A Review

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Plastid Genome Engineering in Plants: Present Status and Future Trends

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Abstract
When comes to genome engineering, the nucleus in plant cells gets all attention. Lately, organelles of prokaryotic origin, enslaved by the plant cells, called plastids are predominantly engineered for basic research and biotechnological applications. Expression of transgenes in plastids offers several advantages over their expression in the nucleus, which includes expression of transgenes to high levels as polycistronic units, biological containment of transgenes through maternal inheritance and elimination of position effects that are frequently observed in nuclear transformation because of random insertion of transgenes into the genome. This review summarizes the state of the art in plastid genome engineering and briefly describes how novel approaches facilitate analyzing plastid genes. Further, extension of the technology to other crop plants is discussed.

Keywords
Plastid; Plastome; Biolistic method; Plastid transformation; Biological containment

Introduction
Plant cells in addition to nucleus contain organelles called plastids and mitochondria, which are generally believed to have evolved from formerly free living eubacteria: α-proteobacteria and cyanobacteria, respectively (Bock and Khan, 2004). Extensive sequencing has revealed that, at least in higher plants, plastids contain a remarkably conserved genome, the plastome. The plastome engineering in higher plants is achieved through a gradual process, which results in uniform alteration of 300-10,000 plastid genome copies in a cell. Despite complex process, plastid genome is an attractive target for crop transformation because proteins in chloroplasts may accumulate to high levels; multiple genes may be expressed as polycistronic units, lack of pollen transmission of gene (s) in most of cultivated crops that results in natural gene containment and elimination of positional effects due to targeted homologous recombination.

Plastid transformation was first achieved in 1988 in a unicellular alga, Chlamydomonas reinhardtii (Boynton et al., 1988) to replace a mutant gene from chloroplasts with a wild type to restore its function, followed by stable transformation of chloroplasts of tobacco using a bacterial gene that encodes aminoglycoside 3′-adenyltransferase and confers resistance to spectinomycin and streptomycin (Svab and Maliga, 1993) and a visual reporter gene encoding green fluorescent protein from jellyfish (Khan, 1997), which facilitated the extension of plastid transformation to non-green plastids (Khan and Maliga, 1999). This was the time when chloroplast transformation was started in several other laboratories to study the function of plastid genes and to express genes of agronomic and biotechnological importance (Heifetz, 2000; Bock, 2001; Maliga, 2004; Daniell et al., 2002). This review summarizes the plastid transformation technology in higher plants and salient examples of its application in basic science and biotechnology.
1 Plastid genome engineering: state of the art

Plastid genome encodes approximately 120 protein and RNA genes in various plant species (Sugiura, 1992; Yang et al., 2010), suggesting that most of their ancestral genes have been either lost or transferred to the nuclear genome during evolution. When comes to plastid genome copy number, it reaches to 10,000 copies per cell because a single leaf mesophyll cell of flowering plants carries 100 chloroplasts, and each chloroplast contains 100 plastome copies. Further, the number of genes, located on the plastome in the inverted repeat region is doubled because of duplication of a large region of plastome in an inverted orientation.

Engineering genome of plastids involves a series of steps to develop homoplasmic transplastomic clones. During the process, transformations vectors that harbor a selectable marker gene, a reporter gene and the passenger gene(s) flanked by homologous targeting sequences, are used. Successful transformation requires; a totipotent explant, an efficient DNA delivery method, selection agents (antibiotics) and a reproducible regeneration protocol to efficiently recover transgenic plants. Transgenes are incorporated into the plastome through homologous recombination events. In practice, the inserted transgene has short DNA sequence tails added at each end, the tails are homologous to sequences on the chloroplast target gene, which thus initiate homologous recombination. Antibiotics used in selecting transgenic plants inhibit chlorophyll accumulation and shoot formation on plant regeneration media, however, cells resistant to these antibiotics regenerate into green shoots, and resistance to these antibiotics is conferred by the expression of genes in plastids. Lethal (kanamycin) and non-lethal antibiotics (streptomycin, spectinomycin) are used to select transformants. As different species have differing sensitivities to selective agents, hence, the successful recovery of the transplastomic lines is dependent on selection concentration that how carefully the concentration of the selection agent was titrated that permitted growth and development of the transformed cells while limiting the growth of the non-transformed cells, and the regeneration strategy adopted. Further, same selective agent cannot be universally used in selecting the transformation events.

