Advancing organelle genome transformation and editing for crop improvement

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ABSTRACT

Plant cells contain three organelles that harbor DNA: the nucleus, plastids, and mitochondria. Plastid transformation has emerged as an attractive platform for the generation of transgenic plants, also referred to as transplastomic plants. Plastid genomes have been genetically engineered to improve crop yield, nutritional quality, and resistance to abiotic and biotic stresses, as well as for recombinant protein production. Despite many promising proof-of-concept applications, transplastomic plants have not been commercialized to date. Sequence-specific nuclease technologies are widely used to precisely modify nuclear genomes, but these tools have not been applied to edit organelle genomes because the efficient homologous recombination system in plastids facilitates plastid genome editing. Unlike plastid transformation, successful genetic transformation of higher plant mitochondrial genome transformation was tested in several research group, but not successful to date. However, stepwise progress has been made in modifying mitochondrial genes and their transcripts, thus enabling the study of their functions. Here, we provide an overview of advances in organelle transformation and genome editing for crop improvement, and we discuss the bottlenecks and future development of these technologies.

Key words: organelle, transformation, genome editing, homologous recombination, crop improvement

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INTRODUCTION

The world population is estimated to exceed 9 billion in 2050, and the global demand for crops will increase by 100%–110% compared with 2005 (Tilman et al., 2011). To feed the rapidly rising population in the face of uncertain climate change and decreased arable land, food production must grow in parallel with reduced inputs. Conventional plant breeding is often considered to be a relatively labor-intensive and time-consuming process, whereas plant genetic engineering is believed to boost crop productivity (Christou, 2013). Nuclear genome transformation is now performed widely in most economically important plant species. However, it has several drawbacks, including but not restricted to unpredictable expression of the gene of interest and gene silencing due to the random location of transfer DNA integration (Meyers et al., 2010). In addition to nuclear transformation, plants also provide the opportunity to transform the small genomes of their two DNA-containing organelles, plastids (chloroplasts) and mitochondria, which are derived from cyanobacteria and α-proteobacteria, respectively (Zimorski et al., 2014; Table 1).

Plastid genomes of higher plants are conserved in size, typically 150 kb, and in coding only about 130 genes (Table 1; Daniell et al., 2016; Scharff and Bock, 2014). They consist of four parts, with a large single-copy region (LSC) and a small single-copy region (SSC) separating two inverted-repeat regions (IRs; Bock, 2015). Compared with conventional nuclear genetic engineering, the accommodation of a transgene in the plastid genome offers several highly attractive advantages. These include (i) highly precise transgene insertion by efficient homologous recombination (Figure 1A); (ii) the potential for expressing foreign proteins to extraordinarily high levels, up to 70% of the total soluble protein (TSP; Oey et al., 2009); (iii) the possibility of multigene engineering through stacking transgenes in synthetic operons in a single transformation event; (iv) the absence of epigenetic effects (e.g., gene silencing and positional effects); and (v) increased biosafety due to the exclusion of plastids from pollen transmission in most crops (Bock, 2015; Boehm and Bock, 2019). In view of these advantages, multiple economic and agronomic traits of interest have been engineered into plastids, including the production of recombinant pharmaceutical proteins (Cardi et al., 2010;
In addition to the transgenic approach, engineered sequence-specific nucleases, such as zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system, have been used to edit nuclear genomes for crop improvement (Chen et al., 2019; Manghwar et al., 2019; Veillet et al., 2020). However, there are no reports on the use of these technologies to target the plastid genome in higher plants. Despite the dearth of nuclease-mediated genome editing strategies for plastome engineering, very precise manipulations of the plastid genome, such as the introduction of point mutations at defined positions, can be achieved via homologous recombination-based plastid transformation (Bock, 2015; Figure 1B). Moreover, plastid transformation is also used extensively to analyze RNA editing (Lutz and Maliga, 2007; Ruf and Bock, 2011; Figure 1C), which creates RNA products that differ from their DNA templates.

