Review

PLASTID GENOME ENGINEERING: A NEW ROAD TO DEVELOP ENVIRONMENT FRIENDLY TRANSGENIC PLANTS

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Abstract: The advancement in our understanding to engineer plant genome to tailor multigenic traits makes it necessary to express multiple genes, in parallel with the development of a better understanding of factors that control the concerted expression of such transgenes. Multiple gene expression in the same recipient cell/plant favors the engineering of organelle genome of the plants. After nucleus, plastids provide the targets to carry out routine transformation. The ability to engineer plastid genome in higher plants is extremely attractive and important to the development of transgenic traits that may be difficult or impossible to achieve by nuclear transformation. Moreover, plastome engineering is advantageous, since proteins in chloroplasts may accumulate to high levels, multiple genes may be expressed as polycistronic units, and lack of pollen transmission in most cultivated crops results in natural gene containment offering the development of bio-safe transgenic plants -- a major concern of many agencies today. Although limitations are always there, engineering the plastome will remain an excellent choice to develop environment friendly transgenic plants that may offer a site to overexpress products of pharmaceutical interest; highly pure, safe and cheap source to reach the consumers.

Keywords: Proplastid, plastome, *ycf*, RNA polymerase, NEP, PEP, chimeric tissue, phenotypic masking, transplastomic, heteroplastomic, homoplastomic

Introduction

Progress in plant genetic engineering has been spectacular since the recovery of the first transgenic plant in 1980s, and has now been applied to a variety of species, resulting in the generation of numerous transgenic plants. A number of techniques, like electroporation, PEG-treatments of protoplasts, *Agrobacterium* and microprojectile bombardment have been used to deliver foreign DNA into plants. Genetically modified crops have provoked some environmental concerns. One common concern is the possibility of gene escape through pollens or seed dispersal from crop plants engineered for herbicide resistance to contaminate their weedy relatives. Another concern is the toxicity of the transgenic pollens to non-target insects such as the monarch butterflies. Still another concern is sub-level expression of transgenes like *Bt*, resulting in increased risk of pests developing *Bt*-resistance. One possible solution to such problems or threats is to express such genes at high levels and compartmentalize their expression. Most of the cultivated crop plants transmit their plastids to progeny plants through female part rather than pollens, thus providing natural containment of transgenes.

Chloroplasts known to have evolved from free-living microorganisms are double membrane-bound intracellular structures containing their own genetic material functioning

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independently or in association with master organelle, the nucleus. Chloroplasts either develop from proplastids in meristematic tissues or from other differentiated plastids such as chromoplasts, amyloplasts and leucoplasts on exposure to light. They contain the entire enzymatic machinery for photosynthesis playing important roles in many essential metabolic processes, including synthesis of starch, amino acids and lipids. The transformation of proplastids into viable chloroplasts is one of the most important changes that take place during plant development. These developmental changes lead to the formation of distinctive features in the chloroplasts. One of the most important features is the presence of extensive internal thylakoid membrane system that is responsible for photosynthetic energy transduction. The four major components of the thylakoid membrane are photosystem I, photosystem II, cytochrome *b/f* complex and the ATP synthase complex [1]. These complexes together with a number of enzymes housed in the stromal fraction of chloroplasts carry out conservation of molecules such as water and carbon dioxide into carbohydrates on energy utilization derived from light. In addition, many biochemical pathways essential for plant growth and development are found in plastids. They include biosynthesis of fatty acids, amino acids and carotenoids. Beneficial alteration of such processes can be explored by incorporating heterologous gene (s) into the plastome. Keeping in view such ideas scientists have tailored a number of characteristics in tobacco model through plastome engineering. Moreover, work is in progress to extend this technology to other crop plants, salient examples of which are discussed in detail in this review.

Plastid Genome Organization

Plastid number varies from 10-20 pro-

plastids in meristematic cells to several hundre
in a leaf cell and provides more targets fo
transformation. Besides, the number and type
of plastids vary, each plastid carries the sam
genetic material, a double stranded circula
molecule of 120 to 160 kb divided into smal
and large single copy regions separated b
inverted repeats. Plastids generally contain 50
150 copies of circular DNA molecules. Plastic
genome has been sequenced from a number o
organisms (Table 1). It encodes about 120
proteins both for genetic as well as photosynthetic
systems (Table 2). A number of open reading
frames called *ycfs* (hypothetical chloroplast open
reading frames) are unknown for their functions
(Table 3). In addition to these several
polypeptides encoded in the nuclear genome,
synthesized on cytoplasmic ribosomes, are
imported into the plastids.