For example, spectinomycin is routinely used in plastid transformation experiments to recover green shoots on regeneration media from tobacco, and has also been used for tomato, potato, cabbage, oil rape seed and carrot (Table 1). But, rice, sugarcane and few other monocots are naturally resistant to spectinomycin; therefore selection for transplastomic lines was carried out on medium containing streptomycin, which inhibits growth of embryogenic cells on regeneration media, because aadA gene confers resistance to streptomycin as well.

Initially, only few plastome copies are transformed, and stable homoplasmic lines are produced with continued selection pressure in which each and every plastome contains the transgene. The process involves cultivation of the cells on a selective medium, during which the cells divide at least 20 times, transformed and non-transformed plastids and plastome copies gradually sort out, yielding chimeric shoots consisting of sectors of transgenic and non-transgenic cells. Both transgenic and non-transgenic cells in a chimeric shoot are green in color because of phenotypic masking by the transgenic cells (Khan and Maliga, 1999), referring that antibiotic resistance is not cell autonomous. Nevertheless, transgenic and non-transgenic sectors can be readily identified in knockout transgenic plants lacking a photosynthesis gene (Figure 1A–D; Khan et al., 2007) or by green fluorescent protein (GFP) accumulation (Khan and Maliga, 1999). The GFP allows direct imaging of the fluorescent gene product in living cells without the need for prolonged and lethal histochemical staining procedures. Its chromophore forms autocatalytically in the presence of oxygen and fluoresces green when absorbing blue or ultraviolet (UV) light (Khan, 1997). The preferred strategy to develop homoplasmic shoots is to regenerate subsequent shoots from the homoplasmic sector, which are rooted, shifted to soil and hardened for growth and seed setting. Homoplasmic transgenic plants can be recovered from embryogenic cells from suspensions or calli but plant regeneration is delayed until plastome and plastid segregation is complete. However, extended propagation of cells on regeneration media is undesirable because it results in either scanty or no regeneration of embryogenic cells into shoots.
Table 1: Plastid transformation in flowering plants: methods of transformation and gene targeting sites in the plastomes