Unlike the conserved size of plastid genomes, the size of mitochondrial genomes in land plants varies from about 208 kb to 11 Mb and is larger than that of animals (15–17 kb; Gualberto and Newton, 2017; Sloan et al., 2012). Despite the great variation in mitogenome size, the number of mitochondrial genes is relatively conserved, with about 60 known genes found in different terrestrial plant species (Unseld et al., 1997; Kubo and Newton, 2008). Most mitochondrial DNA is non-coding, and this explains the observed differences in mitogenome size (Gualberto and Newton, 2017). The similarity between plastids and mitochondria raises the possibility that the mitochondrial genome could be transformed by designing suitable constructs. However, reliable methods for the transformation of mitochondria using a biolistic device currently exist only for yeasts (Fox et al., 1988; Johnston et al., 1988) and green algae (Remacle et al., 2006; Larosa et al., 2012), and no successful transformation of mitochondria in plant systems has been reported to date.

Although the mitochondrial genomes of higher plants are not transformable, targeted modification of the mitochondrial genomes of rice and Arabidopsis has been achieved using TALENs with mitochondrial localization signals (mitoTALENs; Arimura et al., 2010; Ahmad et al., 2016).
et al., 2020; Kazama et al., 2019). Therefore, it is theoretically possible to engineer changes into any part of a plant’s genome.

As the fundamentals of plastid transformation and the toolbox available for this technique have been extensively reviewed (Verma et al., 2008; Bock, 2015; Wani et al., 2015), we highlight recent advances in crop improvement via organelle genome engineering, including organelle transformation and genome editing.

**ENGINEERING THE PLASTID GENOME TO IMPROVE AGRONOMIC TRAITS**

**Plastid genetic engineering for improved photosynthesis**

Improvements in agricultural photosynthetic efficiency and crop productivity are increasingly needed to support the food requirements of a growing global population (Bailey-Serres et al., 2019). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the central enzyme of the Calvin–Benson cycle, is responsible for the assimilation of atmospheric CO₂ and is recognized as the most abundant protein in nature (Hayer-Hartl and Hartl, 2020). Rubisco is composed of eight large catalytic subunits (rbcL) and eight small subunits (rbcS) that are encoded by the plastid genome and the nuclear genome, respectively. Given its slow catalytic rate and poor ability to discriminate between CO₂ and O₂, Rubisco has long been considered a limiting step for photosynthesis and crop productivity (Pottier et al., 2018).

Thus, Rubisco genes are among the first targets for genetic engineering (Parry et al., 2013). One approach is to relocate an rbcS gene back into its pre-endosymbiotic location within the chloroplast genome in order to assemble functional Rubisco in chloroplasts (Whitney and Andrews, 2001; Zhang et al., 2002; Dhingra et al., 2004). Most recently, Martin-Avila et al. (2020) generated a so-called tobRrD tobacco line by silencing all endogenous rbcS genes and substituting the tobacco rbcL gene with the *Rhodospirillum rubrum* rbcM gene, which encodes the L2 form of Rubisco that does not assemble with the small subunit (Martin-Avila et al., 2020). Using the tobRrD line, the authors tested the effects of different sequence features on Rubisco production from synthetic rbcL-rbcS operons transformed into the chloroplast genome to replace rbcM (Martin-Avila et al., 2020). Although the maximum Rubisco amounts obtained reached only ~50% of wild-type levels (Martin-Avila et al., 2020), this work enables the engineering of different Rubisco subunit assemblies and contributes to efforts to engineer greater Rubisco amounts.

