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Plastid genome engineering: potentials and challenges

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Introduction

Advances of molecular biology and genetic engineering tools have provided human kind with unprecedented power to manipulate and develop novel crop genotypes towards a safe and sustainable agriculture in the 21st century. Plant genetic engineering extends numerous opportunities for developing genetically modified crops with an array of desirable traits like, resistance to various biotic stresses such as insect, disease and virus resistance and abiotic stress tolerance, herbicide resistance as well as improvement of nutritional values. Among the transgenics developed majority of them have been developed *via* nuclear transformation techniques or engineering of nuclear genome with a transgene of interest stably integrated into the nuclear genome. However, the nuclear transgenic technology is very useful but also it is associated with certain drawbacks like; insertion of the transgene randomly into the nuclear genome which may result in to silencing of gene, low level of gene expression and uncontrolled spread of the transgene *via* pollen to the neighboring weedy or wild species.

In order to overcome the limitations, posed by the nuclear transformation technology, an alternative approach is fast emerging by which a transgene is delivered and stably integrated into the chloroplast genome. This novel technique is termed as plastid/ chloroplast genetic engineering. The possibility to directly manipulate chloroplast genome- encoded information has paved the way to study of nearly all aspects of plastid gene expression. Transgene expression in the chloroplast (plastid) genome offers several attractions to plant biotechnologists, including high-level accumulation of foreign proteins, transgene stacking in operons and a lack of epigenetic interference with the stability of transgene expression. In addition, this technology provides an environmentally benign method of plant genetic engineering, because plastids genetic information are maternally inherited in most crops and thus are largely excluded from pollen transmission.

Plastid and Plastid genome:

The genetic information of plants is distributed among three cellular compartments: the nucleus, the mitochondria and the plastids. Both the organelles mitochondrion and the chloroplast are believed to have originated from eubacterial cell that became intracellular symbionts of a host cell. The mitochondria and chloroplasts both are prokaryotic in nature, as they have originated from free living eubacteria by a process called endosymbiosis. The concept of endosymbiosis is central to understanding the cellular evolution. The movement of these eubacteria into the prokaryotic cell by endosymbiosis was followed by reduction in their genome size due to massive gene loss and gene transfer to the nuclear genome of the host. Over the time, genes got transferred from the endosymbiont genome to the host nucleus, and some acquired targeting sequences that enabled their products (now synthesized on cytoplasmic ribosomes) to be retargeted back to the organelle. Plastid is the general organelle category encompassing proplastids, the progenitors of all plastid types and chloroplasts (green plastids), chromoplasts (yellow or red, found in some fruits and flowers), and different types of white plastids such as the amyloplasts found in starch containing and elaioplasts found oil containing bodies and leucoplasts are colourless especially found in the roots.

Plastids are plant cellular organelles with their own genome transcription and translation machinery. The plastid genome (plastome or ptDNA) is a highly polyploid, circular double-stranded DNA 120 kb to 180 kb in size, encoding for approximately 120 -130 genes. A salient feature of the plastid genome, as it has a quadripartite organization consisting of two copies of inverted repeats (IRs) of 20–28 kb in size, which divides the rest of genome into a large-single-copy region (LSC; 80–90 kb) and a small-single-copy (SSC; 16–27 kb) region. The most remarkable feature of the plastid genome is its high ploidy level to the extent that the chloroplast DNA constitutes as much as 10-20 % of the total cellular DNA content. The chloroplast DNA is localized in nucleoids each of which contains 5-10 copies of the genome. A typical mesophyll cell of a plant contains about 100 chloroplasts and each chloroplast about 100 genome copies, thereby giving rise to an extraordinarily high ploidy level of up to 10000 chloroplast genome copies per cell.

Usually, the gene content of angiosperm plastid genome is rather conserved, encoding for 4 rRNAs, 30 tRNAs, and 80 unique proteins. These genes mainly fall into two major categories. (i) photosynthesis-related genes; and (ii) genetic system genes, which includes genes for rRNAs, tRNAs, ribosomal proteins and RNA polymerase subunits. It also participates in the numerous essential metabolic and cellular processes including photosynthesis, lipid and amino acid metabolism, host defence and cell signalling in plants. Other biological activities also take place in chloroplast including the production of starch, certain

amino acids and lipids, vitamins, certain pigments in flowers and several key pathways of sulfur and nitrogen metabolism. In addition to this most plastid proteins are encoded in 2100–3600 nuclear genes, the products of which are translated on cytoplasmic ribosomes and imported into plastids.

