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REVIEW

Chloroplast Genomics and Genetic Engineering for Crop Improvement

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Abstract Chloroplast genome sequence information is crucial for understanding the evolutionary relationship among photosynthetic organisms and in chloroplast (plastid) genetic engineering for agricultural biotechnology applications. Plastid transformation technology in crop plants offers numerous advantages over nuclear transformation, including high transgene expression, multiple transgene stacking through operon transfer to plastid genome, lack of epigenetic gene silencing and transgene containment due to maternal inheritance of plastids. More importantly, this technology permits expression of native bacterial genes at much higher level than the levels achievable in nucleus. However, only a handful of crops are amenable to routine plastid transformation due to technical difficulties. The plastid transformation in plants necessitates development of species-specific transgene delivery vector, which ideally should consist of homologous recombination sequences and endogenous plastid regulatory elements for efficient transgene integration and stable protein expression. However, inadequate plastid genome sequence information in majority of agriculturally important species has limited the development of transplastomic crops with desired traits. The recent advancement in high-throughput genome sequencing has resulted in the availability of complete plastid genome sequences in more than 230 photosynthetic organisms, including more than 130 higher plants. The availability of genome sequence data of more crop plants will offer an opportunity to construct species-specific plastid vectors, thus provide a newer platform for efficient plastid genetic engineering with a variety of agronomic applications, including high insect and pathogen resistance, herbicide resistance, tolerance to drought, salt and cold stresses, cytoplasmic male sterility, metabolic pathway engineering, production of antigens, biopharmaceuticals and bio-fuels. However, the major challenges ahead are to develop and implement this novel toolkit efficiently in most major crops for desirable agronomic applications.

Keywords Chloroplast genome sequences · Phylogenomics · Plastid transformation · Plastid genetic engineering

Introduction

Chloroplasts are plant-specific cellular organelles with autonomous genome and play key role in many essential metabolic processes such as photosynthesis, amino acid and fatty acid biosynthesis and production of several secondary metabolites. Chloroplasts, also referred to as plastids, evolved from endosymbiosis of ancestral cyanobaterium in an eukaryotic cell, and resulted in considerable amount of gene exchange between nucleus and cholorplasts [36, 37, 79, 80]. Thus, chloroplast has been the subject of research for phylogenetics of land plants and other photosynthetic organisms for years. Besides, chloroplasts are also conceptualized as specialized 'tool box' for genetic engineering of variety of agronomic traits in higher plants [24]. A number of beneficial features are associated with plastid transformation such as targeted transgene integration, enhanced transgene expression, reduction in epigenetic inactivation of transgene and gene containment due to maternal inheritance [6, 12, 70, 73]. The above advantages have led to popularization of the concept of transplastomic crops as an

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alternative approach to nucleus-derived transgenic crops to address the concerns of 'gene pollution' and to express unmodified bacterial genes at unprecedented high levels. However, there are technical challenges that need to be addressed before we embark upon deploying this technology for crop improvement.

Chloroplast genomes of higher plants are circular, small size (up to 200 kb) with bipartite double stranded DNA. Chloroplast genome is present in numerous copies producing an amplification of approximately 10,000 copies per cell. Chloroplast genome is less prone to recombination and retained most of the ancestral genes, that is why chloroplast genomes serves as an excellent tool for phylogenetic and evolutionary studies [34, 39, 79]. In a quest for understanding the whole genome of several photosynthetic organisms, chloroplast genome sequencing was the focus of research for chloroplast phylogenomics. The efforts gained momentum in the recent years with the availability of high-throughput genome sequencing technologies [34]. Although the gene content and order are largely conserved within a particular group of photosynthetic organism, the genome studies have revealed significant amount of genomic changes in chloroplasts, including gene loss, and inversions, which might serve as important phylogenetic markers [34]. In applied research, the genome sequences of several higher plants and crop plants serve as the basic platform for successful plastid transformation. The targeted integration of transgene in plastid genome through homologous recombination and specific endogenous regulatory sequences needed for stable transgene expression highly depends on the availability of chloroplast genome sequence information of the host plant species [12, 106]. Thus, chloroplast genomics hold enormous significance in further progress of the plastid transformation system in major crops.

Plastid transformation in higher plants has been established in the recent past to engineer several agronomic traits including herbicide resistance, insect and pathogen resistance, abiotic stress tolerance, increased photosynthesis and also production of edible crops engineered to produce 'biopharmaceuticals' [21, 52, 71, 106, 108]. However, till date these engineered traits have been feasible and restricted to only tobacco or few other solanaceous crops. Nonetheless, it is encouraging that these research efforts led to the development of this technology in several crops plants, like potato [75, 93, 104], tomato [2, 83], brinjal [95], rice [50, 58], wheat [16], oilseed rape [10, 41, 96], soybean, [30, 116] and in lettuce [45, 60, 85]. The progress reported thus far has helped raise hopes for generating transplastomic crops in the near future with engineered agronomic traits. Numerous laboratories are engaged worldwide in plastid genomics and genetic engineering; we review here the progress made during the last two decades, i.e. since the time the first report on stable plastid transformation by [100]. We also discuss the major obstacles associated with implementing this technology in crop plants and the challenges ahead with reference to engineering valuable agronomic traits.

Plastid Genomics

Chloroplasts are specialized organelles of plants cells and few eukaryotic algae, which possess their own genome or plastome, besides nuclear genome [98]. The presence of genetic material in plastids of land plants was reported way back by Sager and Ishida in 1963. In 1980s, the genome sequencing of plastids and transformation techniques has gained momentum, especially in tobacco, and became a part of the then ongoing functional genomics program [99]. Since then, a plethora of information on genome organization of plastid, gene expression and phylogeny have been generated till date. These loads of information became necessary as a prerequisite to achieve genetic engineering of plastid in higher plants. The genome information of plastid is absolutely required for successful plastid transformation. It relies on efficient homologous recombination events for integration of transgenes in the plastid genome using the flanking sequences of the intergenic spacer regions. Moreover, the information on endogenous regulatory system and genes is also essential for desirable expression of foreign genes in plastids [12]. Thus, a detail understanding on the plastid genome structure and its gene expression governed by the regulatory genes present within is of prime importance in plastid genetic engineering.