<table>
<thead>
<tr>
<th>Crop</th>
<th>Targeted tissues</th>
<th>Targeting sites</th>
<th>Gene transfer methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>Leaves</td>
<td>rbcL-accD</td>
<td>Bombardment</td>
<td>Svab and Maliga (1993)</td>
</tr>
<tr>
<td>Oilseed rape</td>
<td>Cotyledon petioles</td>
<td>rps7-ndhB</td>
<td>Bombardment</td>
<td>Huo et al., 2003</td>
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<td>Cabbage</td>
<td>Leaves</td>
<td>rnr16S-rnr23S</td>
<td>Bombardment</td>
<td>Liu et al., 2007</td>
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<tr>
<td>Sugar beet</td>
<td>Leaf petioles</td>
<td>rnl16-tps12</td>
<td>Bombardment</td>
<td>DeMarchis et al., 2009</td>
</tr>
<tr>
<td>Lettuce</td>
<td>Protoplasts</td>
<td>trnI-trnA</td>
<td>PEG</td>
<td>Lelivelt et al., 2005</td>
</tr>
<tr>
<td>Cotton</td>
<td>Embryogenic calli</td>
<td>trnI-trnA</td>
<td>Bombardment</td>
<td>Kumar et al., 2004a</td>
</tr>
<tr>
<td>Petunia</td>
<td>Leaves</td>
<td>rbcL-accD</td>
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<tr>
<td>Soybean</td>
<td>Embryogenic tissues</td>
<td>trnV-tps12/7</td>
<td>Bombardment</td>
<td>Dufourmantel et al., 2004</td>
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<td>Eggplant</td>
<td>Green stem segments</td>
<td>trnV-3′tps12</td>
<td>Bombardment</td>
<td>Singh et al., 2010</td>
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<td>Tomatoes</td>
<td>Leaves</td>
<td>trnM-trnG</td>
<td>Bombardment</td>
<td>Ruf et al., 2001</td>
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<tr>
<td>Carrot</td>
<td>Embryogenic cells</td>
<td>trnI-trnA</td>
<td>Bombardment</td>
<td>Kumar et al., 2004b</td>
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<tr>
<td>Wheat</td>
<td>Immature embryos and immature inflorescences</td>
<td>trnV-tps12/7</td>
<td>Bombardment</td>
<td>Cui et al., 2011</td>
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<tr>
<td>Rice</td>
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<td>trnV-tps12/7</td>
<td>Bombardment</td>
<td>Khan and Maliga, 1999</td>
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<tr>
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<td>Bombardment</td>
<td>Sikdar et al., 1998</td>
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<td>Calli</td>
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<td>Bombardment</td>
<td>Wei et al., 2011</td>
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<tr>
<td>Alalfa</td>
<td>Leaves and calli</td>
<td>trnI-trnA</td>
<td>Bombardment</td>
<td>Mustafa (2011)</td>
</tr>
</tbody>
</table>

Note: Only first report on each plant species is reported in the table

Transgenes are generally introduced into plastids by biolistic DNA delivery (Svab and Maliga, 1993; Khan, 1997; Daniell et al., 1998; Khan and Maliga, 1999; Sidorov et al., 1999) or polyethylene glycol treatment (O’Neill et al., 1993; Golds et al., 1993). Microinjection is another approach for the plastid transformation (Knoblauch et al., 1999), which yields transient gene expression. Of these methods, biolistic delivery of DNA into the intact cells and tissues is a reliable approach that allows breaching of cell wall and membranes, principal barriers to DNA delivery, in a non-lethal manner (Sanford, 1990). In this method, high-density metals like tungsten and gold particles are coated with DNA and propelled into the target cells through acceleration provided by high pressure helium gas as propellant. It was experienced that tungsten and gold particles work equally well, but, oxidized layer on tungsten particles is removed before coating DNA. In most laboratories, biolistic delivery method is preferred over other methods to develop transplastomic plants because unlike polyethylene glycol (PEG) treatment of protoplasts, fully expanded leaves, cotyledons, or cultured cells in tissue culture are used (Svab and Maliga, 1993; Khan and Maliga, 1999; Ruf et al., 2001, G. Mustafa and M.S. Khan, unpublished). Further, PEG-treatment of protoplasts is restricted to tobacco only. Alternate methods for DNA delivery to plastids have also been tried. Microinjection, which looks promising for transient gene expression (Knoblauch et al., 1999), has not yet yielded stable transplastomic clones.
2 Functional analyses of plastid genes using omics approaches

The complete nucleotide sequence of an ever-increasing number of plastid genomes have revealed that they are remarkably conserved in their coding capacity and organization in higher plants and algal species. The genome organization and plastid gene expression has been described elsewhere in detail (Daniell and Khan, 2003). Plastids have their own transcription and translation machineries (Gruissem and Tonkyn, 1993),
which were conserved through the evolutionary steps.