Table 1. Fully sequenced plastid genome of organisms

Organism	Genome size (bp)	References
Algae		
Chlorella ellipsoidea	150,613	Wakasugi *et al.* [62]
Cyanophora paradoxa	135,599	Stirewalt *et al.*[63]
Euglena gracilis	143,172	Hallick *et al.* [64]
Odontella sinensis	119,704	Kowallik *et al.*[65]
Porphyra purpurea	191,028	Reith and Munholland [66]
Land plants		
Epifagis virginiana	70,028	Wolfe *et al.* [67]
Marchantia polymorpha	121,024	Ohyama *et al.* [68]
Nicotiana tabacum	155,844	Shinozaki *et al.* [69]
Oryza sativa	134,525	Hiratsuka *et al.* [70]
Pinus thunbergii	119,707	Wakasugi *et al.* [71]
Zea mays	140,387	Maier *et al.* [72]

Table 2. Genes in land plant chloroplast genomes

Genes for the genetic system	
RNA polymerase	*rpoA, B, C1, C2*
Ribosomal RNAs	*rrn (16S, 23S, 4.5S, 5S)*
Ribosomal proteins	*rpl2, 14, 16, 20, 21, 22, 23, 32, 33, 36* *rps2, 3, 4, 7, 8, 11, 12, 14, 15, 16, 18, 19*
tRNAs	*trn* (30 tRNA genes)
Protease	*clpP*
Genes for the photosynthetic system	
Photosystem I	*psaA, B, C, I, J, M*
Photosystem II	*psbA, B, C, D, E, F, H, I, J, K, L, M, N, T*
Cytochrome b/f complex	*petA, B, D, G, L*
ATP synthase	*atpA, B, E, F, H, I*
Ribulose-1, 5-bisphosphate carboxylase	*rbcL*
Protochlorophyllide reductase	*chlB, chlL, chlN*
NADH-dehydrogenase	*ndhA, B, C, D, E, F, G, H, I, J, K*
Acetyl CoA carboxylase	*accD*
Genes of unknown function	*ycf1, 2, 3, 4, 5, 6, 9, 10*

Table 3. Hypothetical Chloroplast Open Reading Frames (ycfs) (The size of the open reading frame in tobacco chloroplast DNA is given)

ORFs	*YCFs*	Possible function/structure
Orf1901	*ycf1*	highly basic, essential for cell survival
Orf2280	*ycf2*	contains ATPase domain
Orf168	*ycf3*	contains intron
Orf184	*ycf4*	photosystem regulation in *Synechocystis*
Orf313	*ycf5*	*ccl1* homology-cytochrome *C* synthesis
Orf29	*ycf6*	small membrane-spanning protein
Orf62	*ycf9*	small membrane-spanning protein
Orf229	*ycf10*	chloroplast inner envelope membrane protein

Gene Expression in Plastids

Plastid DNA of higher plants contains genes for the genetic and photosynthetic functions of the organelle. Chloroplast gene expression has many similarities to gene expression in prokaryotes, from which plastids are believed to have originated [2,3]. Chloroplast genes are transcribed by two separate RNA polymerases. The photosynthesis genes are transcribed by an RNA polymerase containing plastid-encoded subunits homologous to the α, β, and β' subunits of *E. coli* RNA polymerase [4,5]. This RNA polymerase activity was characterized in its soluble and DNA-bound forms [2,3] and the expression of the *rpo* genes was confirmed by detection of the corresponding subunits in highly purified enzyme preparations from maize chloroplasts [6,7] and by western blotting of crude extracts from spinach chloroplasts with antisera raised against individual subunits [8]. Evidence for the expression of the *rpo* genes in the form of the corresponding RNA [9,4] and of specific proteins in soluble chloroplast extracts [4,5] confirmed that the core subunits of a chloroplast RNA polymerase are encoded in the chloroplast genome.

