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Recent advances in plastid transformation

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Abstract

Plastid transformation offers a viable alternative to nuclear transformation because of its numerous advantages. It was against this backdrop that various groups of researchers have been exploiting this group of sub-cellular organelles, over the last two decades, for the genetic engineering of agronomic traits and metabolic pathways, as well as for molecular farming for the production of plant-derived high-valued biopharmaceuticals and industrial proteins. In this short review, we discuss the evolution and development of this technology with respect to the current state-of-the-art, which places it in high pedestal as a cost effective and safe production technology for high quality molecular farming products as well as a highly efficient method to create new metabolic pathways and improve the agronomic traits.

Keywords: Chloroplast transformation, molecular farming, metabolic engineering, agronomic trait engineering

Evolution and development of plastid transformation

Chloroplasts were considered as endosymbionts derived from ancestral free-living photosynthetic cyanobacteria. However, the genomes of present day land plants encode only about 5-10% of genes that are present in the cyanobacteria genome, indicating a massive transfer of genome into the nucleus. However, the proteins encoded by the migrated genes come back to plastid for their function through various mechanisms. A comparison of proteins encoded in the *Arabidopsis* genome with the proteins from the cyanobacterial genomes, suggests that approximately 4,500 of *Arabidopsis* protein-coding genes (~18% of the total) were acquired from the cyanobacterial ancestor of plastids (Martin *et al*. 2002).

The plastids in higher plants include chloroplasts (green plastids), chromoplasts (yellow or red plastids, found in some fruits and flowers), amyloplasts (containing starch) and elaioplasts (containing oil) (Gillham, 1994). Plastids of higher plants are semi-autonomous organelles with a small circular double stranded DNA with a high copy numbers and have their own transcription-translation machinery. Chloroplast gene expression is regulated at both transcription and translation level and requires a coordinate expression with the nuclear genome. The plastid genome is transcribed by two different RNA polymerases: one encoded by the nucleus (NEP) and the other plastid encoded (PEP). While some plastid genes are transcribed by both NEP and PEP, some are transcribed only by the PEP. While PEP is a prokaryotic-type enzyme, NEP is a T7 RNA polymerase-like enzyme (Hess & Börner, 1999). There are several sigma factors encoded by the nuclear genome, which are part of the PEP transcription system. The translational apparatus of chloroplasts is similar to that of bacteria, but has adopted to translate mRNAs in the organelle within a eukaryotic cell (Marín-Navarro *et al.,* 2007).

The plastid transformation (PT) technology was birthed as a result of the quest for low cost, safer and more flexible scale-up expression system than the established systems that use bacterial, fungal and animal cells as production platforms for recombinant proteins, especially pharmaceutical proteins. The first stable chloroplast transformation was reported in *Chlamydomonas* using high velocity microprojectiles by biolistic delivery of naked DNA that integrated into the genome through homologous targeting (Boynton *et al*., 1988). Although transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors was reported by Daneill *et al*. (1990), the first stable plastid transformation in higher land plant was achieved in tobacco by Pal Maliga's group, using same biolistic delivery system (Svab *et al*., 1990). They used transformation vectors that contain a mutated plastid 16S rRNA (*rrn16*) gene that confer spectinomycin and streptomycin resistance. However, a year later, a more efficient selectable marker gene, *aadA*, encoding aminoglycoside 3'-adenylyltransferase, which inactivate spectinomycin and streptomycin, was discovered (Goldschmidt-Clermont, 1991). This PT marker has since been prominently used for selection of cells containing transformed plastid genomes (ptDNA), even though, an alternative marker, *aph*A-6, which confers kanamycin resistance, was later found to be of comparable efficiency with *aad*A (Hunag *et al*., 2002). Stable plastid transformation in tobacco by an alternate transformation protocol based on polyethylene glycol (PEG) treatment of leaf protoplasts in the presence of plasmid DNA was also reported (Golds *et al*., 1993). Since the first high-level foreign protein expression of 2.5% of the total soluble protein was achieved with the bacterial β-glucuronidase