The availability of complete genome sequences, a large number of expressed sequence tags and introduction of high-throughput methodologies such as transcriptomics and proteomics has enabled the analyses of gene expression in photosynthetic organisms from transcription to post-translational levels, and to study the protein-protein interactions (Duggen et al. 1999; Cahoon and Timko, 2000; Yamamoto et al., 2009; Armbruster et al., 2011). DNA microarray technology has been used successfully to functionally analyze the gene expression at transcriptomes level in various plants (Schaffer et al., 2000). Further, ‘RNA-seq’, a deep sequencing approach has been successfully used to investigate the transcriptional control of ectopic chloroplast development in plants, considering the approach a powerful tool to compare gene expression on a genome-wide scale in a species, without a reference genome (Zhou et al., 2011). Recently, transplastomic tomato transcriptomes during fruit development and chloroplasts to chromoplasts conversion are analyzed (Kahlau and Bock, 2008). When compared with leaves, most but not all genes were strongly down regulated in fruits. However, such large changes in RNA accumulation during chloroplast-to-chromoplast differentiation at the time of fruit ripening were not observed; perhaps most plastid genes were translationally down regulated. It is well documented that the transcriptional activity varies from one gene to another as a result of its important role in plastid differentiation and the control of gene expression in response to environmental cues. The transcription in plastids is largely dependent on nuclear genes, encoding polymerases, δ factors and various other transcription regulators (Shiina et al., 2005).

Customized DNA microarrays have also been developed for studying photosynthesis related gene expression profiles (Kurth et al., 2002) because genes with similar functions often display similar transcriptional profiles. Lately, multiple approaches have been used to characterize genes, for example, ‘guilt-by-association’ concept is coupled with reverse genetic approach and a number of genes of unknown functions that exhibit photosynthesis genes like transcription profile are characterized (Dalcorso et al., 2008). Genome-wide characterization of psaA and psbA genes deletion mutants using transcriptomic and quantitative proteomic analyses have revealed that the nuclear and chloroplast genes involved in photosynthesis, energy metabolism and chloroplast biogenesis, and genes transcribed by PEP are down regulated whereas genes transcribed by the nuclear encoded polymerase (NEP) are up-regulated (Khan et al., 2007; Leelavathi et al., 2011), indicating simultaneous activation of multiple signaling pathways in response to disruption of PSI and PSII complexes in ApsaA and ApsbA plants (Leelavathi et al., 2011). Hence, these studies have paved the path to study functional analysis of plastid–nuclear interactions.

However, from proteomics perspective, two-dimensional gel electrophoresis, mass spectrometry and bioinformatics techniques are used to identify proteins and to record their expression profiles, compartmentalization and protein-protein interaction. Though, a number of problems including detecting low abundance proteins and capturing the dynamics of plastid proteomes are highlighted in the literature (Baginsky and Gruissem, 2004) but recently, proteome dynamics is carried out during plastid differentiation, using quantitative two-dimensional gel electrophoresis and tandem mass spectrometry in rice (Kleffmann et al., 2007). Additionally, proteomes from stroma (Rutschow et al., 2008; Ferro et al., 2010), thylakoid (Peltier et al., 2000; Giacomelli et al., 2006; Rutschow et al., 2008; Ferro et al., 2010) and envelope membranes (Froehlich et al., 2003; Ferro et al., 2010), and from thylakoid lumen (Peltier et al., 2002; Schubert et al., 2002) are studied. Hence, these high-level system biology analyses techniques are future technologies to functionally characterize the genes, to study coordinated expression of genes from nuclear and plastid genomes and to study the quality traits affected by temperature (Yun et al., 2012) during lengthy post-harvest storages.

3 Functional analyses of plastid genes using novel approaches
In addition to characterization of plastid genes at transcriptional and translational levels using omics
approaches, the plastid genome engineering has facilitated the functional characterization of plastome-encoded genes and open reading frames. Hence, the functions of most of the major open reading frames have now been studied through simple knockout strategy, as reviewed by Bock and Khan (2004). Reverse genetics, contrary to forward genes, entails known DNA sequences that contain an open reading frame of unknown function(s) to delete or disrupt, resulting in lack of functional gene product with a characterizable phenotype. The strategy involves construction of a transformation vector in which a selectable marker gene is cloned, between the flanking DNA sequences, in such a way that it either replaces or disrupts the target gene/ycf from the plastome via two events of homologous recombination (Figure 2A) followed by selection on antibiotic containing regeneration medium. Using the strategy a