An alternative strategy to improve plant photosynthesis is the integration of a CO₂-concentrating mechanism from cyanobacteria into transplastomic plants to maximize their carbon fixation. Introduction of a foreign Rubisco from *Synechococcus elongatus* PCC7942 into tobacco chloroplasts enabled the functional assembly of Rubisco with higher rates of CO₂ fixation efficiency but slowed growth at elevated CO₂ levels (Lin et al., 2014; Occhialini et al., 2016). Co-expression of the Rubisco assembly chaperone RbcX or CcmM35 (a β-carboxysomal protein) had
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little influence on *S. elongatus* PCC7942 Rubisco assembly in tobacco leaf chloroplasts (Occhialini et al., 2016). Similarly, co-expression of *Arabidopsis* rbcL with the cognate assembly chaperone RAF1 from the tobacco plastid genome generated a functional hybrid Rubisco and produced a two-fold increase in photosynthesis compared with plastomastic lines that expressed *Arabidopsis* rbcL alone (Whitney et al., 2015). Recently, Long et al. (2018) successfully produced a simplified carboxysome within the tobacco chloroplast by swapping the endogenous large subunit with the cyanobacterial Form-1A Rubisco large and small subunits, together with the linker CsoS2 and the shell component CsoS1A (Long et al., 2018). However, as was the case for transplastomic plants that produced cyanobacterial Rubisco alone (Lin et al., 2014), the resulting plants were capable of autotrophic growth only at high CO₂ concentrations (Long et al., 2018). Going forward, the next challenge will be to incorporate the remaining components required for a fully functional carboxysome, such as carbonic anhydrase and vertex proteins, as well as bicarbonate transporters (Hennacy and Jonikas, 2020).

Beyond carbon conservation, the introduction of an alternative pathway for utilization of the photosynthetic substrate glycolate is another effective strategy for improving the Calvin–Benson cycle. Very recently, three distinct alternative glycolate metabolic pathways were introduced into tobacco chloroplasts to reduce photorespiration (South et al., 2019). Coupling the alternative photosynthetic pathway with reduced expression of a glycolate and glyceraldehyde transporter to limit glycolate flux out of the chloroplast raised biomass productivity by >40% under field conditions (South et al., 2019). In this study, a total of 17 constructs were designed for nuclear transformation; these multienzyme pathways could potentially be introduced into the chloroplast by plastid transformation in the form of operons. Similarly, Shen et al. (2019) introduced a newly designed photorespiratory bypass, including native glycolate oxidase, oxalate oxidase, and catalase, into rice chloroplasts (Shen et al., 2019). The transgenic rice plants exhibited marked enhancement of photosynthetic efficiency, biomass yield, and nitrogen content under both greenhouse and field conditions (Shen et al., 2019). In addition, Chen and colleagues generated transgenic *Arabidopsis*, tobacco, and rice plants by expressing an *Arabidopsis psbA* gene, which encodes the D1 polypeptide of photosystem II (PSII), under the control of a heat-inducible promoter in the nuclear genome. These transgenic plants were able to maintain high photosynthetic efficiency, thus increasing crop productivity under heat-stress conditions (Chen et al., 2020). It would be interesting to investigate whether further improvement in photosynthetic efficiency could be achieved through direct overexpression of the above-mentioned genes in chloroplasts by plastid engineering (Bock, 2015).

Metabolic engineering

The plastid harbors a large number of biosynthetic pathways, such as the shikimate, *de novo* fatty acid synthesis, and methylerythritol 4-phosphate pathways, and provides various precursors for a broad range of important secondary metabolites, including tocopherols, pigments, and several phytohormones (Nielsen et al., 2016). The possibility of transgene stacking in synthetic operons is one of the greatest attractions of plastid transformation technology for the engineering of complex metabolic pathways. In general, each cistron within the polycistronic transcript should be cut into monocistronic mRNAs to facilitate efficient translation (Drechsel and Bock, 2011). Introduction of an intercistronic expression element (IEE), a short 50-bp cis-element between the *psbT* and *psbH* genes, into the spacer region between the individual cistrons greatly facilitates polycistronic cleavage into translatable monocistronic mRNAs (Zhou et al., 2007). The IEE contains a binding site for an RNA-binding protein from the pentatricopeptide repeat (PPR) family, which ensures that the different transcribed cistrons are stabilized and can be translated (Legen et al., 2019). Inclusion of the IEE in synthetic operon constructs comprising three genes for the key enzymes of vitamin E (tocochromanol) biosynthesis resulted in an increase of up to 10-fold in total tocochromanol accumulation in transplastomic tobacco and tomato plants (Lu et al., 2013).

