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BIOTECHNOLOGY

Plastid Genome Engineering and its Potential Applications: A Review

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ABSTRACT

Plastid genome engineering is a credible tool for the basic biotechnological research and various innovative techniques have led to the better understanding of the complex processes involved in the plastid transformation. Plastids in higher plants are the major biosynthetic centers for photosynthesis which is the main source of energy requirement. Plastids have their own genome i.e. plastome which is maternally inherited in most angiospermic plant species. Although production of transgenic plants has traditionally been through expression of transgene in the nucleus, but plastid transformation is considered more attractive and efficient target for genetic engineering due to several advantages over nuclear transformation including high level of foreign protein, eliminating the risk of cross pollination with weeds, absence of silencing mechanism and ability to engineer multiple genes rather than a single gene. The potential utility of plastid genome engineering has been explored in development of crops with various agronomic traits, development of vaccine, biopharmaceuticals, therapeutic proteins, biomaterials and industrial enzymes, which will definitely prove beneficial in near future. Plastid transformation is still to be fully utilized for product commercialization, because of the problems associated with protein purification and expression level control. This review article highlights the various possibilities and potential applications of plastid genome engineering for generation of marker free transplastomic plants, improvement in agronomic traits and role of plastids in the production of cost effective biopharmaceuticals and biomaterials.

Highlights

- Plastid genome engineering is the potential emerging biotechnological tool for generation of marker free transgenic plants, agronomic traits improvement and providing affordable biopharmaceuticals and biomaterials.

Keywords: Plastid transformation, genetic engineering, transgene, transplastomic plants.

The plant cell's genetic information is localized in the nucleus along with DNA in chloroplast and mitochondria for DNA replication, transcription and protein synthesis. Plastids are the major biosynthetic centers for photosynthesis in plant cells and eukaryotic algae, which is the primary source of food production (Wang *et al.* 2009). The plastids genome called as plastome is a circular double-stranded DNA molecule of size 120 to 160 kb, present in 1,000-10,000 copies per cell in different plant species and contains 100-120 highly conserved unique genes which are maternally inherited in most angiosperm plant species. The expression of transgenes in the nucleus led to the production of transgenic plants for basic and applied purposes worldwide, but the possibility that transgenes may escape via pollen, contaminating non-transformed plants has given scope for a new field of genetic engineering i.e. plastid transformation (Ruf *et al.* 2007; Daniell 2007). These plants with transformed plastid genomes are termed as transplastomic (Maliga 1993). Plastid transformation is a tissue culture dependent

process which involves integration of transgene that encodes a selectable marker by two homologous recombination events, followed by exposure of plastids to the selective agent and finally elimination of untransformed plastid genome copies in the tissue culture medium containing antibiotics (Bock 2001). The challenge of plastome engineering is to uniformly alter all the genome copies, as genetically stable plants are obtained only if all the genome copies are identical.

Although, plastid transformation was reported in cultured tobacco cells by Daniell *et al.* in 1990 but it was transient foreign gene expression, the first stable plastid transformation in tobacco was reported by Svab *et al.* in the same year. Till today, plastid transformation has been reported in many other higher plants including edible crops for different traits by different researchers Worldwide. Plastid transformation is routinely done in tobacco and the efficiency of transformation is much lower in other plants than in tobacco (Maliga 2004). The possibilities and obstacles to extend this technology to higher crops which regenerate through somatic embryogenesis has been discussed by many authors (Daniell *et al.* 2002; Lee *et al.* 2006; Clarke and Daniell 2011). The chloroplast genome of closely related plant species was not found conserved. Due to the lack of conservation of intergenic spacer regions of the chloroplast and the species specificity of this regulatory sequences have put forward the process of development of highly efficient species specific transformation vectors for integration and expression of transgenes in chloroplast (Daniell *et al*. 2016). First commercial development of an oral drug produced in commercial lettuce cultivar using species specific chloroplast transformation vector was published by Su *et al*. in 2015.

This may open up new era in plastid genome engineering to introduce and express novel genes in the engineered plants for oral delivery of pharmaceuticals and vaccines, which will reduce expensive purification, cold storage, transportation and short shelf life of current protein drugs. In this review, we will discuss advances made so far for generation of marker free transplastomic plants, which is the need of hour for public acceptability of the genetically modified crops, transplastomic plants for expression of agronomically important traits and role of plastids in the production of cost effective biopharmaceuticals and biomaterials in plants.