![Figure 2 Strategies for deletion of chloroplast ycf/genes](image)

Note: A: Standard reverse genetics approach where a gene or ycf is deleted with a selection gene through two events of homologous recombination between the flanks of a designed cassette/vector and the corresponding sequences of the plastome, explained elsewhere (Bock and Khan, 2004); B: The CRE recombinase based strategy used to delete clpP1 gene from tobacco (Kuroda and Maliga, 2003); Transplastomic plants were developed using a transformation vector harbouring *clpP1* gene flanked with lox sites on either side linked with *aadA* gene for selection; The transplastomic plants were pollinated with CRE gene containing pollens from a nuclear transformant; The recombinase from nuclear genome imports into the chloroplasts and catalyzes the excision of *clpP1* gene and thereby develops ΔclpP1 plants; C: The copy correction based strategy employed to functionally analyze *psbA* gene (Khan et al., 2007); The chloroplast transformation vector targets both the *aadA* gene expression and *psbA* gene deletion cassettes into the inverted repeat region through two events of homologous recombination; Sectors of homoplasmic gene deletions were made visible on leaves of the purified homoplasmic shoots as a result of copy correction mechanism
number of genes have been disrupted or replaced. Obviously, homoplasmic phenotype of the transplastomic plants is an absolute requirement for clear interpretation of the data. However, deletion of few genes/yefs has developed heteroplasmic genotypes because of the gene product is essential for cell viability, in which homoplasmic transgenic genotypes cannot be obtained. It is reported elsewhere that both genomes, wild type and transformed, co-exist in a relatively constant ratio. This shows that both types of genomes are required for cell to survive on selection medium and to provide the gene product of the essential gene. Growing heteroplasmic cells on antibiotic-free medium allows sorting of plastid genomes that eventually results in the appearance of homoplasmic wild-type cells (Figure 1B-D; Khan and Maliga, 1999; Bock, 2001; Khan et al., 2007).

Therefore, the development of suitable alternate strategies would facilitate the investigations of the deletion(s). For example, the CRE-loxP site-specific recombination system was used to effectively delete clpP1 gene from tobacco plastids (Kuroda and Maliga 2003). The strategy requires the introduction of loxP sites flanking the clpP1 gene in the plastid genome followed by the availability of CRE gene product, targeted after expressing the gene from nuclear genome. The nuclear-encoded, plastid-targeted CRE enters the plastids and excises the clpP1 copies (Figure 2B). And thus the loss of the clpP1 gene product, the ClpP1 protease subunit, arrests shoot development in tobacco.

Another strategy was developed by Khan and colleagues, which efficiently deletes targeted plastid-encoded gene by exploiting the homologous copy correction mechanism (Figure 2C), and was used to delete psbA gene from plastid genome of tobacco (Khan et al., 2007). Thus, this strategy simplifies the engineering of the final transformation vector compared to standard deletion constructs, thereby providing a high through-put approach for the systematic deletion of plastid-encoded genes. Using the strategy, gene knock out is achieved in two steps. Firstly, the chloroplast transformation is achieved by integrating the aadA gene into the inverted repeat region of the plastome via two events of homologous recombination. Secondly, spontaneous replacement of the endogenous gene by homologous copy correction between sequences flanking the target gene and the sequences integrated in the inverted repeat region. Hence, in case of essential gene product the homoplasmic sectors will appear on leaves/shoots with distinct phenotype, which may be analyzed further to assign function to the gene. In case gene product is not essential for cell survival, the chimeric sectors may be used to regenerate homoplasmic shoots for further analysis (Khan et al., 2007).