Comparison of plastid and nuclear genome engineering techniques:

S.No.	Parameters	Nuclear genome	Plastid genome
1.	Ploidy status (Copy number of Transgene)	Chromosome number is species-specific, but two copies of each of many chromosomes present per cell results in a few copies of the transgene per cell.	10–100 plastid genome (single circular chromosome) copies per plastid and 10–100 plastids per cell depending upon age and type of tissue, resulting in many transgene copies (up to 10000) per cell.
2.	Position effect of transgene insertion	Transgene is inserted at random site resulting variable levels transgene expression	Site-specific insertion of transgene through two homologous recombination events eliminates position effects on transgene expression.
3.	Arrangement/ organisation of genes and Transcription process	Each transgene is independently inserted into the chromosome and transcribed into a monocistronic mRNA.	Genes are often arranged in operons and transcribed into polycistronic RNA so that multiple transgenes can be introduced and expressed in a single transformation event.
4.	Level of gene expression	Gene regulation which determines the rate of transcription and accumulation of transgenes product / foreign protein is often a limitation.	Due to polyploidy level produces abundant transgene transcripts and a high accumulation of foreign proteins (up to 47% of total soluble protein)
5.	Silencing of gene (s)	Consequence of gene silencing resulted in decrease or elimination of transgene expression. Highly variable gene expression.	Gene silencing is not reported. It provides uniform level of gene expression
6.	Homogeneity at ploidy level	Majority of nuclear transgenic lines are either heterozygous or homozygous. The homozygosity in these lines is attained either by selfing or crossing.	Transgenic lines developed through plastid transformation are mostly homoplasmic (all genome copies are homogeneous for the transgene). Homoplasmy is mostly achieved by process of repetitive selection and regeneration.
7.	Toxicity/ harmful effect of foreign proteins	The toxic proteins accumulating within the cytosol might produce some serious pleiotropic effects.	In chloroplast the adverse effects of toxic proteins could be minimized due to compartmentalization
8.	Proper folding of protein and disulphide bond	For formation of disulphide bonds, proteins are targeted to the endoplasmic reticulum.	Chloroplasts have machinery to form disulfide bonds and correctly fold human proteins, making them

	formation		ideal for development of edible vaccines, pharmaceuticals and plantibodies.
9.	Gene Flow/ Gene containment	Inheritance of transgene is paternal results in out-crossing among weeds and crops.	It provides an environmentally benign method of plant genetic engineering, because plastids and their genetic information are maternally inherited in most crops and thus are largely excluded from pollen transmission.

Methods/ Techniques of Plastid Transformation:

Transformation refers to the process of introducing DNA into the genome of an organism. For genetic transformation two approaches have been applied to stable genetic modification of plastids: integration of transforming DNA by homologous recombination/targeting and introduction of independently replicating shuttle vectors.

Stable genetic transformation in plants consists of three phases: 1) delivery of cloned DNA to the appropriate target tissue; 2) selection of cells that have taken up the transforming DNA and integrated it into the genome; and 3) regeneration of transformed plantlets capable of continued propagation or sexual reproduction. This process is now routine for generating nuclear transformants of most plants of agronomic interest. However, the unique structural and metabolic characteristics of plastids pose a novel set of challenges for each step of the transformation process.

Earlier chloroplast transformation was considered to be virtually impossible because it contains double membrane as physical barrier and there are no bacteria and viruses known to infect chloroplasts that could be used as a vector for gene transfer. The development of gene gun as a device for transformation made possible chloroplast transformation. There are three methods used for chloroplast transformation, (i) biolistic gun, (ii) polyethylene glycol (PEG) – mediated transformation and; (iii) galistain expansion fematosyringe. But biolistic gun is the method of choice due to higher success rate as compare to other methods.

The genetic transformation of chloroplast is achieved by transferring a transgene, which is first inserted in a specialized vector to the chloroplasts by Biolistic gun. Inside the chloroplasts, the transgene gets integrated at a specified pre-determined location within the chloroplast genome. This is called gene targeting and is made possible as a result of homologous recombination - a phenomenon prevalent in prokaryotes. Thus it is necessary to have the transgene flanked on either side by homologous sequences

ensuring the integration of the transgene at its defined position in the chloroplast. The homologous region of the genome is first cloned from the chloroplast genome which needs to be transformed and later the gene of interest (the transgene or the selectable marker gene or both with suitable 5' and 3' regulatory sequences) is inserted in the middle using a unique restriction enzyme site.

