plants (Figure 6). However, we acknowledge that despite careful controls during experimentation, the observed data for PolIA<sup>-/-</sup> plants may not be completely accurate. Despite this difficulty in making highly precise measurements, the data suggests that there is an increase in photosynthesis in PolIA<sup>-/-</sup> plants, although it cannot be accurately quantified at this time.

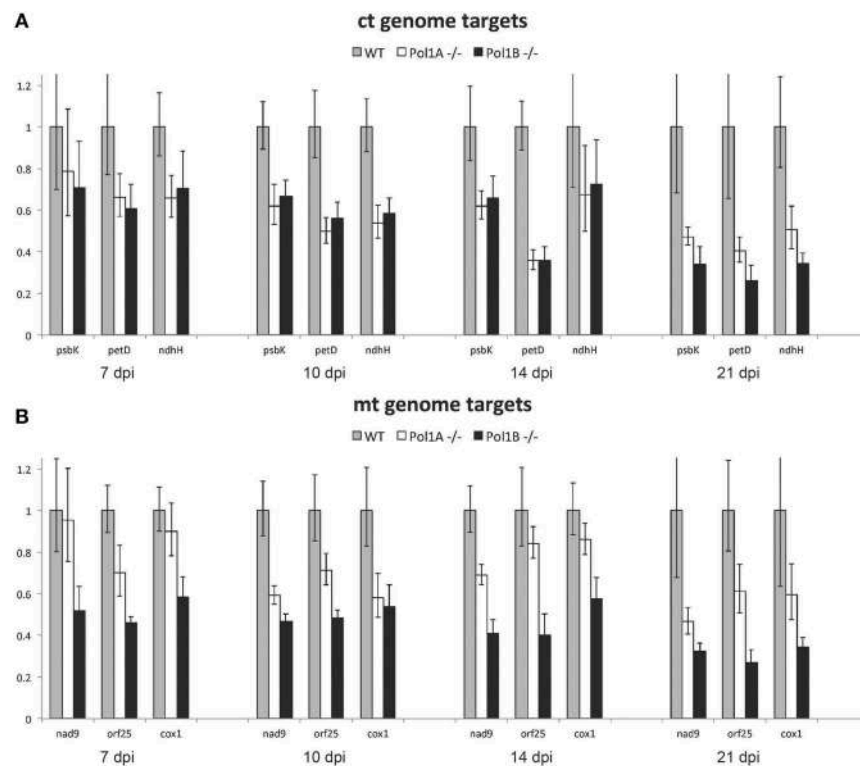
## Analysis of PolIA × PolIB Partial Double Mutants

The results of qPCR analysis and previous genotyping experiments led us to believe that certain genotypes would

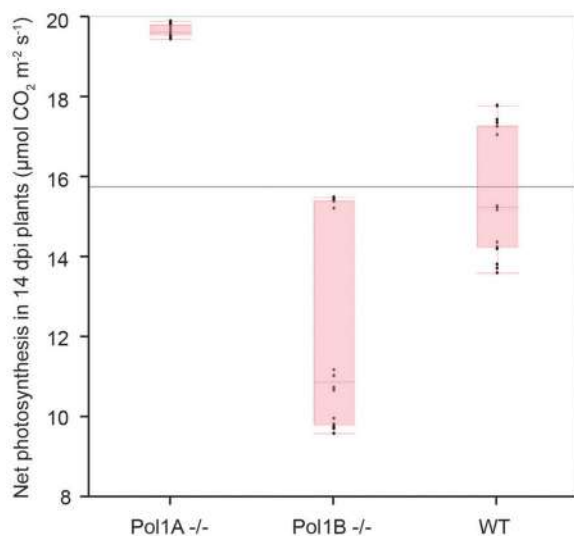
be more beneficial to plant survival than others. To test this theory, we planted seeds on soil in the same manner described above and genotyped all plants that were able to successfully germinate and grow. As expected, none of the surviving plants were homozygous for T-DNA insertions in both DNA polymerase genes as this most likely is lethal to the plant (Figure 7). We also noticed that survival for plants possessing only one functioning DNA polymerase gene was poor. Interestingly we observed strong pressure to maintain both copies of PolIB with at least one functioning copy of PolIA. The pressure to maintain both copies of PolIB suggests higher levels of this polymerase are required to maintain healthy plants.