To expand the use of IEEs, Lu et al. (2017) inserted a synthetic operon consisting of three genes that redirect lycopene into the synthesis of β-carotene and ultimately astaxanthin, a high-value ketocarotenoid. Astaxanthin content in the transplastomic tobacco plants accumulated to 1% of the plants’ dry weight. Intriguingly, grafting the transplastomic tobacco onto the non-transformable species *Nicotiana glauca* facilitated the horizontal transfer of the transgenic chloroplast genomes across the graft junction (Lu et al., 2017). This mode of transfer will help to expand the range of plant species that are not amenable to transplastomic engineering.

Transplastomic tobacco that expressed the core artemisinin acid biosynthetic pathway from two synthetic operons accumulated only low levels of the metabolite (Fuentes et al., 2016). To increase the production of artemisinic acid, a so-called COSTREL (combinatorial supertransformation of transplastomic recipient lines) approach was used to supertransform these lines. An increase in artemisinic acid content of up to 77-fold was achieved in COSTREL lines compared with recipient lines using this strategy (Fuentes et al., 2016). This study provided a proof of concept for combining plastid and nuclear transformation to optimize product yields from complex biochemical pathways in chloroplasts.

Insect resistance

The first biotechnological application of transplastomic technology for pest control was the expression of the *Bacillus thuringiensis* (Bt) *cry1A(c)* gene in the tobacco plastid. These transplastomic tobacco plants accumulated large amounts of the Bt insecticidal protein (3%–5% of TSP) and displayed high levels of resistance to herbivorous insects (McBride et al., 1995). When *cry2Aa2* was expressed as an operon together with two small open reading frames, the *Cry2Aa2* protein accumulated to up to 45% of TSP and led to the formation of crystals (De Cosa et al., 2001). However, high expression levels (10% of TSP) of *cry9Aa2* from the tobacco plastid genome caused severe growth retardation of the transplastomic plants (Chakrabarti et al., 2006), suggesting that the transgene expression level must be carefully optimized in order to provide sufficient protection without imposing a yield penalty. Apart from the model species tobacco, transplastomic soybean expressing the *cry1Ab* gene was also generated and showed strong insecticidal activity to
the velvetbean caterpillar (Dufourmantel et al., 2005). Recently, it was reported that the expression of cry1C from the poplar plastid genome caused high mortality of two lepidopteran caterpillars, Hyphantria cunea and Lymannia dispar (Wu et al., 2019). In another study, transplastomic poplar that expressed cry3Bb caused high mortality of a leaf-eating beetle, Plagiophora versicolora (Xu et al., 2020).

In addition to the expression of Bt genes in transplastomic plants, Zhang et al. (2015) recently developed the novel pest control strategy of expressing long double-stranded RNA (dsRNA) in plastids to target an essential insect gene (Zhang et al., 2015). Because plastids do not have the RNAi machinery, transplastomic potato plants expressing a 297-bp dsRNA against the β-Actin gene of the Colorado potato beetle Leptinotarsa decemlineata accumulated high levels of unprocessed dsRNA in leaves (up to 0.4% of total cellular RNA) and induced a much stronger RNAi response in the beetle compared with their nuclear-transformed counterparts (Zhang et al., 2015). More recently, He et al. (2020) revealed that the length of the dsRNA is an important parameter that affects its plastid accumulation and thereby influences the insecticidal RNAi effect (He et al., 2020). Compared with its effects on Coleoptera (e.g., L. decemlineata), the effects of plastid-mediated RNAi for the control of Lepidopteran insects were much lower, probably due to a refractory RNAi response in this insect order (Zhu and Palli, 2019). Furthermore, unlike that observed for chewing insects, the expression of dsRNAs for management of the sap-sucking insect Bemisia tabaci is less effective in transplastomic plants than in nuclear transgenic plants, largely due to the inability of B. tabaci to access plastid-expressed dsRNAs (Dong et al., 2020).