Plastid genes are transcribed by two distinct RNA polymerases: the plastid-encoded plastid RNA polymerase (PEP) and the nuclear-encoded plastid RNA polymerase (NEP). The genes of the multi-subunit PEP core are encoded by the plastid genome, and are homologous to the eubacterial (*E.coli*-like) DNA-dependent RNA polymerase α, β, and β' subunits [3]. The δ^{70}–like factors required for promoter recognition [10] are encoded in the nucleus [11]. PEP promoters are similar to eubacterial δ^{70}-type promoters: the core comprises two hexameric sequences corresponding to the eubacterial-35 (TTGACA) and eubacterial-10 (TATAAT) promoter elements. The hexamers are spaced 17-

19 nucleotides apart; transcription initiation 5-7 nucleotides downstream of the eubacterial-10 box sequence [3]. The catalytic subunit of NEP is related to the mitochondria and phage-type T3/T7 RNA polymerases [12]. PEP is derived from the RNA polymerase of the ancestral bacterium. It is assumed that the phage-type plastid RNA polymerase evolved by duplication of the nuclear gene encoding the mitochondrial enzyme, and re-targeting of the gene product to plastid [13]. Several plastid promoters have been shown to direct the transcription of genes in prokaryotic cells [14], and the expression of chloroplast genes in *E. coli* cell-free extracts allows identification and localization of many of the genes for photosynthesis components [15,16,17]. However, there are some cases where transcription initiation sites are not preceded by typical consensus elements [18], e.g. *trnS* and *trnR* genes in spinach and a light-inducible *psbD/psbC* promoter in barley and wheat [19,20,21]. Recently, another non-consensus type promoter in the S' region of the tobacco *atpB/E* operon has been reported [22]. Transcript levels from these promoters have been decreased by cyclohexamide, a cytoplasmic protein synthesis inhibitor, providing further evidence for non-consensus-type plastid promoters [22]. More recently, it has been shown that many plastid genes and operon have at least one promoter each for *E. coli*-like RNA polymerase and nuclear-encoded plastid RNA polymerase [23].

Transcription of plastid genes by one or both RNA polymerases reflects their function. PEP transcribes Photosystem I and II genes; therefore it plays an important role in chloroplast gene expression. In the absence of the PEP, non-photosynthetic proplastid is still maintained indicating that NEP transcribes all essential housekeeping genes. Indeed, most non-photosynthetic genes have promoters for both RNA polymerases. Only a few genes are known to be transcribed exclusively from a NEP promoter: *accD* encoding a subunit of the acetyl-CoA carboxylase in dicots [23] (this gene is encoded by the nucleus in monocots); *clpP* encoding the proteolytic subunit of the *Clp* ATP-dependent protease in monocots, and the *rpoB* operon in all higher plants [24,25]. The *rpoB* operon includes *rpoB*, *rpoC1* and *rpoC2*, the genes for three of the four PEP core subunits. Transcription of the *rpoB* operon is highly regulated (1,000-fold), controlling the availability of the PEP. Thus, the *rpoB* NEP promoter plays a central role in chloroplast development. It is assumed that the phage-type plastid RNA polymerase evolved from a mitochondrial enzyme [13]. We propose that, initially, transcription of plastid genes by the NEP occurred from spurious promoters, generating additional sets of RNAs for plastid genes. NEP probably became indispensable when it took over transcription of essential genes, such as the *rpoB* operon encoding the subunits for the PEP, the RNA polymerase derived from the ancestral cyanobacterium. Transcription of PEP genes by the NEP was probably a critical step of the nucleus indirectly taking control of the transcription of plastid genes, thereby fully integrating plastids in multicellular plants.

Selectable Marker Genes to Manipulate Plastome

The selectable marker genes confer resistance to drugs in plastids. These drugs inhibit chlorophyll accumulation and shoot formation on plant regeneration media. A number of drugs like hygromycin, lincomycin, spectinomycin, streptomycin, kanamycin or G418 and phosphinothricin (PPT) encoded by *hph*, *aadA*, *nptII* and *bar* genes, respectively, have been used to select and screen cells or plants carrying these genes during the process of plant genome engineering and selection. Of these, the genes

encoding spectinomycin, streptomycin, kanamycin and phosphinothricin (PPT) have been successfully used to select cells with transformed plastid genome. During normal selection procedure, marker gene recipient cells go through phases of embryogenesis and organogenesis before regenerating as green shoots. During the time of embryogenesis and organogenesis, the wild type and transformed plastids and plastid genome copies gradually sort out. Extended period of genome and organelle sorting yields chimeric plants consisting of sectors with wild type and transgenic cells. In the chimeric tissue, antibiotic resistance conferred by marker gene (s) is not cell autonomous: transplastomic and wild type sectors are both green due to phenotypic masking by the transgenic tissue. Chimarism necessitates a second cycle of plant regeneration on a selective medium. In the absence of a visual marker, this is an inefficient process and ends up in heteroplastomic tissues or plants in most of the transformation events [26]. To facilitate this system, a gene providing visual selection and screening is required. Availability of such genes is discussed below.