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(GUS), (Staub & Maliga 1993), there have been significant advances in the development of plastid expression technology, which have largely demonstrated that plastid could indeed serve as a safe platform for the large-scale production of recombinant proteins. Similarly, the first proof of agronomic trait engineering via PT, which was demonstrated by McBride *et al*. (1995), actually served as the impetus for the advances made in this area of plant biotechnology to date. Additionally, it was around this time that the foundation for the recent advances in the elimination of selectable markers conferring antibiotic resistance, and which are generally of bacterial origin, and as such constitute biosafety concerns, was laid (Carrer & Maliga, 1995; Fischer *et al*., 1996). Moreover, the advances made to date with respect to increasing the spectrum of higher plants that are amenable to this technology was premised on the possibility of stably transforming *Arabidopsis* chloroplasts in 1998 (Sikdar *et al*., 1998), a feat that doused the notion that the technology may be limited to the Solanaceous species only. The advances in the abovementioned areas of the PT technology, which have now placed it in high pedestal as a cheap and safe production technology for high quality molecular farming products, and also as a highly efficient method for agronomic traits- and metabolic engineering, are thus the focus of this review paper.

Table 1: Advantages of PT over nuclear transformation.

Plastid transformation	Nuclear transformation
Plastid genome is highly polyploid leading to high accumulation of protein	Nuclear genome is not highly polyploidy hence low level of protein expression
Plastids possess prokaryotic gene expression system, which facilitate the expression of several genes simultaneously from single operons	The nucleus does not possess prokaryotic gene expression system, hence cannot express several genes simultaneously
Facilitates the expression of multi-subunit complex proteins from polycistronic mRNAs, under a single promoter	The expression of multi-subunit complex proteins from polycistronic mRNAs is not practicable, hence several promoters are needed to drive the expressions of the individual genes encoding the respective subunits
Polycistronic multigene expression enables enhanced sequential metabolic reactions in a single transformation procedure	Multiple transformation procedure will be required to achieve multigene expression
The use of a single operon to express several genes removes the burden of using several selection markers	Several selection markers will be used to independently select for integration events of these individual genes
The plastid genome is versatile in codon usage for recombinant protein production	Widespread codon usage bias exist, hence the codon optimization is common, in order to optimize translation efficiency
Provides substantial degree of natural biocontainment of transgene flow by out-crossing, as plastids are inherited through maternal tissues in most species	There is always the risk of out-crossing through pollination
No positional effects and epigenetic interference because integration is guarded into the functional region of the genome through homologous targeting.	There are positional effects and epigenetic interference because integration is random
Absence of transgene instability and gene silencing	Presence of transgene instability and gene silencing
Eliminates the need for transit peptide for the transgene	transit peptide is needed for genes destined for the plastid genomes

Information from Staub et al.(2000), Maliga (2004), Daniell et al. (2005), Bock (2007), Moeller and Wang (2008), Hasunuma et al. (2008), Cardi et al., 2010, Meyers et al. (2010, Obembe et al. (2010).

Rationale for the PT technology

The diversely numerous advantages of PT over its nuclear counterpart are the incentive for its adoption as the preferred stable transformation strategy. These advantages are presented in Table 1. Thus, PT has an unparalleled leverage as a tool for production of valuable compounds through metabolic engineering and molecular farming as well for achieving enhanced efficiency in agronomic trait engineering.