**PLASTOME EDITING FOR GENETIC ANALYSIS**

Most genome editing approaches require a targeted DNA double-strand break (DSB) at the DNA sequence that is to be edited (Chandrasegaran and Carroll, 2016). Repair of these DSBs via error-prone non-homologous end joining (NHEJ) leads to random mutations, whereas error-free homology-directed repair creates precise sequence changes when a homologous DNA substrate is provided (Chen et al., 2019). In the plastids of Arabidopsis, and probably other land plants, DSBs are predominantly repaired by microhomology-mediated end joining, although NHEJ may occur in a few cases (Kwon et al., 2010). The Chlamydomonas chloroplast can use both DSB repair and synthesis-dependent strand-annealing pathways to fix DSBs (Odom et al., 2008), Yoo et al. (2020) first applied CRISPR-Cas9-mediated genome editing to Chlamydomonas chloroplasts by introducing two plasmids: one carried both a Cas9 and a guide RNA (gRNA) expression cassette, and the other carried the donor DNA fragment for integration between the two DSB sites created by the action of CRISPR-Cas9 (Yoo et al., 2020). No insertions or deletions were detected at one of the Cas9 cleavage sites in the chloroplasts. These data suggest that the NHEJ repair system is absent in Chlamydomonas chloroplasts. Nevertheless, the possibility that the CRISPR-Cas system was not activated in Chlamydomonas chloroplasts cannot be ruled out.

Currently, CRISPR-Cas systems have not been successfully used to manipulate organelle genomes in higher plants because the double membranes of the organelles prevent the import of most nucleic acids (Gammage et al., 2018). The availability of plastid transformation technology leads us to ask whether DNA editing would occur if both Cas9 and gRNA were directly expressed from the plastid genome. In this case, a specific challenge for CRISPR-Cas9-mediated plastome editing would lie in the high degree of polyploidy of the plastid genome; for example, there are approximately 10 000 copies of plastid DNA in a single tobacco leaf cell (Shaver et al., 2006). If plastomes with small indels (insertions and/or deletions) created by CRISPR-Cas9 do not reach a stable homoplastic state, there is a risk of gene conversion between the disrupted and wild-type genomes (Khakhlova and Bock, 2006). Gene conversion can eliminate the indels so that the desired edited plastomes are ultimately not obtained despite the initial presence of the intended base changes.

Owing to the active homologous recombination system in plastids, it is relatively easy to disrupt a plastid gene by inserting or replacing with a selectable marker gene cassette (Figure 1B). More subtle changes such as point mutations and small indels can be achieved by co-transformation of the selectable marker gene and a target gene with a specific mutation (Krech et al., 2013). Over the years, the functions of most plastid genes have been characterized by targeted knockout or mutation (Scharff and Bock, 2014). The availability of alternative selectable markers and the development of marker recycling techniques enable the construction of double or triple knockouts and the introduction of mutations in multiple plastid genes, thereby facilitating the discovery of possible molecular interactions or synergistic effects in a photosynthetic complex (Fleischmann et al., 2011; Ehrnthaler et al., 2014).

**EMPLOYING RNA EDITING TECHNOLOGY IN AGRICULTURE**

RNA editing refers to a post-transcriptional process that changes bases in the transcript sequence relative to their corresponding gene. C-to-U RNA editing in the mitochondria of flowering plants was first discovered 30 years ago (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989) and was reported in chloroplasts 2 years later (Hoch et al., 1991). Typical angiosperms edit 20–60 sites in their chloroplast genomes, whereas 300–600 editing sites are seen in mitochondrial RNAs (Ichinose and Sugita, 2017). Most editing sites are located in protein-coding genes, with a few sites in non-coding regions, tRNAs, introns, or UTRs (Eder et al., 2018).

RNA editing is performed by an editosome, a multiprotein complex that contains multiple protein factors. Although a variety of editing factors have been identified as components of the plant RNA editosome, PPR proteins are its core components (Small et al., 2020; Sun et al., 2016). PPR proteins are characterized by a modular organization of a tandem array of 35-amino-acid helix–turn–helix motifs (Barkan and Small, 2014). Angiosperms contain hundreds of different PPRs that can be separated into the P and PLS subfamilies. The P-type PPRs
contain only canonical motifs composed of 35 amino acids, whereas the PLS-type PPRs also contain longer L-type (35–36 amino acids) and shorter S-type (31–32 amino acids) motif variants. Whereas P-type PPRs are associated with RNA end maturation, intron splicing, and transcript stability, proteins of the PLS subfamily function almost exclusively in C–U RNA editing in plant organelles (Barkan and Small, 2014; Small et al., 2020). The RNA editing-associated PLS-type PPR proteins feature additional C-terminal extensions: the E1 motif, the E2 motif, and the DYW domain, named for its distinctive Asp–Tyr–Trp C terminus (Cheng et al., 2016; Lurin et al., 2004; Figure 2A). The DYW domain harbors a sequence similar to a conserved cytidine deaminase motif (Salone et al., 2007) and has been proposed to catalyze C-to-U conversion. Two recent studies have shown that the expression of a single DYW-type RNA editing factor from Physcomitrium patens is sufficient to catalyze C-to-U RNA editing in Escherichia coli and in vitro (Oldenkott et al., 2019; Hayes and Santibanez, 2020).