Development of marker free transplastomic plants

The marker genes are required for the selection of transplastomic plants. After selection of transplastomic plants, the marker genes are eliminated for the biosafety concern to release antibiotic resistant gene in the field crops and the high level expression of marker gene will increase metabolic burden on the plant (Lutz and Maliga 2007). The marker free plants can be obtained by direct repeats or Cre-lox recombination approaches. In the Cre-loxP site specific recombination, marker gene (flanked by two directly oriented lox sites, 34 bp) and gene of interest are introduced into the plastid genome without Cre activity. When marker elimination is required, a gene encoding nuclear plastid targeting Cre activity is introduced into the nucleus and subsequent import in plastids excises sequences between two lox sites (Corneille *et al.* 2001). Another site specific recombinase (phiC31 phage integrase) have been used for the excision of *aadA* marker gene, flanked by directly oriented non identical phage *attP* (215 bp) and bacterial *attB* (54 bp) attachment sites. The marker gene thus removed after nuclear transformation of transplastomic plants with integrase gene encoding a plastid targeted integrase enzyme (Kittiwongwattana *et al.* 2007).

Both the systems (Cre-lox and Int-att) are equally efficient for obtaining marker free plants, but Int-att appears to be better choice as plastid DNA contains pseudo lox sites recognized by Cre (Corneille *et al.* 2003; Lutz *et al.* 2004; Kittiwongwattana *et al.* 2007). Alternatively, the removal of marker gene via directly repeated sequences (Iamtham and Day 2000), transient co-integrative (Klaus *et al*. 2003; 2004) and cotransformation-segregation (Kindle *et al.* 1991; Ye *et al.* 2003) approaches may be used to obtain marker free plants, but due to some limitations, these approaches are not commonly used to obtain marker free plants. Recently, removal of *aadA* marker gene was achieved by using mycobacteriophage Bxb1 recombinase and *attP*/*attBII* recognition sites (Shao *et al.* 2014). Several antibiotic-free selectable markers such as D-amino acid oxidase (Gisby *et al.* 2012), isopentenyl transferase (IPT) (Dunne *et al.* 2014) and anthranilate synthase α-subunit (ASA2)

(Baronne *et al.* 2009) have also been developed for selection of transplastomic plants in recent years.

Engineering of plastid genome for agronomic traits

The engineering of plastid genome for agronomic traits is important to feed worldwide increasing population (Clarke and Daniell 2011). Hence several agronomic traits for crop improvement, including herbicide resistance, insect resistance, draught tolerance, salt, water and temperature tolerance have already been engineered via plastid transformation (Verma and Daniell 2007; Repkova 2010). The major advances have already been made by expressing heterologous *cry* genes for delta-endotoxin from *Bacillus thuriengiensis* via engineering chloroplast genome. Plastid expression of Bt gene in important major crops has not yet reached commercial development, as market is saturated with Bt crops that avoid the use of expensive chemical pesticides (Jin and Daniell 2015). Different *cry* genes have been expressed in different crops against a range of pests by plastid transformation (Kota *et al.* 1999; De Cosa *et al.* 2001; Gatehouse 2008; Chakrabarti *et al.* 2006; Liu *et al.* 2008; Kim *et al.* 2009; Dufouramantel *et al.* 2005).

The authors suggested that targeting of *cry* genes to chloroplast confers a high level plant resistance to different insects, thus providing an efficient strategy for crop insect management. The RNA interference (RNAi) concept was also used for engineering chloroplast genome for insect resistance (Jin *et al.* 2015; Zhang *et al.* 2015). The study conducted by Jin *et al*. (2015) used lepidoptern chitin synthase (Chi), cytochrome P450 monooxigenase and V-ATPase as RNAi targets, which are essential proteins required for insect survival. The transcripts level of targeted genes were reduced to almost undetectable levels in the insect midgut, which resulted in significant reduction in net weight of larvae and population rate. In another study, Zhang *et al*. (2015) introduced dsRNA via chloroplast genome to target insect β-*actin* gene to provide resistance against Colorado potato beetle. The expression of dsRNAs via chloroplast genome explore the possibility of use of RNAi approaches to confer desired agronomic traits or to downregulate dysfunctional genes following oral delivery of dsRNA bio-encapsulated within the plant cell (Jin and Daniell 2015).