Reporter Genes to Manipulate Plastome

In addition to selectable marker genes, vital reporter genes undoubtedly contribute to the development of transformation technology by serving as tools for visual monitoring of transgene expression in transformed cells, tissues and organisms. A number of genes have been used to study gene expression, in plants as well as animals, as reporters. For example, the genes encoding β-glucuronidase, *uidA* [27] and β-galactosidase, *lacZ* [28], chloramphenicol acetyl transferase, *cat* and neomycin phospho-transferase, *nptII* [29,30], nopaline synthase, *nos* [31,32], octopine synthase, *ocs* [33] and luciferase, *luc* [34] have been used as reporter genes for transformation. From these genes, *uidA* has successfully been expressed transiently and is stable in a variety of organisms [35,36]. However, histochemical detection of GUS in plant organelles requires prolonged incubation because the envelope membranes of the organelles act as a selective barrier to substrate penetration. Moreover, chlorophyll bleaching in plants is required to make GUS staining more effective using either ethanol or chloral hydrate [37]. Furthermore, chemicals and physical procedures used in staining disrupt cell ultrastructure [38].

The use of a non-toxic marker to identify transgenic cells after transformation is an effective procedure for discerning transformed cells/organs and removing untransformed or non-expressing cells, tissues or organs. The green fluorescent protein (*gfp*) of the jellyfish, *Aequorea victoria*, has recently been used as a reporter gene in plants and animals [38,39,40, 41,42,43,44] which provides an easily scored cell-autonomous genetic marker in plants and has major uses in monitoring gene expression, protein localization and screening of transformation events at high resolution. The green fluorescent protein has successfully been expressed in *E. coli* and chloroplasts of tobacco, rice [26], different plastid types [45] and potato using chloroplast as well as bacterial-specific expression signals. It was therefore expected that genes would be expressed from bacterial promoters in chloroplasts. This protein has been expressed in plastids transiently [45] as well as stable expression of *gfp* in chloroplasts under the control of such bacterial promoters has been obtained successfully [46]. The development of a gene encoding bifunctional proteins can minimize the use of different set of promoters and terminators and may result in plastid DNA fragment deletion through homologous recombination due to homology with plastid

DNA, and physical separation of the genes. Moreover such a gene will facilitate both selection and visual screening of recipient cells. Such a gene has been developed through translational fusion of *aadA* (a selectable marker gene) and *gfp* (a visual reporter gene from jellyfish) genes called FLARE-S (Fluorescent antibiotic resistance enzyme conferring resistance to spectinomycin and streptomycin). This bifunctional protein facilitates plastid transformation to rice beside tobacco, where plastid transformation is not associated with a readily identifiable phenotype [26].

Techniques to Manipulate Plastome

The prerequisite of plastome engineering in plants is the availability of efficient tissue culture system to provide mature chloroplasts as targets. Different explants such as leaves in tobacco [47], *Arabidopsis* [48], potato and embryogenic cells in rice [26] provide different plastid types and proplastids to mature chloroplasts as targets during plastid transformation process. Other prerequisites include a method to deliver foreign DNA through the double membrane of the plastids, efficient integration of the heterologous DNA without interfering with the normal functions of the plastid genome and efficient selection and screening of the transplastome. Work on transient gene expression in plastids in several laboratories has provided different means to introduce foreign DNA into the plastome. These include *Agrobacterium* [49], polyethylene glycol (PEG) treatment [50,51,52], biolistic DNA delivery [53,54] and microinjection [55] methods. Of these methods, only PEG treatment [51,52] and biolistic DNA delivery [47,56,57] have yielded stable plastid transformants. Of these two, only biolistic DNA delivery method is being used to extend plastid transformation to different plastid types in different plants including dicots [45,47,56] as well as monocots [26]. A pictorial protocol for plastid transformation to integrate DNA into the plastid genome in higher plants is given here (Fig. 1) and summarized below.