Selection for the transformed chloroplast genomes makes use of the prokaryotic nature of the chloroplast's genetic system and thus depends on sensitivity of the chloroplasts to antibiotics known to inhibit specific steps in prokaryotic gene expression. Genes conferring resistance to such antibiotics are used as chloroplast-specific selectable marker genes in the selection process.

Potential Advantages of Plastid Genome Engineering:

Plastid genetic engineering offers various unique advantages, including the multi-gene engineering in single transformation event, high-level of transgene expression, transgene containment by maternal inheritance, as well as a lack of pleiotropic effects and position effects. However, this technology offers opportunities for genetic engineering of crops:

1. It offers site-specific transgene integration into spacer regions of the plastid genome which eliminates the concerns of position effects and introduction of vector sequences, which are potential concerns in nuclear transformation.
2. Chloroplast has an ability to accumulate any foreign proteins or their biosynthetic/metabolic products, which could be harmful if they were in the cytoplasm. The adverse effects of toxic proteins might be minimized by chloroplast compartmentalization.
3. All the chloroplast transgenic lines express same level of foreign protein within the range of physiological variations.
4. Plastids are better compartments for the expression of bacterial genes than the nucleus due the prokaryotic nature of an organelle.
5. Hyper-expression of vaccine antigens or therapeutic proteins in transgenic chloroplasts (leaves) or chromoplasts (fruits/roots) facilitates efficient oral delivery which can significantly reduce their cost of production, purification, cold storage and transportation and also minimize complications associated with intravenous delivery.
6. The stability and accumulation of a particular RNA or protein may be greater in a plastid compartments than the nuclear cytoplasmic compartment due to differences in proteases, ions and other constituents.

7. Maternal transmission of chloroplasts is an important feature to avoid transgene flow *via* pollen to the closely relative crops and weedy species.
8. Chloroplasts have unique machinery to form disulfide bonds and correctly fold human proteins, making them ideal for development of edible vaccines, pharmaceuticals and plantibodies.
9. The ability of chloroplasts to form disulfide bonds and to fold human proteins has opened the door to high-level production of biopharmaceuticals in plants.
10. Chloroplast offers unique feature for the multi-gene engineering in a single transformation event and simultaneous expression of multiple transgenes (``transgene stacking'') which helps in the manipulation of biosynthetic pathways, and induction of biosynthesis in plants.
11. Chloroplast transformation vectors use two targeting sequences that flank the foreign genes and insert them, through homologous recombination, at a precise, predetermined location in the organelle genome.
12. This approach has been used to investigate plastid DNA replication origins, RNA editing elements, promoters, RNA stability determinants, processing of polycistrons, intron maturases, translation elements and proteolysis, import of proteins and several other processes.

Applications of Plastid Genome engineering:

Chloroplast transformation technology has the potential for generating transgenics in major cereals and horticultural crops with traits of agronomic importance includes, (i) the engineering of resistance traits; (ii) the modification of metabolic pathways and; (iii) the production of pharmaceuticals in plants. The potential applications of this technology are as follows:

1. Chloroplast transformation is a powerful tool for the study of plastid biogenesis and function.
2. The application of this technology is increasing in the areas related with improved productivity, such as increased rate and duration of leaf photosynthesis and plant metabolic pathways.
3. This technology is better suited for hyper-expression of vaccine antigens and production of valuable therapeutic proteins.
4. The major application of this technology is the Multigene transfer (MGT) for the modification of metabolic pathways, which can include a large number of genes and feedback mechanisms simultaneously.
5. Development of cytoplasmic male sterile lines using this technique opened up new possibility for production of hybrid seed in crops.