## DISCUSSION

Analysis of mutations in the genes encoding the organellar DNA polymerases can provide helpful information for understanding their role in chloroplast DNA replication and genome maintenance. However, at the current time analysis of organelle DNA polymerase mutants has apparently only been done for Arabidopsis (Parent et al., 2011; Cupp and Nielsen, 2013) and maize (Udy et al., 2012). In maize it was shown that a single nuclear-encoded chloroplast-localized DNA polymerase (encoded by the w2 gene) is responsible for nearly all ctDNA



**FIGURE 5 | Change in relative chloroplast and mitochondrial genome copy number.** Note that mutations in Pol1A and Pol1B affect chloroplast genome copy number equally (**A**) however mutation of Pol1B causes a more severe drop in mitochondrial genome copy number (**B**). In both mutants, genome copy number gradually decreases but remains lower than wild type as the plants age.



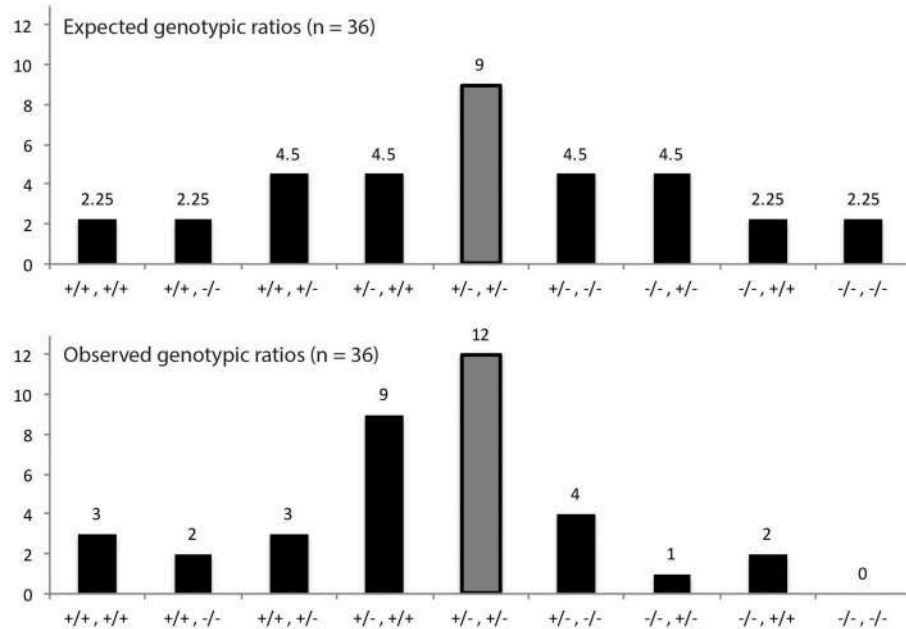
**FIGURE 6 | Net photosynthetic rates in mutant plants.** Observed photosynthesis rates appear to increase in Pol1A<sup>-/-</sup> mutants.

replication (Udy et al., 2012). In contrast, our results show that both Pol1A and Pol1B are required to maintain normal growth of *A. thaliana* (Figure 2, Supplementary Movie 1, Cupp and Nielsen, 2013).

Both of the previous reports on Arabidopsis focused on Pol1B, which indicated effects on mtDNA copy number and mitochondrial structure (Cupp and Nielsen, 2013) and on plastid DNA repair (Parent et al., 2011). In this paper, we have focused on Pol1A, and show that it plays a role along with Pol1B in ctDNA replication as measured by copy number analysis. This analysis also indicates that Pol1A contributes to a lesser extent in mtDNA maintenance. Mutants in each DNA polymerase gene have a limited effect on phenotype, with Pol1B plants growing the slowest, while Pol1A plants grow only slightly slower than wild type plants.