**Figure 2.** PPR proteins involved in organelle editing and their application in plastid engineering.

(A) A model for the association of a single PLS-type PPR editing factor with its target RNA substrates in organelles. A presumptive RNA target is recognized by the PPR–RNA binding code proposed by Barkan et al. (2012) based on amino acid identities at the 6 and 1' amino acids positions of the S and P repeats (T/S + N: A; T/S + D: G; N + N: C/U; N + S: C > U; N + D: U > C). The role of the L repeat is not yet clear. The three carboxy-terminal PPRs of PLS-type PPR proteins generally differ in their amino acid conservation and are labeled P2, L2, and S2 (Cheng et al., 2016). The DYW domain with cytidine deaminase activity catalyzes the C-to-U conversion in plant organelles.

(B) Expression of a gene of interest controlled by a synthetic 5' UTR that is specifically stabilized by a designed PPR protein. The nuclear-encoded PPR protein is regulated by a tissue-specific or inducible promoter (Rojas et al., 2019; Yu et al., 2019). cTP, chloroplast transit peptide; GOI, gene of interest; L(R)B, left (right) border of transfer DNA; PPR, pentatricopeptide repeat; SMG, selectable marker gene; UTR, untranslated region.
confirming the cytidine deaminase activity of the DYW domain. PPR proteins directly bind the RNA sequence in a one-PPR-per-nucleotide manner. The combination of di-residues at the 6 and 1’ amino acid of P-type and S-type PPRs is responsible for specific RNA base recognition (Barkan et al., 2012). Their modular and predictable interactions with RNA make them amenable to custom modification and design (Yagi et al., 2014; Shen et al., 2016; Colas des Francs-Small et al., 2018).

Plastid transformation technology has facilitated the study of RNA editing, including the mapping of cis-acting elements for RNA editing-site recognition and the introduction of heterologous editing sites in order to test evolutionary conservation of trans-acting specificity factors (Ruf and Bock, 2011). In general, there is limited evolutionary conservation of plastid RNA editing sites between species. Editing sites have been frequently lost during evolution by the acquisition of C-to-U mutations, and the loss of a chloroplast editing site is usually accompanied by the loss or degeneration of its cognate PPR protein in the nucleus, probably because of the absence of selective pressure for its maintenance. Consequently, heterologous RNA editing sites are usually not recognized when introduced into species that lack them (Hayes et al., 2012; Hein et al., 2016; Figure 1C). For example, when the spinach psbF-26 editing site was introduced into the psbF gene of tobacco, which naturally lacks a corresponding editing site, tobacco plastids were incapable of editing the spinach site, resulting in strongly impaired PSII function (Bock et al., 1994). Interestingly, introduction of the Arabidopsis DYW-type PPR editing factor LPA66 was sufficient to reconstitute editing of the spinach psbF-26 site in tobacco chloroplasts and rescued the PSII-deficient phenotype caused by the loss of the editing site in tobacco plastids.
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by the unedited psbF-26 site (Loiacono et al., 2019). These results demonstrate that although additional non-PPR transacting factors also contribute to editing reactions (Bentolila et al., 2012; Hackett et al., 2017), new RNA editing sites can be established through introduction of the co-evolving editing site and its corresponding PPR protein. Moreover, new engineered editing sites in plastid genomes can be used to develop inducible expression systems for biotechnology and to design efficient on/off switches for gene expression. Recently, Rojas et al. (2019) developed a regulatory system by introducing a modified maize PPR10-encoding gene in conjunction with a cognate binding site upstream of a plastid transgene that encoded green fluorescent protein (GFP; Rojas et al., 2019). Driving the modified PPR10 expression with an ethanol-inducible promoter resulted in an approximately 20-fold induction of GFP (Rojas et al., 2019). Likewise, when the maize PPR10 variant was driven by the tuber-specific patatin promoter, the level of GFP increased by 60-fold, up to 1.3% of TSP in the amyloplasts (non-green plastids) of potato tubers (Yu et al., 2019; Figure 2B).