The size of the plastid genome in land plant and photosynthetic organisms generally varies from 120 to 217 kb [37, 43]. The genome of plastid in most of the land plants is conserved. It consist of a double stranded, single, circular chromosome with two inverted repeats (IR_A and IR_B) separating the large single copy and small single copy regions. These IRs are populated with rRNA genes (16S, 23S and 5S) and some other genes. The difference in the plastid genome size in different plants is mainly due to the number of genes in the IRs that are duplicated. The plastid genome consists of approximately 120 genes, which are basic set of genes related to organelle gene expression and reproduction. The genes present in the plastid genome are of three broad categories [79, 98], which comprise of genes (i) for photosynthesis (photo-system I and II –*psaA*, *psaB*, *psbA*, *psbB*, *cytb6f*, ATP synthases, *rbcL* and NAD(P)H genes etc.), (ii) regulatory genes for gene expression (tRNA genes-*trna H*, *trnK*; rRNA genes—*rrn16*, *rrn5*; RNA polymerase *rpoA*, *rpoB*; ribosomal subunit genes-*rps2*, *rps3*, *rpl2*, *rpl16* etc., and (iii) conserved ORFs such as *ycfs* and protein coding genes like *matK*. The copy number of plastid genome per

plant cell is very high; each chloroplast consists of 50–100 copies of plastid genome and each cell consists of more than hundreds of chloroplasts making the copy number of ~10,000 per cell [5]. This feature of high copy number of genomes is exploited in genetic engineering of plastids for over expression of transgenes, thereby allowing the recombinant proteins to accumulate at high concentrations of over 10% of the total soluble proteins [22].

Chloroplast Genome Sequences

The advancement of sequencing technology including the next generation sequencing (NGS) has facilitated rapid sequencing of plastid genome of thousands of plants from various groups [34]. Till date approximately 230 plastids have been sequenced (NCBI organelle genomes, http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2759&type=4&name=Eukaryotae%20Organells. This includes species mostly from flowering plants, and others like bryophytes, lycophytes, gymnosperms, green and red algae, photosynthetic *dinoflagellate chromalveolates*, and other photosynthetic organisms. The plastid genome sequences of approximately 130 higher plants and over 30 different crop plants are available (Table 1). The availability of large number of plastid genomic sequences and the huge genomic information have facilitated understanding the extensive genomic changes in plastid genome due to symbiotic evolution of this organelle. The whole genomic features and sequence information are also being exploited to resolve the long standing phylogenetic quest of 'tree of life' [34, 37]. The first two complete genome sequences of plastid that was made publicly available were from tobacco and liverwort [76, 92], which were basically sequenced through Sanger's di-deoxy method of sequencing. Since then there was an exponential increase in genome sequences during the last 5 years [34]. Approximately 67 plastid genome sequences were made available during 2010–2011 alone most of which were sequenced through high-throughput NGS platforms like 454-Roche or Solexa-Illumina platforms. Some of the recent plastid genomes of *Pinus* spp. were sequenced through massively parallel sequencing (MPS) system like Solexa-Illumina Genome Analyzer system [78, 109]. The latest plastid genome sequence available was from *Cucumis melo* (melon), which was made publically available on 13th September, 2011 and was sequenced using whole-genome shotgun assembly and bacterial artificial chromosome—end sequencing technology [81]. The massive amount of information on plastid genome generated in the recent years from different plant families and green algae would fancy the chances of understanding phylogenetic relationships at very low taxonomic levels and also exploit in developing more efficient transplastomic technology for crop improvement.

Plastid Genomics to Resolve Phylogeny and Evolution

The 'endosymbiosis' event between cyanobacterium and eukaryotic cell about few billion years ago led to the evolution of the present day land plant and changed the world's food chain since then. During the process of evolution from an endosymbiont to cellular organelle most of the cyanobacterium genes were either lost or transferred to the host nucleus [103]. The process of gene transfer from the endosymbiont and the host cell nucleus is the central theme of organelle genome studies and resolving the 'tree of life' [56, 80]. Unavailability of sufficient genome sequences in the past has affected the understanding of reconstructing the detail phylogeny and evolution of organisms. However, in the recent past the explosion of genome sequences through high-throughput genomic technologies has made it possible to conduct phylogenic studies of photosynthetic organism in an efficient manner and elucidate evolutionary history of organisms.

Analysis of the plastid whole genome sequences of several plant clades and photosynthetic organisms and the genomic features revealed extensive restructuring of the genome between them, although the structure of land plant plastid is generally conserved. Few inversions of genomic regions in the vascular plants were used as potential phylogenetic markers. The plastid genome sequences enabled comparative genome studies and revealing the facts of genome shuffling, genome reduction and gene function loss (pseudogene, e.g. *trnRCCG*) throughout the plant evolutionary process [34]. The plastid genome sequences also enable utilization of more genomic features and gene information to reduce error rates in phylogenetic reconstruction of organisms. The plastid genome size, nucleotide composition, gene content and order, intron loss or gain and codon usage pattern are few genomic features used as tool to resolve phylogenetic relationships even at the deep-level in angiosperms [34]. Thus, the availability of large scale complete genome sequences of plastids has increased the efficiency of resolving phylogenetic studies and evolution of land plants, and is expected to improve further with more number of genome sequences in near future.