Analysis of partial double mutants indicates a strong preference for at least one copy of the Pol1B gene. As expected, no viable homozygous double mutants were observed, indicating that at least one copy of one of the DNA polymerases is required for growth, although growth is progressively affected by the loss of either the second Pol1A or Pol1B allele. As mentioned previously, there is a strong pressure to maintain at least two functioning copies of either DNA polymerase gene, and an even stronger pressure to maintain both Pol1B genes with at least one functioning Pol1A gene. This suggests that Pol1B is much more essential to plant survival and may also be needed at higher expression levels to support a healthy plant. This is in line with our previous report that Pol1B mutants are haploinsufficient while Pol1A is not, which suggests an additive effect of functional Pol1B gene copy number (Cupp and Nielsen, 2013).





**FIGURE 7 | Proportion of genotypes from DNA PolIA × PolIB crosses.** The results come from 36 plants that were able to successfully grow on soil. The horizontal axes represent the possible genotype combinations starting with PolIA and followed by PolIB (e.g., +/-, +/- represents PolIA<sup>+/-</sup>, PolIB<sup>+/-</sup>, respectively). The middle bar represents the heterozygous combination of genes and is highlighted gray for convenience. Because the results are only from surviving plants, certain genotypes were not observed, such as PolIA<sup>-/-</sup>, PolIB<sup>-/-</sup> as this combination most likely is lethal to the plant. A particularly interesting genotype was PolIA<sup>+/-</sup>, PolIB<sup>+/-</sup> which was present in an uncharacteristically high number of plants.

**TABLE 1 | Prediction of PolIA and PolIB organelle localization.**

Prediction program	PolIA*		PolIB*	
	Ct	Mt	Ct	Mt
TargetP	0.928	0.314	0.588	0.741
PCLR	0.995	–	0.915	–
Predotar	0.950	0.100	0.600	0.450

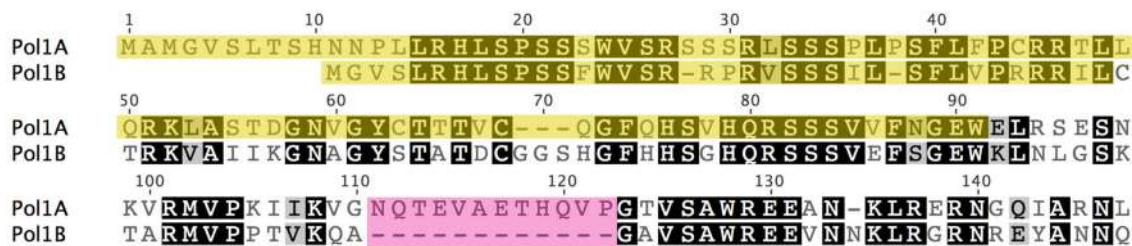
\*Each prediction program returns the likelihood of each resulting protein localizing to either chloroplasts (Ct) or mitochondria (Mt). This prediction is made based on the amino acid sequence of each polymerase.

Expression of the DNA polymerase genes appears to be very high in young developing tissues, especially in meristems (Kimura et al., 2002). PolIA is expressed most abundantly in developing and rosette leaves (Figure 4; Cupp and Nielsen, 2013), which agrees with the data available from online expression databases. In contrast, PolIB is expressed highly (relative to PolIA) in non-photosynthetic tissues (Cupp and Nielsen, 2013). However, both are expressed in all tissues. The higher expression of PolIA in leaves suggests that it may play an important role in ctDNA replication. However, the small effect of a homozygous insertion mutant for this gene on plant growth indicates that the PolIB gene can at least partially complement the PolIA mutation.