6. Escape of foreign genes from dispersal through pollen or seed from the transgenic crops to their weedy relatives to avoid environmental concern and genetic pollution to other crops.
7. Due to natural containment, plastome engineering found to be successful strategy to develop herbicide-resistant genes plants.
8. The availability of transgenic technologies for chloroplasts has facilitated the functional characterization of plastid genome-encoded genes and open reading frames using reverse genetics approaches.
9. In chloroplast transformation, the desired expression levels can be adjusted by choosing appropriate combinations of plastid expression signals (e.g. promoters, Shine–Dalgarno sequences, 3' untranslated regions), but this is often a trial and- error process and requires multiple rounds of construct optimization.
10. Advancement in genomic tools led to the availability of completely sequenced chloroplast genomes; provide a valuable source of phylogenetic data for resolving relationships among angiosperms.
11. Exploring the advantages of plastomics for the expression of genes coding for insecticidal proteins or allowing for herbicide resistance. Crops expressing the *Bacillus thuringiensis* (Bt) toxin from the nucleus may only produce suboptimal amounts of toxins giving rise to an enhanced risk of pests developing Bt resistance.
12. It is hoped that plastid-mediated molecular farming will lead to the bio-fabrication of a range of biopolymers and pharmaceutical proteins.

Challenges of plastid genome engineering:

Despite such significant progress, this technology has not been extended to major crops. However, highly efficient soybean, carrot and cotton plastid transformation has recently been accomplished through somatic embryogenesis using species-specific chloroplast vectors. Major obstacles to extend this technology to crop plants that regenerate through somatic embryogenesis include inadequate tissue culture and regeneration protocols, lack of selectable markers and inability to express transgenes in non-green plastids.

1. First challenge is to introduce foreign DNA into non green tissues, containing several kinds of plastids, namely proplastids, leucoplasts, amyloplasts, etioplasts and chromoplasts, in which gene expression and gene regulation systems are quite different from mature green chloroplasts.

2. Another barrier to developing plastid transformation for crop plants, including cereals has been their regeneration from non-green embryonic cells (containing proplastids) rather than leaf cells (containing chloroplasts).
3. The ability to regenerate chloroplast transgenic plants through somatic embryogenesis and achieve homoplasmy, which lacks the benefit of subsequent rounds of regeneration offered by organogenesis, because segments of somatic embryos cannot be regenerated into plants.
4. Identification of appropriate regulatory sequences, which function in non-green plastids, is necessary to achieve foreign gene expression.
5. Find and standardize appropriate method for delivering foreign DNA through the double plastid membrane. Development of new methods of gene delivery systems into chloroplasts should be developed to overcome this problem.
6. Lack of information on chlorplast genome sequences for several important crop species to locates intergenic sequences which are essential for integration of transgenes through homologous recombination.
7. The need to identify plastid expression signals (e.g. promoters, 50 and 30 untranslated regions; suitable to direct efficient transgene expression in non-green tissues and organs therefore represents a vital task.
8. Need a much better understanding of plastid gene expression and its control in non-green plastid types as found in most fruits, tubers and seeds.

Conclusion:

Transformation of the plastid genome has a number of inherent advantages for the engineering of gene expression in plants. These advantages include: 10–50 times higher transgene expression levels; the absence of gene silencing and position effect variation; the ability to express polycistronic messages from a single promoter; uniparental plastid gene inheritance in most crop plants that prevents pollen transmission of foreign DNA; integration via a homologous recombination process that facilitates targeted gene replacement and precise transgene control; and sequestration of foreign proteins in the organelle which prevents adverse interactions with the cytoplasmic environment.

Plastid transformation holds great potential for the introduction of important agronomic traits to plants, as well as the production of biomaterials and therapeutic proteins such as antibodies, biopharmaceuticals and vaccine antigens. Higher level of transgene expression in transgenic and ability to properly fold human blood proteins with proper disulfide bridges; and prokaryotic nature to express

native bacterial genes are attractive features for the generation of large quantity but low-cost therapeutic proteins in chloroplasts. Recent success in engineering the chloroplast genome for resistance to diseases, herbicides and insects and for production of biopharmaceuticals has opened the door to a new era in biotechnology. The availability of sequencing and high-throughput gene expression data have promoted comparative analysis with genome wide studies of the duplication, loss and transfer events in chloroplast genomes. This could be considered a major step forward towards exploiting the usefulness of chloroplast genetic engineering. Using the genomic information will increase our understanding of plastid biochemistry and molecular biology and need to identify plastid expression signals (e.g. promoters, 5' and 3' untranslated regions) suitable to direct efficient transgene expression in non-green tissues and organs.

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