TOWARD MOLECULAR BREEDING THROUGH MITOCHONDRIAL GENOME MODIFICATION

Although much is known about the sequences of higher plant mitochondrial genomes, detailed and direct molecular analyses of plant mitochondrial genomes have been limited by the lack of viable mitochondrial transformation tools. However, nuclear transformation using TALEN technology has made the editing of plant mitochondrial genomes possible. Kazama et al. (2019) pioneered the use of mitoTALENs to precisely knock out cytoplasmic male sterility-associated genes in rice and rapeseed (Kazama et al., 2019; Figure 3A). Most recently, mitoTALEN technology was used to disrupt two mitochondrial genes, atp6-1 and atp6-2, in Arabidopsis (Ariruma et al., 2020). Attempts to modify the mitogenome using CRISPR-Cas systems have been hindered by the challenge of transporting the gRNA into the mitochondrial matrix (Gammage et al., 2018). Nevertheless, steps toward the development of CRISPR machinery for mitochondrial genome editing have been described in mammalian cells. The first report of mitochondrial genome manipulation using CRISPR-Cas9 in HeLa cells was controversial, as the authors showed that Cas9 could be imported into the mitochondrial matrix without a mitochondrial targeting signal and that the gRNA could be spontaneously imported into the mitochondria without any additional modification (Jo et al., 2015). A recent study showed that gRNAs with short hairpin structures can promote mitochondrial import and specific cleavage of mitochondrial DNA, albeit at low levels because of limited import into the mitochondrial matrix (Loutre et al., 2018). More recently, Hussain et al. (2020) demonstrated that gRNA could be efficiently imported to the mitochondria of mammalian cells with an additional 20-bp stem–loop element of nuclear RNase P in a polynucleotide phosphorylase-dependent manner (Wang et al., 2010). This modified single guide RNA could functionally interact with mitochondria, targeting Cas9 to mediate sequence-specific mitochondrial DNA cleavage (Hussain et al., 2020; Figure 3B). In a breakthrough, Mok et al. (2020) developed an RNA-free base editor (DdCBE) for human cells by fusing a non-toxic half of an interbacterial toxin with transcription activator-like effector array proteins and a uracil glycosylase inhibitor. DdCBE can catalyze C-G-to-T-A conversions in the human mitochondrial genome with high target specificity (Mok et al., 2020; Figure 3C). Successful application of CRISPR-based technologies and DdCBEs in mammalian cells raises the possibility of precisely manipulating mitochondrial genomes in crop plants (Figure 3).

In addition to mitochondrial DNA modification, it is also possible to block or edit targeted plant mitochondrial transcripts using designed PPR proteins. It has been demonstrated that modified RPF2 (a mitochondrial PPR protein) binds a new target located within the coding sequence of nad6 (encoding a subunit of respiratory complex I in Arabidopsis) and specifically induces the cleavage of nad6 RNA, almost completely eliminating the translation product (Colas des Francs-Small et al., 2018). This approach opens a novel avenue for reverse genetics studies of mitochondrial gene function and has potential applications in agriculture. The moss P. patens and the angiosperm Arabidopsis share the same editing site in the mitochondrial nad5 transcript, which is corrected by the species-specific editors PPR79 and CWM1, respectively. PPR79 was able to properly edit the nad5 site when expressed in the Arabidopsis cwm1 mutant (Oldenkott et al., 2020). This suggests that PPR79 may be used to introduce specific mutations in organelle genes of various evolutionarily distant plant species.