4 Engineering pathways in chloroplast to improve photosynthesis

Photosynthetic carbon metabolism is a key factor in plant growth and yield. Photosynthetic carbon fixation, catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is a fundamentally inefficient process for most plants; this has been extensively reviewed elsewhere (Cleland et al., 1998; Spreitzer, 1999; Bock and Khan, 2004). Major limitations on the efficiency of carbon dioxide fixation are: first, Rubisco works very slowly and it catalyzes only a few reactions per second, and second, the ability of oxygen to bind to the active site of the enzyme in a non-productive reaction in which ribulose bisphosphate is broken down and carbon dioxide is released (Wingler et al., 2000). Thus, Rubisco catalyzes two competing reactions, carboxylation and oxygenation, the rates of which depend upon the relative concentrations of CO2 and O2, as well as on temperature. Carboxylation leads to net CO2 fixation, whereas oxygenation generates glycolate that can only be metabolized outside chloroplasts by photorespiratory processes in peroxisomes and mitochondria (Medrano et al., 1995). Therefore, plant growth and yield can be improved by increased photosynthesis and/or by reduced photorespiration. Though, the discovery of an alternate version of Rubisco would improve the efficiency of photosynthesis but despite considerable effort, this aim has yet to be realized. Engineering C3 plants with the C4 pathway seems to be more promising but benefits of concentrating CO2 in the chloroplasts of C3 plants have been questioned because they are known to leak gases (Tolbert, 1997). Kebeish et al (2007) has incorporated glycolate
catabolic pathway in chloroplasts to alleviating photorespiratory losses in *Arabidopsis thaliana*. To establish the glycolyte catabolic pathway in chloroplasts, three vectors harboring five genes that encode subunits of GDH, GCL and TSR, respectively, were developed. The authors first targeted the three subunits of glycolyte dehydrogenase (GDH) to chloroplasts after expressing from nuclear genome and then introduced glyoxylate carboligase (GCL) and tartronic semialdehyde reductase (TSR) to complete the competitive photorespiratory pathway for converting glycolyte to glycerate. Engineered pathway increases photosynthesis and thus promises to widen applicability of the approach to cereals, such as wheat and rice (Khan, 2007).

Another approach to improving photosynthesis in plants is: they can develop chlorophyll and assemble functional chloroplasts in the dark and are ready for photosynthesis upon exposure to light (Yamazaki et al., 2006; Kusumi et al., 2006). Chlorophyll biosynthesis is a multi reactions pathway in which the reduction of protochlorophyllide (PChlide) to chlorophyllide (Chlide) that subsequently converts into the chlorophyll by phytlyation is a major regulatory step. The reduction of PChlide to Chlide is catalyzed by two different enzymes: a light dependent, nuclear-encoded, plastid localized single subunit enzyme (LPOR, Light-dependent protochlorophyllide oxidoreductase) that requires light for its activation in angiosperms, and a light independent plastid-encoded enzyme (DPOR, Dark-operated protochlorophyllide oxidoreductase) which is composed of three subunits, encoded by genes namely, *chlL*, *chlN* and *chlB*. Incorporating DPOR in the genomes of plastids of cereal crops can improve photosynthesis by increasing chlorophyll contents, consequently developing photosynthetically competent chloroplasts in the dark which will be ready for photosynthesis upon exposure to light. Recently, the genes *chlL*, *chlN* and *chlB* from the plastid genome of *Pinus thubergii*, a Japanese Black Pine are introduced into the plastome of *Nicotiana tabacum* (Nazir, 2012). The transformed tobacco plants recovered on spectinomycin-containing regeneration medium were tested using PCR, showing successful integration of expression cassettes into the chloroplast genome and also the homoplasmity was determined through Southern blot analysis. As these genes are involved in chlorophyll biosynthesis affecting photosynthetic performance of the plants, thus homoplasmic transgenic plants carrying *chlB* genes have been first investigated. Two exciting observation are made: one, shoots from the cuttings of the transgenic plants developed early and more roots in the dark whereas shoots developed from wild type cuttings showed etiolated growth with no roots. Upon shifting from dark to light in growth room, leaves of the transgenic shoots showed early development of chlorophyll pigments compared to the wild type shoots. When cuttings were grown in the light, similar to dark, transgenic shoots also showed significant difference in root development from untransformed wild type shoots (S. Nazir and M. S. Khan, unpublished). Two more set of chloroplast transgenic plants were developed by engineering plastome with expression cassettes; one carrying *chlL* and *chlN* genes and the other *chlLN & B* genes in an operon to express under a constitutive promoter from tobacco chloroplast ribosomal RNA operon. Plants are screened and under investigations to generate physiological and biochemical data, supporting the early findings on gymnosperms that three subunit enzyme is required for developing functional green chloroplasts in the dark. Our attempts are first known efforts to engineering the three subunit pathway in plastid genome of tobacco, a representative model plant from angiosperms. This will help understanding the molecular biology of transgenic angiosperms.