Particle bombardment-mediated transformation

The gene transfer technique that is generally being used for plastid transformation is the particle bombardment. This can be performed on a wide variety of cell and tissue explants of most species (including nowadays cereal plants), because it is neither species- nor genotype-dependent, hence it has no biological constraints or host limitation. Besides, this method of transformation can facilitate co-transformation of a cell with two of more transgenes simultaneously (for comprehensive expert review, see Altpeter *et al*., 2005). The DNA transfer by particle bombardment makes use of physical processes to achieve the transformation of crop plants. The particle bombardment-mediated transformation is performed with a device known as the PDS-1000/He. The device uses high-pressure helium-aided acceleration of gold or tungsten microparticles coated with DNA. The built-up helium pressure is released using a rupture disc, which produces a shockwave that travels to the second disc (macrocarrier) on which the DNA-coated are spotted. The macrocarrier is then propelled into a stopping screen, which retain the macrocarrier but ensures the continuous travelling of the

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microprojectiles, which finally hit their target tissues held in a vacuum, facilitating the delivery of the naked DNA into the plant cells and ultimately into the nuclear-, plastid- or mitochondrial genome. Particle bombardment is the most efficient way to achieve plastid transformation in plants and is the only method used to achieve mitochondrial transformation (Maliga & Small, 2007)

Advances in molecular farming and in the engineering of agronomic traits and metabolic pathways

The application of PT has made tremendous advances in the field of molecular farming for the production of varied high-valued biopharmaceuticals including monoclonal antibody (Daniell *et al*., 2004), γ- and and alpha-interferon (Leelavathi & Reddy, 2003; Arlen *et al*., 2007), human alpha 1-antitrypsin (Nadai *et al*., 2008), insulin-like growth factor (Daniell *et al*., 2009), and antimicrobial peptide (Oey *et al*., 2009). Also this platform has been explored for high-level production of a host of vaccine antigens including bacterial antigens such as tetanus and Cholera toxin B (Tregoning *et al*., 2003; Davoodi-Semiromi *et al*., 2009) and bio-defense vaccine antigens, anthrax and plaque (Koya *et al*., 2005; Arlen *et al*., 2008). Viral antigens including hepatitis C, swine fever virus and human papillomavirus (L1) have also been expressed in the chloroplasts (Birch-Machin *et al*., 2004; Fernandez-SanMillan *et al*. 2008; Shao *et al*. 2008). Other antigens successfully expressed in the chloroplast include the protozoan antigens such as the surface antigen of *Entamoeba histolytica* and malaria vaccine candidates (Chebolu & Daniell, 2007; Davoodi-Semiromi *et al*., 2009), and the autoantigens for diabetes-type 1, the cholera toxin B-proinsulin fusion protein (CTB-pins) and human glutamic acid decarboxylase (hGAD65) (Ruhlman *et al*., 2007; Wang, X. *et al*., 2008). Other valuable chloroplast-derived products include elastin-derived polymer and monellin (Guda *et al*., 2000; Roh *et al*., 2006).

With respect to metabolic engineering, the PT platform has made remarkable advances in increasing the yield of useful compounds such as p-hydroxybenzoic acid (25% dry weight) (which is in hot demand as a major monomer in liquid crystal polymers) (Viitanen et al., 2004) and astaxanthin, which is a highly valued red pigment that is being used as feed supplement in poultry farming and aquaculture (Hasunuma *et al*., 2008). Additionally, the technology has been used to achieve overproduction of tryptophan, a very important amino acid (Zhang *et al*., 2001; Tsai *et al*., 2005) and a polyester, polyhydroxybutyrate (Lössl *et al*., 2003).

Moreover, several transgenes have conferred useful agronomic traits to the plants including drought resistance (Kumar *et al*., 2004), bacterial and fungal pathogen resistance (DeGray *et al*., 2001), herbicide resistance (Ye *et al*., 2001), and insect resistance (De Cosa *et al*., 2001; Dufourmantel *et al*., 2005). Additionally, the TP platform has recently found relevance in bioremediation (Hussein *et al*., 2007).