A significant increase in PolIA expression was observed in homozygous mutant PolIB plants (Cupp and Nielsen, 2013). In contrast, in homozygous PolIA mutants there is no significant

change in PolIB gene expression (Figure 3). PolIA homozygous mutants show an increase in net photosynthesis (Figure 6). Photosynthesis was also affected in PolIB mutants (Cupp and Nielsen, 2013). There may be an inverse relationship between mtDNA levels and net photosynthesis. It may be a decrease in mtDNA, which would affect mitochondrial function, causes a compensatory increase in chloroplast function, including photosynthesis. Thus, while mutants in both genes share some similarities (reduction in growth rate and organelle genome copy numbers and effect on photosynthesis), there are differences in the levels of these effects that strongly suggest different functions for the two DNA polymerases.

Although, both DNA polymerases have been shown to be dual targeted to chloroplasts and mitochondria, we hypothesize that chloroplasts rely more on PolIA whereas mitochondria rely more on PolIB for DNA replication. We hypothesize that a mutation in PolIB causes increased expression of PolIA to make up for the loss of function of PolIB proteins. In the reverse scenario, mutation of PolIA has a less severe effect, and PolIB may compensate for loss of function of PolIA without the need for higher PolIB expression. Further supporting this hypothesis are localization predictions based on protein sequence analysis. When the protein sequences for PolIA and PolIB are analyzed by localization prediction programs Target P (Emanuelsson et al., 2007), PCLR, (Schein et al., 2001) and Predotar (<https://urgi.versailles.inra.fr/predotar/predotar.html>), PolIA is consistently predicted to localize to chloroplasts more strongly than mitochondria while PolIB is



**FIGURE 8 | Predicted signal peptides of PolIA and PolIB and sequence homology in the early region of each polymerase protein.** Predictions of each protein's signal peptide was made using ChloroP. Residues highlighted in yellow represent the predicted signal peptide to be cleaved after localization. Note that despite a much shorter predicted signal peptide, PolIB continues to maintain high homology with PolIA for ~60 more residues. The first region of dissimilarity between the two polymerases is highlighted in pink.

most strongly predicted to localize to mitochondria. A summary of these results can be found in **Table 1**. A more detailed analysis using ChloroP (Emanuelsson et al., 1999) predicts that the first 91 residues of PolIA whereas only the first 36 of PolIB serve as a signal peptide for PolIB, which may help explain the differences in preferred localization. However, PolIB maintains high homology with PolIA beyond its predicted signal for ~60 residues (**Figure 8**). Thus, while the genes and protein products are highly homologous, they have some significant differences at the N-terminal and other internal regions, contributing to the observation that the two DNA polymerases are not fully redundant to each other.

In contrast to the computer predictions, both PolIA and PolIB have been shown to be dual-targeted to chloroplasts and mitochondria (Christensen et al., 2005). However, the two DNA polymerases may not be equally localized to both organelles at all stages of plant development. It was reported that plastid localization of PolIA was only obtained when the entire 5'UTR was included in the GFP fusion construct. When the UTR was deleted, initiation of protein synthesis occurred only at the annotated start codon and localization became dual-targeted. The 5'UTR lacks an in-frame upstream start codon, suggesting that an alternate non-AUG start codon was used (Christensen et al., 2005). Localization may vary depending on growth conditions, which could dictate which form of the protein is translated and thus which organelle it is targeted to. This may also play a role in the localization of the proteins when one of the DNA polymerase genes is knocked out in the T-DNA insertion lines. The absence of one DNA polymerase may trigger signal(s) for expression of a form of the other DNA polymerase that can compensate for the mutated enzyme. This could explain some of the slight differences in growth rate and other characteristics between the two mutants. The proposed presence of an alternate mechanism for ctDNA replication could also explain why disruption of one or both of the mapped origins (ori) is not lethal, while some of the linear fragments generated still map near the mapped ori regions (Mühlbauer et al., 2002; Scharff and Koop, 2006). The confirmation and characterization of different replication mechanisms and differential localization of the organellar DNA polymerases during plant development or in response to mutation or stresses deserves further study.