Plastid Genomics for Efficient Plastid Transformation

The sequence information of higher plant plastid is very crucial for developing efficient plastid transformation technology in crop plants. Unavailability of adequate genomic information in the past has limited and delayed establishment of this technology in agricultural crops [86]. Efficient plastid transformation vector necessitates specific flanking DNA sequences for successful homologous recombination event and endogenous regulatory sequences

Table 1 Chloroplast genome sequences of few model plant systems and crop plants available at NCBI (till Oct' 2011)

Sl. No.	Scientific name	Common name	Plant family	Accession No.	Chloroplast genome size (in bp)	Year of genome sequence
1.	*Nicotiana tabacum* L.	Tobacco	Solanaceae	NC_001879	155,943	1986
2.	*Oryza sativa* subsp. japonica	Japanese rice	Poaceae	NC_001320	134,525	1989
3.	*Zea mays* L.	Maize	Poaceae	NC_001666	140,384	1995
4.	*Arabidopsis thaliana* (L.)	Thale-cress	Brassicaceae	NC_000932	154,478	1999
5.	*Spinacia oleracea* L.	Spinach	Amaranthaceae	NC_002202	150725	2000
6.	*Triticum aestivum* L.	Wheat	Poaceae	NC_002762	134,545	2000
7.	*Medicago truncatula* Gaertn.	Barrel Clover	Fabaceae	NC_003119	124,033	2001
8.	*Saccharum officinarum* L.	Sugarcane	Poaceae	NC_006084	141,182	2004
9.	*Cucumis sativus* L.	Cucumber	Cucurbitaceae	NC_007144	155,293	2005
10.	*Lactuca sativa* L.	Lettuce	Asteraceae	NC_007578	152,765	2005
11.	*Solanum lycopersicum* L.	Tomato	Solanaceae	NC_007898	155,461	2006
12.	*Solanum tuberosum* L.	Potato	Solanaceae	NC_008096	155,296	2006
13.	*Glycine max* (L.) Merr.	Soybean	Fabaceae	NC_007942	152,218	2006
14.	*Daucus carota* L.	Carrot	Scandiceae	NC_008325	155,911	2006
15.	*Gossypium barbadense* L.	Egyptian cotton	Malvaceae	NC_008641	160,317	2006
16.	*Gossypium hirsutum* L.	Cotton	Malvaceae	NC_007944	160,301	2006
17.	*Helianthus annuus* L.	Sunflower	Asteraceae	NC_007977	151,104	2006
18.	*Hordeum vulgare* subsp. vulgare	Barley	Pooideae	NC_008590	136,462	2006
19.	*Citrus sinensis* (L.) Osbeck	Sweet orange	Rutaceae	NC_008334	160,129	2006
20.	*Coffea arabica* L.	Coffee	Rubiaceae	NC_008535	155,189	2006
21.	*Oryza sativa* subsp. indica	Indian Rice	Poaceae	NC_008155	134,496	2006
22.	*Sorghum bicolor* (L.) Moench	Sorghum	Poaceae	NC_008602	140,754	2006
23.	*Vitis vinifera* L.	Grape	Vitaceae	NC_007957	160,928	2006
24.	*Lolium perenne* L.	Ryegrass	Pooideae	NC_009950	135,282	2007
25.	*Manihot esculenta* Crantz	Cassava	Euphorbiaceae	NC_010433	161,453	2008
26.	*Brachypodium distachyon* (L.) Beauv	Brachipodium	Pooideae	NC_011032	135,199	2008
27.	*Carica papaya* L.	Papaya	Caricaceae	NC_010323	160,100	2008
28.	*Cicer arietinum* L.	Chickpea	Fabaceae	NC_011163	125,319	2008
29.	*Lathyrus sativus* L.	Grass pea	Fabaceae	NC_014063	121,020	2010
30.	*Pisum sativum* L.	Garden pea	Fabaceae	NC_014057	122,169	2010
31.	*Vigna radiata* (L.) R.Wilczek	Mungbean	Fabaceae	NC_013843	151,271	2010
32.	*Cucumis melo* subsp. melo	Melon	Cucurbitaceae	NC_015983	156,017	2011
33.	*Brassica rapa* var. glabra	Chinese cabbage	Brassicaceae	NC_015139	153,482	2011

for desired transgene expression [106]. The foreign gene to be delivered into plastid genome is flanked by left and right nucleotide sequences from the host plastid genome which determines the site of transgene insertion through homologous recombination [108]. Approximately 16 sites of plastid genome were used for specifically targeting of transgene insertion in plastid genome [70]. Recently, the transcriptionally active spacer region of *trnI/trnA* genes situated between ribosomal operon of plastid genome was found as most effective site for transgene integration through plastid transformation than the previously used *rps12/trnV* and *trnM/trnG* sites. The advantages of *trnI/trnA* site is that it (i) increases the copy number of the transgene due to its location in the inverted repeat region; and (ii) accurately processes the transgene due to its copy correction mechanism and presence of replication origin and intron sequence [12]. Utilization of heterologous intergenic spacer sequence information in the past has resulted into reduced efficiency of successful plastid transformation due to very low nucleotide sequence conservation even among the plants from related family [88]. Thus, the genome sequence information of plastid of different crops plants is absolutely necessary for utilizing species specific flanking sequences in the transformation

vector for allowing effective homologous recombination events during plastid transformation process.

Besides, the information on the endogenous regulatory sequences in the plastid genome of different plant species is also important in regulating transgene expression [86]. The level of transgene expression in plastid is highly regulated by promoter and 5′- and 3′-UTR elements including ribosomal binding sequence [31]. The N-terminal UTR elements are required to stabilize the transgene expression [119]. Most popular promoter for plastid transformation vector is strong plastid rRNA operon promoter (*Prrn*) while most widely used 5′ and 3′-UTR regions are from *psbA/TpsbA* [97, 106, 107]. Other promoter and regulatory sequences used are the eubacterial-type (PEP) and phage-type (NEP) RNA polymerases and *rbcL* 5′-UTR, respectively, which are highly active in non green plastids [104]. Although, heterologous regulatory sequence (tobacco *psbA* 5′-UTR) is utilized till date for transgene expression in plastid, in most of the cases the transgene accumulation failed to express to the satisfactory level. Few other regulatory sequences used in plastid transformation to regulate transgene expression are 5′-UTR of viral T7 system, *rbcL*, *rpl22*, *psbB*, *psbC* and *atpB* genes [52]. Ruhlman et al. [86] have compared endogenous and heterologous regulatory elements of several crop species in lettuce and tobacco transplastomic lines to demonstrate the utility of species specific sequences in plastid transformation vector, thus emphasizing the need for complete genome sequences of plastid of the recipient crop plants for basic and applied research.