During the process of plastid transformation by biolistic delivery, the DNA molecules are precipitated onto the surface of microscopic metal particles either of gold or tungsten that carry at least 20 to 50 copies of the plasmid DNA [58]. However, a single or at the most few copies of the DNA come in contact with plastid genome resulting in the development of transplastomic clone after transformation cells or leaf pieces are placed on regeneration medium containing a drug. In most of the cases, the drug used to select and screen transformed cells is spectinomycin encoded either by mutated 16S rRNA genes [47,59,60] or by bacterial gene *aadA* [56]. Sensitive cells tart regenerating into green plants. During this process, the cells have to go through at least 16 to 17 divisions to regenerate into homoplastomic transgenic clones. Selection, using *aadA* gene, yields 100-fold increase in plastid transformation efficiencies compared to that obtained with mutant 16S rRNA genes [56]. Low transformation efficiency using mutant 16S rRNA genes is explained by elimination of 99 out of 100 copies of the integrated DNA before the recessive selectable marker can be expressed [56].

The foreign DNA integrates into the plastid genome through two homologous recombination events (Figure 2). The selectable and passenger genes in a transformation vector are flanked by chloroplast DNA on either side to integrate foreign DNA at a specific site. The challenge faced by the plastid transformation technology is the introduction and expression of passenger genes in the plastid in the absence of selectable marker and to extend this technology to non-green plastids of cereal crop plants. As discussed later, research is in progress for solving these problems.