It is interesting that of the four species for which organellar DNA polymerase genes have been characterized, Arabidopsis and tobacco, which are dicotyledonous plants, have two organelle localized DNA polymerases that both appear to be essential for normal growth and replication of chloroplast and mitochondrial genomes. In contrast, maize and rice, which are monocots, appear to have a single DNA polymerase that is responsible for substantially all ctDNA replication. Analysis of organellar DNA polymerases in additional species will be required to determine whether this is a consistent pattern, which would suggest significant differences in the replication machinery for plants from these two lineages.

Chloroplast genome copy numbers per cell are highest in young photosynthetically active leaves. Chloroplast genome copy number varies widely between tissues, ranging from 3 to 275 copies per plastid in leaf cells of different developmental stages (Zoschke et al., 2007; Liere and Borner, 2013). For other species there are 10–400 copies of the chloroplast genome per plastid, translating to 1000–50,000 genome copies per plant cell (also see Boffey and Leech, 1982; Tymms et al., 1983). This number has been given as a compelling basis for chloroplast genetic engineering. Such high copy numbers could theoretically lead to high expression of introduced genes. Indeed, high yields of gene products in engineered chloroplasts have been reported (Grevich and Daniell, 2005; Maliga and Bock, 2011).

## CONCLUSION

In summary, there are two closely related organelle-localized DNA polymerases in *A. thaliana*. While mutants in either gene have only a slight effect on plant growth and net photosynthesis, the two enzymes do not appear to be fully redundant. Mutation of PolIB causes a more drastic effect on growth compared to the effect of mutation in PolIA. This is supported by genome copy number analysis. Mutation of either DNA polymerase causes a similar decrease in ctDNA copy number, while mutation of PolIB causes a more substantial reduction in mtDNA genome copy number than PolIA mutation. While knockdown of PolIB resulted in increased expression of PolIA, suggesting compensation for the loss of PolIB (Cupp and Nielsen, 2013), knockdown of PolIA did not lead to any significant change in PolIB expression (this work). However, PolIA mutants exhibit a

small increase in net photosynthesis, suggesting some adjustment in plants to the reduction in organelle DNA levels. Analysis of double mutants suggests that while homozygous mutants of either DNA polymerase are still viable, there is a strong pressure to maintain two functioning copies of PolIB or at the least two functioning copies of either DNA polymerase. These findings indicate that both are important for plant organelle genome replication and plant development, and suggest distinct roles for PolIA and PolIB in Arabidopsis. A better understanding of the dynamics and controls of ctDNA copy numbers are important to improve chloroplast genetic engineering to overexpress introduced genes, which is relevant to this special topic issue.

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## AUTHOR CONTRIBUTIONS

SM and BN designed the experiments and wrote the manuscript. SM performed most of the experiments, analyzed data, prepared figures, and edited the manuscript. BN obtained funding, directed the experiments, performed some of the computational gene analysis, and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00057>

**Supplementary Movie 1 | Time lapse of WT, Pol1A<sup>-/-</sup>, and Pol1B<sup>-/-</sup> plants growing side by side. In this video, plants can be seen growing at slightly different rates based on their mutation.** The plants in the first frame are 7 dpi (days post-imbibition). Black frames represent times when lights were turned off to simulate the night cycle. In total, the plants can be seen growing from 7 dpi to 32 dpi. In this video, no difference is noted between WT and Pol1A<sup>-/-</sup> plants. However, Pol1B<sup>-/-</sup> plants can be seen lagging slightly behind WT and Pol1A<sup>-/-</sup>. This delay is most pronounced when plants begin to put up shoots.

**Supplementary Table 1 | Primers used for qPCR analysis of mitochondrial and chloroplast genome copy number.**

**Supplementary Table 2 | Primers used for RT-PCR analysis of PolIA and PolIB expression.**

**Supplementary Table 3 | Primers used for zygosity testing.**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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