Genesis of Transplastomic Crops

For plastid genetics and molecular biology, the eukaryotic green algae *Chlamydomonas reinhardtii*, served as the model organism. Integrating a foreign gene stably into plastids of *C. reinhardtii* by Boynton and his group [8] led to the genesis of plastid transformation, and soon the avenues opened up with the results of first stable plastid transformation in higher plants such as tobacco with chimeric *aad*A gene [24, 69, 71, 100, 108]. The technique of using selectable marker *aadA* [35] and marker removal techniques [32] were also developed in *C. reinhardtii* for the first time. Since then the plastid transformation was extended to other higher plants such as *Arabidopsis* [94] and poplar [77]. Till date more than 100 transgenes have been transformed stably in plastids of higher plants [13]. The increasing debate on potential environmental risks associated with genetically modified (GM) crops developed through nuclear transformation, has led to perception of the transplastomic technology as a safe alternative [3, 89]. Since chloroplasts are maternally inherited, the GM crops with transgene integrated into plastid genome would potentially negate the chances of pollen escape into environment thus effecting a biological containment [12, 20, 84, 101]. As a result, development of plastid transformation in crop plants was emphasized in the past few years to address the GM debate issues. Although at present plastid transformation technique is established in a number of crop plants, such as rice [50, 58], tomato [83], potato [75, 93, 104], oilseed rape [10, 41], *Lesquerella* [96], lettuce [60], soybean [30], carrot [54], cotton [55], cabbage [63], cauliflower [74], sugarbeet [27], brinjal [95], no transplastomic crops are commercially available till date. A major challenge ahead is to implement this technology in crops with agronomic applications, especially in monocots to enhance food security of the burgeoning world population.

Plastid Genetic Engineering

Plastid Transformation Process

Chloroplast or plastids in plant cells are usually enveloped by inner and outer membranes [36], thus for plastid transformation the transgene needs to pass through these membranes in addition to the cell wall and cell membrane [24]. Plastid transformation involves several crucial steps. Unlike nuclear transformation process where *Agrobacterium* is most efficient transgene lodging tool, the delivery method in plastids of higher plants is possible only through biolistic bombardment particles [73]; although other methods like polyethylene glycol (PEG), *Agrobacterium* and microinjections were also reported for plastid transformation [52]. The transgene integration in plastids is target-specific due to homologous recombination, which also differs in respect of random insertion through the classical *Agrobacterium*-mediated nuclear transformation process. The targeted gene delivery and the prokaryotic nature of chloroplast genome necessitates specialized designing of plastid transformation vector to include both homologous sequences flanking the transgene and endogenous regulatory sequences for stable transgene expression [73]. Thus, the plastid transformation technology in higher plants or crops includes following important stages: (a) construction of species-specific plastid targeting vector(s), which is usually an *E. coli* vector, consisting of gene of interest (GOI), a single strong promoter and terminator for single or multiple GOI, flanking homologous recombination sequences, selectable marker (streptomycin–spectinomycin and kanamycin) or reporter gene (GUS or GFP) and regulatory 5′ and/or 3′ UTR regions; (b) standardizing delivery procedures (biolistic or PEG) for introducing the transgene into plant cells and integration into chloroplast genome by two homologous recombination

events; (c) stringent selection of heteroplastomic cell lines on appropriate selection medium gradually to obtain homoplastomic cultures after successive cell divisions; and (d) regeneration of fertile transplastomic plants capable of inheriting offspring with engineered plastome for successive generations [6, 24, 40, 52, 70, 106]. In higher plants, the most successful approach of regenerating transplastomic plants was achieved through organogenesis from leaf tissues although few reports are also available to utilize somatic embryogenesis for the regeneration of chloroplast transgenic in rice [12]. Despite the reasonable progress made in plastid transformation in crop plants, there is a need to address few specific challenges, such as requirement of species-specific plastid vector, efficient marker removal techniques, embryogenic regeneration system for monocot crops, rapid and an efficient method of achieving homoplasmy, and regulated expression of transgene as only when and where desired, so that the plastidial genetic engineering is efficiently utilized for crop improvement. Lossl et al. [66] have demonstrated the efficacy of ethanol inducible transgene expression system in tobacco plastids thereby opening the possibility of regulating transgene in GM crops with greater precision and security.

Benefits of Plastid Transformation

Several beneficial features associated with plastid transformation techniques have generated interests among researchers to choose it as an alternative tool over nuclear transformation. Some of these promising advantages are: (i) high tissue specific transgene expression and foreign protein accumulation (5–25% of total soluble protein) due to polyploid nature of the plastid genome and high stability of transgene; (ii) highly regulated transgene expression helped by well defined promoter set and expression cassettes available from studies with model plant tobacco; (iii) possibility of simultaneous introduction of multiple traits ('transgene stacking') facilitated by inherent polycistronic translation mechanism of plastid genetic system and co-transformation [53]; (iv) unwanted position effects due to absence of high order chromatin structure in plastid DNA and transgene integration by homologous recombination process; the latter is also important for generating only one type of transplastome; (v) absence of DNA methylation and epigenetic gene silencing or co-suppression in plastid genes; (vi) recent availability of selectable marker recycling or elimination techniques to address GM risk issues; (vii) 'transgene containment' possible due to strict maternal transmission of plastid genes in most crop plants resulting into less ecological risk [7, 73, 108]. Although, few reports citing of evidences of plastid gene transfer to nucleus and possibility of pollen transmission to wild plants [23, 42, 102] are available, the level of transgene containment offered by plastid is always higher than nuclear transgene. Thus, plastid transformation in crop plants offers a satisfactory platform for expressing transgenes of various agronomic traits.