### 5 Engineering plastids of monocotyledonous plants

Chloroplast transformation is recently achieved in a number of plant species (Table 1) in which transformed cells has been regenerated from tissues containing green plastids, the chloroplasts. Salient examples are: tobacco, potato, cotton, tomato, carrot, oilseed rape, petunia, sugar beet, lettuce, cabbage, egg plant and soybean. However, extending plastid transformation to monocotyledonous sugar and cereal crops including rice, wheat and sugarcane is still at its early stage of development. Amongst major hurdles, one barrier to developing plastid transformation in cereals, has been their regeneration from non-green embryonic cells,
containing undifferentiated plastids (the proplastids) rather chloroplasts. Other impediment in developing homoplasmic transgenic plants, particularly of rice, might be the low level of marker gene expression in non-green plastids in embryogenic cells because of low genome copy number and low rates of protein synthesis, this has been discussed at length elsewhere (Daniell et al., 2002). Identification of promoters and UTRs active in non-green tissues can help to overcome this limitation. The rRNA operon has two promoters, one for the eubacterial-type plastid-encoded plastid RNA-polymerase (PEP) and one for the phage-type nuclear-encoded plastid RNA polymerase (NEP). Expressing transgene under ribosomal RNA promoter, recognized by both polymerases may accumulate transprotein to high levels, leading to development of stable homoplasmic lines.

Yet, another limitation is the availability of a single dominant marker gene, aadA that encodes aminoglycoside adenyllyltransferase and confers resistance to spectinomycin and streptomycin. The aadA has been used predominantly to transform plastids and the selection was carried out on spectinomycin in dicotyledonous plants. Cells from monocotyledonous or cereal crops are naturally resistant to spectinomycin but are sensitive to streptomycin. Hence, streptomycin can be used as a selection agent to recover transgenic clones on media, as demonstrated in rice (Khan and Maliga, 1999). In these studies, embryogenic suspensions were developed from calli derived from scutellum of rice seeds. Embryogenic cells were bombarded using chloroplast transformation vector harboring aadA and gfp (encoding a modified green fluorescent protein) genes which were translationally fused and expressed under ribosomal RNA promoter recognized by plastid encoded RNA polymerase (PEP) and heteroplasmic shoots were recovered. Encouraged from these results; Kim and his colleagues (2006) transformed rice plastids using same selectable marker aadA gene and a reporter gene, gfp. Both genes were expressed under ribosomal RNA promoter in an operon. Again, heteroplasmic shoots were recovered on streptomycin containing regeneration medium. Whether heteroplasmic progeny plants could further be purified to develop homoplasmic plants was not reported. These results have reconfirmed the findings of Khan and Maliga (1999). It may be argued that streptomycin may delay the isolation of resistant shoots, hence it may hinder in the recovery and purification of homoplasmic plastid transformants in cereals when used as a primary selection means. Hence, adding a second marker for dual or stepwise selection on streptomycin and on a second antibiotic may facilitate selection and purification of transplastomic cells/shoots of cereals.