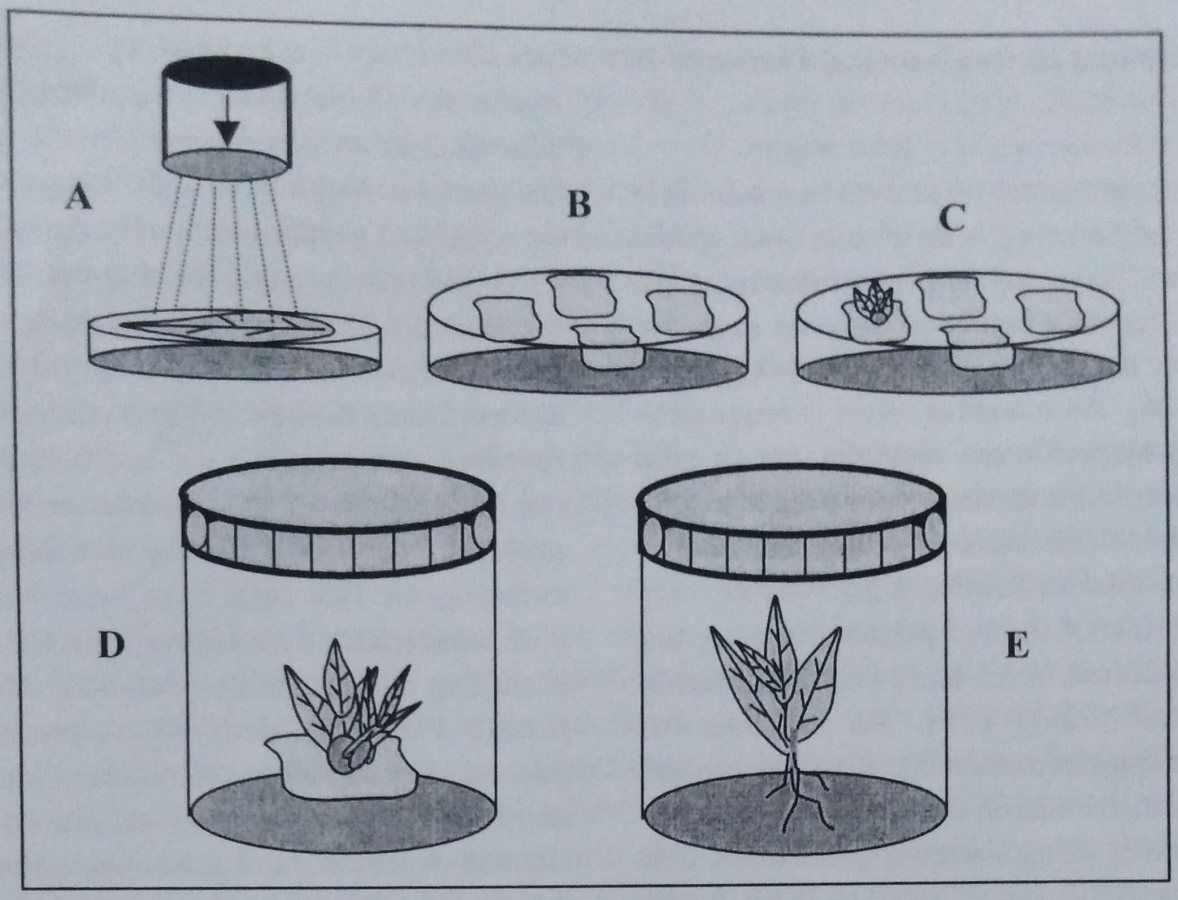

Figure 1. Plastid transformation. **A**. Introduction of DNA using PDS 1000 He biolistic gun. **B & C.** Selection of plastid transformants on regeneration medium containing spectinomycin that results in regeneration. **D.** Regeneration and proliferation of regenerated shoots on selection medium. **E.** Regenerated shoot was excised and placed for rooting and transfer to soil after obtaining homoplasmicity.

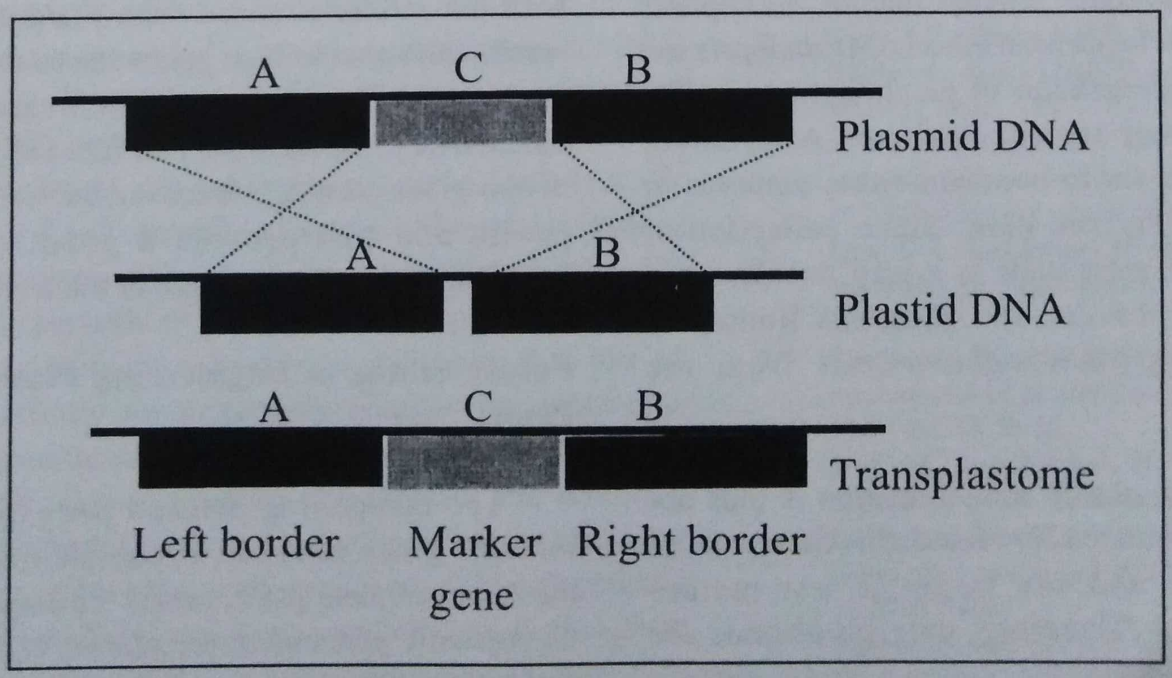

Figure 2. Gene insertion into the plastome. A and B are plastid DNA fragments used to introduce a marker gene (C) into the plastome through homologous recombination events between A & B in the plasmid DNA and plastid DNA.

Present Status of Engineering Plastome in Plants

Although successful and reproducible plastome engineering is limited to such model species as *Chlamydomonas* and tobacco, it has also been recently achieved to some extent in many other plants like *Arabidopsis* [48], potato and rice [26]. An intensive effort is required to make this system more reproducible in crop plants other than tobacco to get deeper insight into the molecular biology of plastids than is currently available. Among higher plants, very low transformation through plastome engineering has been reported in *Arabidopsis* with unfertile regenerated plants [48]. As in tobacco, chloroplast transformation in potato has resulted in stable transformation events after bombardment of leaves using bacterial gene *aadA* that encodes aminoglycoside 3"-adenylyltrans-ferase conferring resistance to spectinomycin and streptomycin which inhibit translation of mRNAs. However, plastid transformation has remained a mystery in cereals such as rice due to the unavailability of efficient tissue culture system that could provide mature chloroplasts as targets as happens in tobacco, *Arabidopsis* and potato. Regeneration of plants is not possible through leaf tissue *in vitro*. Alternative approaches are to bombard either embryos or scutellum-derived calli. Since collection of embryos at mass scale is a very intensive and laborious process, the choice is limited to developing embryogenic cells from the scutellum.