Applications of Plastid Transformation Towards Engineering Agricultural Traits

The beneficial features of plastid transformation in crop plants not only promise hyper expression of foreign protein but also offer an opportunity to tinker with several agronomic traits without triggering much of the environmental risks. Although, no commercial crops have been developed till date through transplastomic approach, the researchable agronomic traits have been established in model plant system tobacco and few crop plants. The present section deals with the status of major agricultural applications, the pitfalls and the perspectives for utilizing this technology in addressing future crop biotechnology challenges.

Herbicide Detoxification

Genetic engineering of crop plants for imparting herbicide resistance, especially for broad spectrum Glyphosate, through overexpression of mutant 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS) gene is one of the important transgenic traits. However, the risk of gene escape from transformed nuclear genome through pollen dispersal and creation of 'super weed' had led to an alternative approach of expressing modified or split EPSPS in plastid genome for gene containment [11, 18, 19, 114]. Similarly, the *bar* gene expression has been demonstrated in tobacco plastids to confer efficient resistance against herbicide phosphinothricin (PPT) [67]. Other herbicide resistance genes expressed in model plant tobacco include *crtI* gene encoding phytoene desaturase from *Erwinina carotova*, *bxn* gene encoding bromoxynil specific nitrilase from *Klebsiella pneumoniae* exhibiting tolerance to norflorazon and bromoxynil, protoporphrinogen IX oxidase tolerant to many bleaching type herbicides [40], recombinant 4-hydroxyphenylpyruvate dioxygenase gene for isoxaflutole [29], and mutated acetolactate synthase gene for sulfonylurea herbicides [91].

Biotic Stress Resistance

Insect Pest Resistance

Transgenic approach of controlling insect pest is one of important strategies, which involve expression of crystal protein genes (*Cry*) from bacteria *Bacillus thuringiensis*. *Cry1Ab* and *Cry1Ac* are most utilized against target pests

of corn and cotton, respectively at the commercial level. However, the phenomenon of 'codon biasness' of these prokaryotic genes (AT rich) in the eukaryotic nucleus (GC rich) drastically reduces expression level providing a chance of developing resistance against this toxin. Even the ecological risk posed by the escape of transgene through pollen [65] needs a consideration for indiscriminate utilization of *Cry* genes under nuclear background. Alternatively, expressing native Bt *Cry1Ac* gene in tobacco chloroplasts under 16S rrn promoter with chimeric ribosome binding site of *rbcL* and 3′ UTR of *rps*16 gene exhibited high (3–5% TSP) accumulation of Bt toxins in leaves [72]. Overexpression of *Cry2Aa2*, a smaller size Bt protein gene, in tobacco chloroplasts has resulted into very high level of toxin protein accumulation with relatively less possibility of developing resistance against Bt. Leaves from transplastomic tobacco plants proved to be 100% lethal against tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*) without developing resistance unlike *Cry1A* genes [51]. Subsequently, high CRY protein expression (>10% TSP) in plastids was obtained in tobacco using *cry9Aa2* gene [9] and in cabbage using *cry1Ab* gene [64]. In an interesting study in tobacco, polycistronic nature of chloroplast genome was utilized for 'transgene staking' of *Bt Cry2Aa2* operon, which comprises 3 operon systems. *Cry2Aa2* gene is the distal toxin gene with 2 *orfs*; the *orf* immediately upstream to *cry2Aa2* gene codes for a chaperonin involved in folding of Bt crystal proteins and preventing it from proteolytic degradation. Driven by this *Cry2Aa2* operon, toxin proteins accumulated to an unprecedented high level (45.3% TSP) in the leaves and persisted stably even for the later stages of leaf development (during senescence). Leaves showed 100% mortality against tobacco budworm, cotton bollworm and beet armyworm [25]. In a recent study by Jin et al. [44], an elevated expression of β-glucosidase (*Bgl-1*) in the tobacco plastids has ensured protection against aphids and whiteflies due to increase in sucrose ester levels, besides an increase in biomass and trichome density.

Disease Resistance

Plastid genetic engineering also promises combat against phytopathogenic microbes. Hyper expression of a synthetic microbial lytic peptide (MSI-99) in tobacco chloroplasts have resulted into high level of peptide expression (21.5% TSP) and resistance against *Pseudomonas syringae*, and spores of fungal species *Aspergillus* and *Fusarium* [26]. MSI-99 is an antimicrobial peptide (AMP) with an amphipathic α helix that binds to outer membrane phospholipids of bacteria and fungi. In consequence, these peptides aggregate to form pores and results into bacterial lysis. Since AMPs function at high dose, chloroplast expression of these peptides was conceptualized [21]. In a separate study, *argK* gene of *P. syringae* pv. *Phaseolicola*, coding for toxin-resistant enzyme ROCT, was introduced into tobacco chloroplasts using a plastid transit peptide (pea *rbcS*) and *Agrobacterium* transformation. The transgenic plants exhibited enhanced level of salicylic acid and resistance to fungal and viral pathogens. Similarly, biolistic introduction of other salicylic acid producing genes, such as *entC* and *pmsB* has exhibited enhanced accumulation of salicylic acid in plastids and resistance to pathogenic fungi *Oidium lycopersicon* [105]. In a recent study, overexpression of AMPs Retrocyclin-101 (RC101) and Protegrin-1 (PG1) in tobacco plastids enhanced protein production and plants exhibited resistant to tobacco mosaic virus infections [59]. The above results although were not sufficient to harness plastid genetic engineering for resistance to pathogen in crop plants, nonetheless provide a possibility to establish the technology in higher plants.