Advances in strategies for enhancing protein expression

The strategies for enhancing protein expression in the chloroplast have been based on ensuring protein stability rather than transcript abundance due to the lack of correlation between increased transcript level and translation efficiency (Verma & Daniell, 2007). For example a chaperon for the CRY protein was used to fold an insecticidal protein, Cry2Aa2, into cuboidal crystals, which protected the foreign protein from degradation from chloroplast proteases, thereby leading to remarkable increase in protein accumulation (De Cosa *et al*., 2001). Similarly, a striking 500-fold increase in expression of the human serum albumin was achieved when regulated in the light for protection inside inclusion bodies (Fernandez-San Millan *et al*., 2003). Also, some proteins such as insulin are unstable in the chloroplast; as such can only gain stability when expressed as fusion proteins. For example the fusion of insulin with Cholera toxin B-subunit led to high expression of up to 16% of the total soluble protein (Ruhlman *et al*., 2007). Additionally, codon optimization has also been recently found to enhance protein expression, to a certain extent, in the chloroplasts (Zhou *et al*., 2008; Oey *et al*., 2009).

Besides, Oey *et al*. (2009) were able to accumulate a phage-derived bactericidal protein to an unprecedented level of over 70% of the plant's total soluble protein (the highest foreign protein expression level obtained in plant so far). They exploited the evolutionary resistance of phage lysins against prokaryotic proteases for achieving a very high stability of the protein inside the chloroplasts, which retain prokaryotic gene-expression machinery. This unrivalled foreign protein expression further validates the PT technology as an expression platform for cost-effective large-scale production of proteinaceous pharmaceuticals. It is also worthy of note that the protein antibiotic killed the pathogenic group A streptococci without any purification, an advantage that is especially relevant when the protein antibiotic is to be produced for topical application (Oey *et al*., 2009).

Advances in marker excision strategies

The main reason for the clamor for the removal of the maker genes from transgenic plants is because of the antibiotic resistance they confer, which poses appreciable biosafety concerns. Besides, their presence will no longer be necessary to maintain the transplastomic state when plants with a uniformly transformed ptDNA population have been obtained, as such their removal will spare the transgenic plant of the unnecessary metabolic burden imposed by high-level expression of the marker gene (Lutz & Maliga, 2007). Four strategies being used for plastid marker gene excision include homology-based excision through directly repeated sequences, excision by phage site-specific recombination system, transient cointegration of the marker gene and the cotransformation-segregation approach.

The homology-based excision through directly repeated sequences relies on loop-out elimination of

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sequence between two directly oriented repeats. There have been a lot of advances since the first demonstrations of this approach in tobacco and *Chlymadomonas* in the mid 90s (Carrer & Maliga, 1995; Fischer *et al*., 1996). These include the interruption of an herbicide resistance gene with excisable selectable marker, such that after marker elimination, the integrity of the herbicide resistance gene can be restored and as such can be used for identifying the marker-free clones (Dufourmantel *et al*., 2007). The second advance is the visual identification of the marker-free plants as a result of a loss of function of a co-excised plastid pigmentation gene, *rbcl* (Kode *et al*., 2006).

However, a second more efficient and widely adopted strategy for marker gene excision is the use of the nuclear-encoded, plastid targeted phage site-specific recombinases. This involves the construction of transplastomic plants, which carry a marker gene flanked by two directly oriented recombinase target sites. The marker gene is then removed by the introduction of a gene encoding a plastid-targeted recombinase in the plant nucleus, which eventually enters all plastids and simultaneously excises the marker genes flanked by directly oriented target sites (Lutz & Maliga, 2007). Two of such recombinases, the Cre recombinase, derives from the P1 bacteriophage, and which excises target sequences flanked by directly oriented 34 bp loxP sites (Corneille *et al*., 2001; Kuroda & Maliga, 2003, Tungsuchat *et al*., 2006; Sinagawa-García *et al*., 2009; Zhou *et al*., 2008) and the ΦC31 phage integrase, Int (Lutz *et al*., 2004; Kittiwongwattana *et al*., 2007) have been used successfully to achieve marker-free transplastomic plants.