Currently, two types of selection systems have been reported for plastid transformation in dicotyledonous plant species: non-lethal and lethal selection systems. The aadA gene that confers resistance to spectinomycin and streptomycin is being used as a non-lethal selection marker (Svab and Maliga 1993, Khan and Maliga, 1999; Khan et al., 2007), the nptII (neo) gene from transposon Tn7 that encodes neomycin phosphotransferase and confers resistance to kanamycin and neomycin as lethal selection system (Reviewed in Bock and Khan, 2004; Maliga, 2004). Another gene aphA-6 that encodes aminoglycoside phosphotransferase and confers resistance to kanamycin has been used in plastid transformation experiments as a new dominant selection marker (Huang et al., 2002). Genes comprising lethal selection system confer resistance to antibiotics that enable transformed cells to survive and proliferate on the selective medium, while sensitive cells to die (Jones et al., 1987). Unlike lethal antibiotics, spectinomycin and streptomycin inhibit chlorophyll accumulation and shoot regeneration on media of non-transformed cells and allow transformed cells to regenerate into green shoots. A dual selection system has been developed and using the system transplastomic plants are recovered as green shoots on regeneration medium, containing streptomycin and kanamycin (Joyia and Khan, unpublished) whereas non-transformed cells turned brown and became dead. Similar to earlier reports on rice plastid transformation, the green shoots appeared to be heteroplasmic, suggesting the inclusion of an efficient regeneration protocol that does not affect embryogenic state of cells during repeated rounds of regeneration but is useful in purification of transformed plastids, leading to the development of homoplasmic plants. Similarly,
chloroplast transformation using dual selection system is achieved in sugarcane where heteroplasmic shoots are recovered on streptomycin and kanamycin (Mustafa, 2011). Nonetheless, these studies warrant the development of a reproducible regeneration system that allows recovery and purification of homoplasmic shoots, without compromising the regeneration with successive rounds. Further, transplastomic sugarcane plants are recovered by selecting calli only on streptomycin-containing regeneration medium, expressing gfp in leaves (G. Mustafa and Khan M.S., unpublished).

With regard to plastid transformation in wheat, stable transformation of chloroplasts from immature scutella and inflorescences is achieved by introducing nptII and gfp genes in the intergenic region between atpB and rbcL (Cui et al., 2011) and selection of transformants on kanamycin. Stable inheritance of transgenes, nptII and gfp in the progeny was confirmed by PCR and by confocal microscopy, respectively. Transformation efficiency reported (two regenerants per 42 bombardments) was extra ordinarily low, however was comparable with that of sugar beet in which one transgenic per 36 bombardments and potato in which three transgenics from 46 bombardments were recovered on selection medium. Where this report encourages the use of lethal selection genes for chloroplast transformation in cereals, the article was retracted by the corresponding author, stating that “After receiving the comments from the readers, we checked the results carefully and decided to repeat all the experiments. As the first author has already left the lab, other members are still trying to repeat these experiments. Because it takes a long time to have these experiments redone, we decide to retract our paper”. Hence, to date, only report on chloroplast transformation in cereals is the chloroplast transformation in rice, carried out by Khan and Maliga (1999) and reproduced by Lee et al (2006).

6 Perspectives of engineering plastids
As explained above, chloroplast transformation after the first report in tobacco using dominant selection gene, aadA (Svab and Maliga, 1993) has been extended to other dicotyledonous plants of agriculture and horticulture importance. Contrary to dicotyledonous plants, plastid transformation in monocotyledonous cereal and sugar plants is at its early development, conceivably because of above mentioned limitations and due to the developmental differences. Hence, developing routine plastid transformation protocols for these crops remains a challenge. Efficient and dominant selection is only one aspect that can be developed, yet it warrants to be combined with developing an in vitro cell culture protocol that allows efficient recovery and purification of homoplasmic shoots without compromising the regeneration with successive subcultures.

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