Abiotic Stress Tolerance

Dehydration is a major abiotic stress affecting most of the crop plants globally due to drought, salinity and freezing. Biotechnology of plastids has been demonstrated in higher plants for tolerance to drought; salt and temperature stresses [17, 108]. Engineering plants for drought tolerance is achieved by expressing yeast trehalose phosphate synthase (*TPS1*) gene in both nucleus and plastids of tobacco. Transplastomic plants produced higher level (25 fold) of trehalose accumulation and high degree of drought tolerance (in 6% PEG) but without any pleiotropic effects when compared to nuclear transgenic plants. Plastid expressed plants with *TPS1* gene also showed phenomenal response in surviving dehydration for 24 days and then rehydration as compared to control plants [57]. In an attempt to engineer plastids for imparting salt tolerance in plants, choline monooxygenase (CMO) from sugar beet and betaine aldehyde dehydrogenase (BADH) from spinach were independently introduced in tobacco plastids. Although, both the enzymes were produced in the plastids, the accumulation of betaine in the plastids was found to be very low in the tobacco plastids expressing CMO due to absence of BADH activity. However, the transplastomic plants exhibited enhanced tolerance to the toxic levels of choline and salt/drought stress compared to wild type plants. The transplastomic tobacco plants also exhibited higher photosynthesis in the presence of salt stress (150 mM NaCl) suggesting the feasibility of improving higher plants against drought and salt stress through plastid genetic engineering [117]. The transplastomic carrot cells expressing high level of BADH exhibited enhanced salt tolerance even in the presence of very high salt stress

(400 mM NaCl) [54]. Engineering fatty acid desaturase gene in transplastomic tobacco plants has indicated a possibility of imparting cold tolerance by manipulating lipid content in vegetative and reproductive tissues [15]. The *codA* gene from *Arthrobacter globiformis* coding for choline oxidase when targeted to chloroplasts of rice plants, they maintained higher photosystem II activity and they showed better physiological performance under water-stress; such as enhanced detoxification of reactive oxygen species compared to wild type plants [48]. Transplastomic expression of *E. coli* enzyme l-aspartate-alpha-decarboxylase encoded by the *panD* gene in tobacco exhibited tolerance to high temperature stress [33]. A recent study demonstrated genetic manipulation of antioxidant enzymes in plastids of tobacco through introduction of three enzymes dehydroascorbate reductase, glutathione-*S*-transferase and glutathione reductase. The homoplasmic tobacco plants were found to exhibit tolerance against oxidative stress, salt, cold and heavy metal when treated with methyl viologen, the environmental stress mimicking agent [61]. All the above studies provide a foundation for research on improving stress tolerance traits to provide effective plant protection in field crops through plastid genetic modifications.

Quality Improvement

Chloroplast genome engineering has also been attempted to engineer nutritionally important metabolic pathways, especially for enhancement of essential amino acid biosynthesis, vitamin content and fatty acid quality in seeds [82]. Overexpression of β-subunit of the two units (α and β) of anthranilate synthase in tobacco plastids exhibited tenfold increase of free tryptophan in the leaves. Although the α subunit expression was high, the functional enzyme activity increased only up to fourfold as both the units need to be expressed consistently for tryptophan biosynthesis [115]. To improve fatty acid oil quality in seeds by increasing lipid quantity, overexpression of *accD* gene encoding acetyl-CoA carboxylase was achieved by a strong rRNA promoter in the tobacco plastids. The plants produced enhanced fatty acid content in leaves resulting into reduced leaf senescence and increased seed production [68]. Overexpression of Δ9-desatuarase gene from *Solanum commersonii* and from *Anacystis nidulans* in tobacco plastids by Craig et al. [15] showed and increased unsaturation of fatty acid in leaves and seeds. Plastid expression of pro-vitamin A in tomato at very high level [2] and astaxanthin, a pigment of human health interest in tobacco plants [38] has raised the hope of metabolic engineering of nutraceuticals through transplastomic plants. Besides, genetic engineering of early steps of fatty acid biosynthesis, plastid transformation can also be harnessed for producing unusual fatty acids such as very long chain polyunsaturated fatty acids, which are usually found in cold water fishes and have potential health benefits [82].

Plastid Genetic Engineering for Improved Photosynthesis

Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) is the central enzyme in chloroplasts, which assimilates atmospheric CO_2 into food through a process known as photosynthesis. Rubisco consists of eight large subunits (LSU or rbcL) and equal number of small units (SSU or rbcS). The rbcS is coded by nuclear genome while the catalytic unit rbcL is coded by the plastid genome. The advent of plastid transformation technology and its recent developments in plastid genomics has motivated genetic manipulation of Rubisco in higher plants, in terms of deletion, mutation and replacement of *rbcL* gene towards improving photosynthetic efficiencies and better carbon fixation in crop plants [113]. In an early attempt to excise *rbcL* gene from tobacco plastome produced non-autotrophic plantlets but survived by supplementation with external sucrose. However, fusing *rbcL* gene along with the pea *rbcS* gene transit peptide sequence and nuclear transformation have complemented this deficiency to some extent and allowed slow autotrophic growth [46]. When a histidine tagged *rbcS* gene was put into tobacco plastid along with *psbA* promoter and terminator they produced transcripts and the products were processed and assembled into Rubisco but with less efficiency [110]. The replacement of tobacco *rbcL* gene with homologous *rbcL* gene from sunflower (*Helianthus annus*) or cyanobacterial (*Synechococcus* PCC 6301) formed inefficient non-autotrophic Rubisco hybrids with large subunits from sunflower or cyanobacteria and small subunits from tobacco [47]. The first ever Rubisco manipulation in higher plant tobacco that resulted into complete autotrophic and fertile plant had utilized *rbcM* gene from a photosynthetic bacteria *Rhodospirillum rubrum* that encodes a different form of Rubisco in place of tobacco *rbcL* gene. The only limitation in such case was to provide carbon dioxide externally [111]. Dhingra et al. [28] used *rbsS* cDNA into transcriptionally active spacer region of chloroplast genome of nuclear rbcS antisense tobacco plant, along with two different 5′-UTRs. The transgenic tobacco plants showed successful expression of Rubisco units and assembly of LSU and SSU to form Rubisco holoenzyme with normal functions of plant growth and photosynthesis [28]. The results promised engineering foreign Rubisco genes *in planta* without affecting the photosynthesis efficiency. Genetic engineering of the complete Rubisco enzyme (L_8S_8) was limited due to the location of the two units in two different genomes. Several efforts were made to express Rubisco

subunits in transplastome and replace higher plant Rubisco units with Rubisco from phylogenetically similar *R. rubrum and Methanococcoides burtonii* [1, 111]. The hybrid Rubisco development was also demonstrated by functional assembling of Rubisco-L subunits from sunflower and Rubisco *S*-subunits from tobacco [90]. To carry out further fundamental studies on 'hybrid Rubisco engineering', a master tobacco plant line was generated through plastid transformation. The transplastomic plant lines expressed Rubisco from *Rhodospirillum rubrum*, whose genes lack sequence homology to re-introduced Rubisco gene and an altered genotype to accelerate obtaining homoplasmic lines [112].