In rice, plastid transformation is thus not associated with readily identifiable tissue culture phenotype and the target is not mature chloroplasts. Therefore, only proplastids are available to access the plastome. Proplastids are smaller than the microprojectiles used in the biolistic transformation protocol, a major drawback with transforming the plastids of agronomically important crop plants. Size of the plastids may not be a problem for the reason that the particles do not have to hit the target plastids directly, as thought previously, because in the PEG (polyethylene glycol treatment) treatment method the DNA is targeted to the cellular compartment where it is imported into the chloroplasts. However, the exact mechanism involved in targeting the DNA into he plastids is not yet known. In the absence of alternative protocols for transforming rice proplastids, embryogenic rice cells have been bombarded with constructs containing FLARE-S [26], excluding the possibility of directly hitting the plastids. Plants obtained with this procedure are genetically unstable. However, our effort continues to be directed at search for an alternative protocol and gene expression signals that could be help in developing fertile and stable transgenic plants of rice. The results are encouraging, especially when GFP is used to express in different plastids types of marigold, carrot roots and pepper fruits, under plastid gene expressing signals, transiently [45]. Together with the results obtained with FLARE-S, our results show that foreign genes can be expressed in non-green plastids and, at least in case of rice, the DNA can be incorporated into the genome of non-green plastids. Moreover, the results with potato and rice provide a good start for broadening the species range of the technology.

Future Status of Engineering Plastome in Plants

The compelling demand for expressing multiple genes controlling multiple traits, into the same recipient plant, favors the engineering of organelle genome of the plants. The ability for genetic transformation of the plastid genome in higher plants is extremely attractive and important for development of transgenic traits

that may be difficult or impossible to achieve by nuclear transformation. Down the road, the ultimate objectives of genetic engineering are to decipher what and how events occur inside the living cells or organisms during the process of tailoring particular characters of economic importance in them. The application of plastome engineering of crop plants will depend on choice or suitability of plant material for cell culture manipulations, a prerequisite of plastid transformation technology, and on an efficient system to detect transformation events.

As plastids of higher plants house many biochemical pathways such as biosynthesis of starch, amino acids and lipids encoded exclusively by nuclear genome, beneficial alteration of these processes can be explored by incorporating heterologous genes into the plastome. Keeping in view such ideas or points, a number of characteristics have been tailored in the tobacco model through plastome engineering. The following are some salient examples of such research activities: (1) high level expression of *Bt* genes to eliminate the possibility of low toxicity effects on insects resulting in the development of resistance against pesticides [61], (2) diversification of resistance to insects of different orders in the same plant by stacking different *cry* genes in an operon form (e.g. *Bt cry2aa2* operon) in tobacco chloroplasts leading to formation of insecticidal crystals [62], (3) expression of anti-microbial peptide (AMP), an amphipathic alpha-helix molecule that has an affinity for negatively charged phospholipids commonly found in the outer-membrane of bacteria in tobacco chloroplasts and retaining biological activity against *Pseudomonas syringae* (a major plant pathogen), and (4) altering plastid genome by utilizing yeast trehalose-6-phosphate synthase gene for developing drought tolerance in tobacco plants (Henry Daniell, personal communication). In addition to these, marker-free selection of chloroplast transformants has also been obtained [63]. Hopefully, plastid genome engineering in plants may become an industry in the near future for expressing highly pure, safe and cheap proteins of pharmaceutical and consumer interest.

Conclusions

Engineering chloroplast genome of land plants is becoming an important Bio-Industry since the first chloroplast transformant was obtained in 1990 by Svab *et al.* [47]. Plastid transformation provides a means of producing bio-safe transgenic plants engineered for different traits such as development of insect-resistant, herbicide-resistant, bacterial-resistant and salt/drought-tolerant plant lines. Moreover, oral vaccines would become available in near future. Although there are always some limitations, engineering plastome remains an excellent choice for developing environment friendly transgenic plants.

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