The transient co-integration approach is more effort-demanding and consequently enjoys less adoption. This is because the removal of the selectable marker gene involves the generation of a loss of function mutant lines generated by knocking down the photosynthetic gene *rpo*A or *pet*A and a subsequent gene complementation for the restoration of the lost green pigmentation capability (Klaus *et al*., 2003; 2004).

The co-transformation-segregation approach relies on co-transformation and segregation of the ptDNA that has been transformed independently with two plasmids, a marker gene and an herbicide resistance gene. The transplastomic clones are identified by selection for spectinomycin resistance, with the resultant heteroplastomic cell carrying the wild-type ptDNA, the ptDNA with marker gene integration, the ptDNA with herbicide gene integration and the ptDNA with both vectors. Subsequent replication and segregation of the ptDNA on non-selective medium eventually yields chloroplasts with homoplastomic ptDNA for both vectors as well as for the single individual vectors. As high as 20% of the desired herbicide-resistant plants, which lack the antibiotic resistance marker was obtained for tobacco (Ye *et al*., 2003).

A recent and promising alternative approach that has been developed for tobacco plastid transformation involves the use of a selection system that is based on the feedback-insensitive anthranilate synathase (AS) α-subunit of tobacco (ASA2) as a new selective marker and the indole analogue 4-methylindole (4MI) or the tryptophan analogue 7-methyl-DL-tryptophan (7MT) as the selection agents (Barone *et al*., 2009).

Advances in widening the range of crop species

Even though tobacco proffers almost infinite advantages as an ideal crop for various applications of the PT, however, the presence of nicotine and other alkaloids has been the single major disadvantage, especially for the production of biopharmaceuticals, the recent use of low-nicotine varieties like LAMD (Arlen et al., 2007) not withstanding (Verma & Daniell, 2007). The need to broaden the crop spectrum for PT is particularly imperative for oral delivery systems. It is therefore exciting to note that the range of the PT-amenable crops has been broadened since the first stable plastid transformation was achieved in a non-tobacco species in 1998 (Sikdar *et al*., 1998). As such, stable PT has been successfully achieved in crops species such as oilseed rape (Hou *et al*., 2003), petunia (Zubko *et al*., 2004) and poplar (Okumura *et al*., 2006), following the same protocols developed for tobacco, with minor modifications though. It is even more excitingly noteworthy that chloroplast transformation has been possible in leafy edible crops like lettuce and cabbage (Kanamoto *et al*., 2006; Liu *et al*., 2007), and other edible crops like potato (Nguyen *et al*., 2005), tomato (Ruf *et al*., 2001), and carrot (Kumar *et al*., 2004a), which offer ideal systems for the oral delivery platform. The range of PT-amenable crops has even been widen to include also the economically important crops such as cotton (Kumar *et al*., 2004b), rice (Lee *et al*., 2006) and soybean (Dufourmantel *et al*., 2004). Not only is the PT technology available for these variety of crop species but also that it employs different plant regeneration approaches including somatic embryogenesis and organogenesis from leaf and protoplast cultures, which makes it all the more promising to still increase the crop species base for the technology.

Future prospects

So far plastid transformation has been accomplished in relatively few species. There are several factors that have limited the expansion of plastid transformation technology to agronomically more important crop species such as cereal crops. A major factor is the lack of good selection marker that can be used in rice, wheat, maize etc. Also lack of reproducible tissue culture procedures that are compatible to plastid transformation is another serious limiting factor. Although some partial success was achieved in rice, generating a homoplastomic lines remained a distant dream. Moreover, it is not possible to generate homoplastomic plants via subsequent rounds of regeneration using leaves as explants. Based on the

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knowledge available, it is possible in the near future to apply plastid transformation to improve the agronomic traits in important crop species. Nevertheless, the available plastid transformation and expression technology in several dicots can be readily exploited for the large scale production of high value products required in bulk quantities useful in human health and technical industry.

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