Cytoplasmic Male Sterility (CMS) Through Plastid Biotechnology

CMS system is very important in hybrid seed production in several crops. In an effort to engineer metabolic pathway for biologically degradable plastic polyhydroxybutyrate (PHB) through expression of three genes, *phaA*, *phaB*, and *phaC* in tobacco plastids [66], the high level of accumulation of PHB in chloroplasts resulted in male sterility and growth retardation. Investigating the above facts by Ruiz and Daniell [87], it was revealed that the β-ketothiloase enzyme coded by *phbA* gene when expressed in tobacco plastids, resulted into 100% male sterile plants without any other pleiotropic effects [87]. The study encouragingly envisaged plastid biotechnology to impart CMS in transplastomic plants for the first time, which might provide advantage in hybrid seed production. However, more research on inducing cytoplasmic sterility through plastid genome engineering is needed before exploiting it in crop plants for increasing productivity through hybrid development.

Plastid Transformation in Crop Species: An Update and Current Bottlenecks

In higher plants, tobacco is the most preferred model plant for research on plastid transformation, either for overproduction of foreign proteins, to act as bioreactors for biopharmaceutical, or to establish genetic engineering potential for important agronomic traits in higher plants. Till date exploitation of plastid transformation in other crop species is elusive in implementing this technology for crop improvement. The plastid transformation technology was established in some dicot crops and very few monocots (Table 2), while it needs be focused on major cereal crops like rice, wheat, maize, barley and sorghum to feed the burgeoning population [12]. One of the major bottlenecks of successful plastid transformation involves the method of transplastomic plant regeneration; i.e. organogenesis versus somatic embryogenesis. High success of plastid transformation was achieved in dicot crops regenerating through organogenesis from leaves in tissue culture medium. As against this, most of the cereal crops follow somatic embryogenetic pathway for in vitro regeneration, thus limiting plastid transformation in major food crops [12, 106]. Plastid transformation through somatic embryogenesis has been worked out in few crops such as carrot, cotton, soybean and rice using species specific plastid vector. The process of achieving homoplasmy in monocot cereal crops becomes difficult with somatic embryogenesis, which is a major challenge that needs to be addressed [12]. Another major bottleneck of expanding plastid transformation technology in crops plants is the challenge to engineer non-green plastids such as amyloplasts, chromoplasts, elaioplasts, leucoplasts and proplasts found in the fruits, tubers, roots and grains of crop plants. The transgene expression gets reduced drastically due to down regulation of most of the plastid encoded genes in plastids other than chloroplasts [49, 54, 118]. Thus, the above limitations along with the requirement of species specific vector and regulatory sequences for stable protein expression are the major hindrances faced in implementing plastid transformation in crop plants, especially in major food crops. Several attempts have been made to implement plastid transformation technology beyond tobacco, but success was achieved with solanaceous crops like tomato, potato, brinjal and few other crops such as soybean, oil

Table 2 Chloroplast transformation established in major crop plants

Sl. No.	Crop plant	Transgene	References
1.	Potato	*aadA* and *gfp*	[75, 93, 104]
2.	Tomato	*aadA*, lycopene β-cyclase	[2, 83]
3.	Oilseed rape	*aadA*, *cry1Aa10*	[10, 41]
4.	*Lesquerella fendleri*	*aadA* and *gfp*	[96]
5.	Carrot	*dehydrogenase (badh)*	[55]
6.	Cotton	*aphA-6*	[54]
7.	Soybean	*aadA*, *Cry1Ab*	[29, 30]
8.	Lettuce	*aadA, gfp*, anthrax protective antigen (PA), human proinsulin (Pins) fused to cholera toxin B-subunit (CTB)	[45, 60]
9.	Rice	*aadA* and *gfp*, *bar*	[58, 62]
10.	Cauliflower	*gus* and *aadA*	[74]
11.	Cabbage	*aadA, uidA,* cry1Ab	[63]
12.	Brinjal	*aadA*	[95]
13.	Sugar beet	*aadA* and *gfp*	[27]
14.	Wheat	*nptII* and *gfp*	[16]

seed rape, etc. Here, we briefly give the progress made so far in developing chloroplast transformation system in crop plants.

Solanaceous crops

Tomato, potato and brinjal are the major solanaceous crop where plastid transformation has been achieved. Transient expression of GFP as a reporter gene was the earliest plastid transformation in amyloplast of potato tissue slices along with other green and non-green plastid types of tobacco, *Arabidopsis*, red pepper fruits and carrot roots [39]. Plastid transformation in potato was further strengthened subsequently through various studies. Two separate tobacco plastid specific vectors pZS197 carrying *aadA* selectable gene and pMON30125 carrying *aadA* and *gfp* genes driven by *Prrn* and *TpsbA* were used to produce homoplasmic plants but neither resulted in fertile plants [93]. Nguyen et al. [75] and Valkov et al. [104] have subsequently improvised potato plastid transformation to achieve homoplasmic lines and expression of high transgene in non green amyloplasts of tubers using strong *rrn* operon promoter and synthetic *rbcL*-derived 5′-UTR [104]. A significant progress was made in successful plastid transformation and generating stably inherited transplastomic plants in tomato [83]. Plastid transformation in tomato was further improved through vector improvisation and following strict selection process to obtain high frequency (1.5–4%) of transplastomic plants, high transgene expression (>45% of TSP), and viable seeds [83]. Plastid transformation in tomato was advanced further to engineer carotenoid biosynthetic genes lycopene β-cyclase from *Erwinia* and daffodil [2] thus, opening up the possibility of nutritional enhancement in crop plants through plastid biotechnology. Recently, plastid transformation technology was also exploited in brinjal (*Solanum melongena*) [95], which promises to develop transplastomic plants resistant to fruit and shoot borer.