Plastid transformation has also been used for the development of plant varieties which are resistant to bacterial and fungal diseases. Disease resistant tobacco was developed by expressing MSI-99, an antimicrobial peptide which conferred resistance to fungal pathogen *Colletotrichum destructive* in tobacco (DeGray *et al.* 2001). Transplastomic plants inhibited the growth of pregerminated spores of *Aspergillus flavus*, *Fusarium moniliforme* and *Verticillium dahlia* and *Pseudomonas syringae* pv *tabaci* bacteria by more than 95% compared with non-transformed control plant, which suggested that MSI-99 expressed in tobacco chloroplasts can provide significant protection from both bacterial and fungal pathogens. The research conducted by Wang *et al.* (2015) showed that MSI-99 expressed in tobacco chloroplast is capable of providing protection against rice blast, one of the most dangerous fungal rice disease.

The possibility of plastid genome engineering for weed control has been explored in several studies. Plastid expression of *bar* gene which encode herbicide inactivating phosphinothricin acetyltransferase (PAT) enzyme led to high level enzyme accumulation and conferred field tolerance to glufosinate (Daniell *et al.* 1998; Lutz *et al.* 2001). Plastid expression of bacterial 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme in transgenic chloroplast of tobacco and soybean resulted in strong herbicide tolerance (Dufouramantel *et al.* 2007). In another study, plastid expression of a variant form of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene conferred higher resistance to the broad-spectrum herbicide, glyphosate (Roudsari *et al.* 2009).

Every plant is exposed to various biotic and abiotic stress factors such as drought, salinity and freezing which affect plant's growth and ultimately crop production. Plastid genetic engineering has successfully been used for the development of abiotic stress tolerance in plants. Trehalose, an osmoprotectant accumulated under stress conditions can play a significant role in protecting plant cells against damage caused by these stresses. The expression of trehalose phosphate synthase 1 (*TPS1*) gene in chloroplast has no phenotypic variation (Lee *et al.* 2003) as compared to nuclear transgenic plants. The study conducted by Kumar *et al.* (2004) clearly showed that transgenic carrot plants expressing *badh* (betaine aldehyde dehydrogenase) gene accumulated glycine betaine which showed

tolerance to high conc. of NaCl up to 400 mmol/l, the highest level of salt tolerance reported among genetically engineered crops. A gene for choline monooxygenase (*BvCMO*) from beet (*Beta vulgaris*) was expressed via plastid genetic engineering in tobacco (Zhang *et al.* 2008). Transplastomic plants accumulated glycine betaine, an osmoprotectant in leaves, roots and seeds and showed tolerance to toxic level of choline and salt/drought stress when compared to wild type plants. Khan *et al*. (2015) also highlighted that expression of *ArDH* gene in tobacco chloroplast increases tolerance to high conc. of NaCl up to 350 mM due to expressed *ArDH* gene encoding enzyme arabitol dehyrogenase, which is responsible for reduction of D-ribulose to D-arabitol.

The chloroplast targeted *codA* gene from *Arthrobacter globiformis* for transgenic rice has been developed for water stress tolerance which showed higher photosystemII activity and better physiological performance under water stress conditions (Kathuria *et al*. 2009). Temperature stress resistance, an important agronomic trait can be successfully achieved by expressing *E. coli panD* gene which catalyses the decarboxylation of L-aspartate to generate β-alanine and CO_2. Transplastomic plants expressing *panD* was able to endure high temperature stress than that of wild type plants (Fouad and Altpeter 2009; Wani *et al.* 2015). Further, Chen *et al*. (2014) showed that by using protease inhibitors and chitinase in transgenic tobacco confer resistance against insects, pathogens and abiotic stress.