Moreover, precise C-to-U modification via DYW-type PPRs could create start codons or remove stop codons in organellar transcripts in order to regulate their translation potential. Furthermore, precise mitochondrial RNA editing might also be achieved by the introduction of a CRISPR-Cas13-mediated RNA editing system (Abudayeh et al., 2016, 2019; Zhan et al., 2019) if a reliable method of gRNA delivery to the mitochondrial matrix could be developed (Figure 3B).

CONCLUDING REMARKS AND PERSPECTIVES

The development of plastid transformation technology has paved the way to transgene expression, genome editing, and RNA editing analysis in plastids. Over the years, plastid transformation in the model systems Chlamydomonas and tobacco has become more and more routine, with a transformation efficiency approaching that of nuclear transformation. Limitations in currently available selection methods and regeneration protocols for transplastomic cells are generally considered to be the bottleneck for extending the species range of the technology (Bock, 2015). Recently, the aminoglycoside acetyltransferase (6”)-Ie/aminoglycoside phosphotransferase (2”) gene that confers resistance to tobramycin has been developed as a new selectable marker for tobacco (Tabatabaei et al., 2017). In combination with other antibiotics, this marker gene is expected to be useful for facilitating the development of monocot plastid transformation (Tabatabaei et al., 2017). Fine-tuning the expression of developmental regulator genes such as Baby boom and Wuschel2 has dramatically increased the
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nuclear transformation efficiency and enhanced the regeneration ability of some monocot crops (Lowe et al., 2016, 2018). However, Baby boom and Wusche12 may be of little use in the improvement of cereal crop plastid transformation efficiency because explants for the regeneration of cereals are non-green tissues/organs such as hypocotyls and immature embryos rather than the leaf explants used for most successful plastid transformations (Tissot-Lecuelle et al., 2014). Although plastid transformation has been achieved in tobacco suspension cell cultures (Langbecker et al., 2004) and soybean embryogenic cultures (Dufournel et al., 2004), successful transformation of plastids in non-green tissues of cereal crops remains elusive. Currently, biotic transformation is the most efficient method for introducing foreign DNA into the plastid compartment (Bock, 2015). In a remarkable recent study, carbon nanotubes were designed to selectively deliver plasmid DNA into the chloroplasts of mature non-model plants through a lipid-exchange envelope-penetration mechanism without the aid of external chemical or mechanical force (Kwak et al., 2019). Whether this nanotechnology can be applied to plastid transformation will require further verification. Collectively, the development of highly efficient selection and regeneration protocols for transformed cells and the exploration of nanotube-mediated DNA delivery will make a significant contribution to the stable plastid transformation of major crops. Because of the unique attractions of transplastomic technology, commercial products from transgenic plastids, such as pharmaceutical and industrial proteins, high-value metabolites, and stress-tolerant plants, are expected to enter the market and feed the world by 2050.

Unlike the tetrapartite genome organization of plastids (an LSC and an SSC separating two IRs) (Bock, 2015), plant mitochondrial genomes vary strikingly in size and structural organization within and among species (Gualberto and Newto, 2017; Sloan et al., 2012; Table 1). The plant mitochondrial genome is typically described as multipartite, mainly comprising a mixture of branched molecules, linear concatemers, and circularly permutable linear molecules. It can evolve rapidly in structure owing to high recombination activities in mitochondria (Gualberto and Newton, 2017; Johnston, 2019; Sloan, 2013). This extremely complex and unstable genome structure contributes to the failure of mitochondrial transformation in higher plants, and the lack of selectable markers for mitochondrial transformation adds to the challenge (Larosa and Remacle, 2013; Li et al., 2011). Plant mitochondria were shown to be sensitive to chloramphenicol, suggesting that chloramphenicol acetyltransferase may be a good candidate selectable marker for plant mitochondrial transformation (Li et al., 2011). With the extension of toolboxes for nuclear genome editing in recent years, several molecular tools have also shown great potential for application to mitochondrial DNA editing (Figure 3), thus opening a new avenue for breeding crops by modifying the mitochondrial genome, for instance by breeding male sterile rice and rapeseed (Kazama et al., 2019).

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