Engineering of plastid genome for production of biopharmaceuticals

One of the most fascinating applications of plastid genetic engineering is being used for the production of biopharmaceuticals. Plant cell expressing therapeutic proteins can be lyophilized and stored indefinitely at room temperature without losing their efficacy (Kwon *et al.* 2013). So, the use of high level expression of particular protein in edible leaves permits oral delivery and hence reduces production cost by eliminating the purification step. Plastid transformation has made enormous advances in the field of molecular farming for the production of high end biopharmaceuticals. More than 40 biopharmaceuticals and vaccine antigens have been expressed in the chloroplast genome by different researchers (Jin and Daniell 2015).

The first therapeutic protein, human somatotropin (hST) was expressed in a soluble, biologically active and disulfide bonded form (Staub *et al.* 2000), since then many researchers have expressed both bacterial and viral vaccine antigen genes in plastid genome. The vaccines developed have only been experimented on mice and developing effective vaccines for human use is still in the progress (Daniell *et al.* 2009). Most therapeutic proteins were expressed in tobacco chloroplast for initial evaluation and for the oral delivery of drugs, its usefulness was limited due to high alkaloid content. After extensive optimization of plastid transformation protocols in lettuce, therapeutic proteins were subsequently expressed in lettuce (Ruhlman *et al.* 2010) which is the only reproducible transplastomic system for oral delivery of biopharmaceuticals and vaccines. Some algae have also been explored for production of vaccine antigens. Dauvillee *et al*. (2010) successfully utilized *Chlamydomonas reinhardtii* to accumulate Apical Membrane Antigen-I (AMA-I) and Meroziote Surface Protein-I (MSP-I) for vaccine against malaria.

Engineering of plastid genome for production of biomaterials

Plastid transformation has been used for the production of many industrially valuable biomaterials such as enzymes, amino acids and polyester. The plastid transformation has been used to produce p-hydroxybenzoic acid (pHBA), which is major monomer in liquid crystal polymers. The transplastomic tobacco plants expressing *ubiC* gene produced highest level of pHBA polymer in normal healthy plant (Vitanen *et al*. 2004). The chloroplast produced enzymes offer several advantages over traditionally produced enzymes including significantly reduced cost, improved stability and no need for enzyme purification. Genes for thermostable xylanase enzyme used in pulp and paper industry were successfully expressed in tobacco chloroplast (Leelavathi *et al.* 2003; Kim *et al*. 2011). Themostable cell wall degrading enzyme Cel9A from *Thermobifida fusca* have been expressed in tobacco chloroplast genome with expression level as high as 40% TSP (Peterson and Bock 2011). Agarwal *et al*. (2011) expressed β-mannanase from

Trichoderma reesei in tobacco chloroplast. Chloroplast produced enzyme showed wider pH optima and thermostability than *E. coli* produced enzyme. Plastid transformation have also been used for the production of amino acid, tryptophan (Tsai *et al.* 2005) and polyester, polyhydroxybutyrate (Lossl *et al.* 2003).

CONCLUSION

The chloroplast genome has become innovative target for plant genetic engineering due to several advantages over nuclear transformation**.** Although more than 100 transgenes have been stably integrated and expressed in tobacco chloroplast genome till date, however, extension of technology to other crop plants is limited by several factors including non-availability of chloroplast genome sequences and optimization of plastid transformation protocols in different crop species. Plastid transformation is routinely carried out in tobacco, while efficiency of transformation is low in other crop plants. Plastid transformation have been used for engineering of several important agronomic traits such as insect resistance, herbicide resistance, draught, salt, water and temperature tolerance in an eco-friendly manner which will definitely enhance crop productivity. Generation of marker free transplastomic plants, which is the need of hour for public acceptability of the genetically modified crops will facilitate public acceptance in near future. The plastid transformation is being used for the production of valuable vaccines antigens and therapeutic proteins.

This technology has not resulted in product commercialization, because of problem associated with protein purification and in nascent stage as experimented only in animal model. Most of the therapeutic proteins and vaccine antigens are produced in tobacco plastid genome which cannot be used for oral administration because of its toxic alkaloid contents. At present lettuce is the only reproducible system used for production of therapeutic proteins. Therefore, it is necessary to use those plant species that can be used as a system for oral delivery of biopharmaceuticals and vaccines. Further studies with edible crops will be needed in near future for successful implementation of plastid genetic engineering for oral administration of drugs.

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