Rice and Other Cereals

Chloroplast transformation has been attempted in non-green plastids of embryogenic cells. A rice plastid specific transformation vector consisting of fusion gene known as FLARE-S (Fluorescent Antibiotic Resistance markers); containing aminoglycoside 3′-adenyl transferase (*aad*A) and green fluorescent (*gfp*) from *Aequorea victoria* enabled visual tracking of transplastomic cells among the chimeric tissues during the second round of selection process. The transplastomic rice plants could not achieve homoplasmy and the resultant rice plants turned sterile [50]. Lee et al. [58] too have achieved stable plastid transformation in rice from mature seed-derived calli and the transformants were able to transmit transgenes to T_1 progeny through viable seeds. However, the transplastomic lines did not achieve homoplasmy despite stringent selection for few generations. Further research on establishing plastid transformation in rice is in progress to achieve homoplasmic herbicide resistant transplastomic lines (unpublished). Cui et al. [16] have recently succeeded to develop a protocol for plastid transformation and regeneration of plantlets from scutella of immature embryo and immature inflorescences of wheat. Out of three transformants one was found to be homoplasmic [16]. Chloroplast transformation has also been attempted in another major cereal crop maize (*Zea mays*), with limited success. The *gfp* and *badh* genes were introduced into maize calli. The GFP expression was found very high at protein level (46% of TSP) in somatic embryos (T_0) of maize but transplastomic plants could not be regenerated (Aseem et al. unpublished). We found maize explants possessed inherent resistance to spectinomycin, hence proving difficult to select the transformed shoots (Rooz and Bansal, unpublished) Although, homoplasmic plants are yet to be achieved in rice and maize, recent plastid transformation efforts have provided leads to resolve the challenges and difficulties of this technology in monocot transformation.

Brassica

Plastids transformation technology was established in few *Brassica* crops besides the model plant *Arabidopsis* [94]. *Lesquerella fendleri*, is a wild oilseed species with desirable seed oil content in which plastid transformation was demonstrated using translational fusion gene *aadA16* and *gfp* [96]. The transplastomic plants produced homoplastomic fertile plants with viable seeds. However, the transformation frequency achieved was very low (one per 25 bombardment) as found with plastid transformation in other crops attempted. Chloroplast transformation was also reported the same year in another important oilseed crop, *Brassica napus* by Hou et al. [41]. A two gene expression cassette, one containing *aadA* gene and another insect resistance gene, *cry1Aa10* was introduced into *Brassica napus* cotyledon petioles through biolistic bombardment [41]. One of the transplastomic lines showed insect resistance against 2nd instar *Plutella xylostella* larvae. However, the transplastomic plants obtained from this study could not achieve homoplasmic state and the frequency of getting transformants was also very low. This could be explained by the requirement of the transgenic protocol of *Brassica* species that relies on the cut ends of petioles, which bears very less chloroplasts [41]. The plastid transformation in *B. napus* was further improved by Cheng-Wei et al. [10]. Chloroplast transformation was also worked out in other *Brassica* crops such as cabbage [63] and

cauliflower [74]. We have recently succeeded in achieving plastid transformation in an elite cultivar of *B. juncea* using crop-specific expression vector [4].

Other Crop Plants

Apart from the above crops, plastid transformation was attempted and success reported in other crops such as in soybean (*Glycine max*). Plastids of leaves and photoautotrophic embryogenic suspension cell cultures were transformed with *aadA* gene and both the *rbcL* and *rbcS* of *Chlamydomonas reinhardtii* with an aim to engineer Rubisco and increase photosynthetic efficiency of soybean plants, which resulted into partial success and no plantlet could be regenerated. However, the results showed preliminary promises of Rubisco engineering through plastid transformation in soybean and emphasized the need for improved methods of transformation and regeneration [116]. Plastid transformation in soybean was further improved by Dufourmantel et al. [30]. Plastid transformation was demonstrated in other crops like carrot [54], cotton [55], lettuce [45, 60, 85], and sugar beet [27].

Conclusions and Future Perspectives of Plastid Transformation in Crop Plants

Plastid transformation has progressed gradually from *Chlamydomonas reinhardtii* to model plant tobacco and slowly towards other higher plants. Most of the agronomic traits targeted for engineering via plastids were established in tobacco with an aspiration that it would be implemented in crop plants. However, till date no transplastomic crop plant could be commercialized due to various technical reasons. Development of efficient plastid transformation technology in wide range of plants has been dependent primarily on available chloroplast genome sequences and tissue culture mediated regeneration from green tissues, preferably leaf explants. Now more than 200 chloroplast genome sequences are available which would facilitate not only our understanding of genome evolution in plants but also the chloroplast genome organization in number of crop plants [106]. Most of the endogenous regulatory regions required for stable expression of plastid expressed transgenes reside within this spacer region. Thus, effective vector construction for plastid transformation in any new crop species will require species-specific chloroplast genome sequence data. Therefore, the future of plastid transformation in unexplored crop plants needs parallel focus on chloroplast genomics [13]. Besides genome sequence information, efficient plastid transformation in crop plants from wide taxonomic groups requires attention of other crucial factors like transgene delivery, selection, regeneration and process of achieving homoplasmy. This was evident from the facts that although genome sequence information was available in several cereal crops such as rice, wheat, maize their plastid transformation and homoplasmic lines could not be made possible until recently. One of the major obstacles of plastid transformation in crop plants is the requirement for targeting transgenes in proplastids, which because of its small size gets physically damaged during biolistic transformation process. The encouraging fact is that the plastid transformation with agronomically important genes are now possible with the advantages of efficient removal of the plastid marker gene through approaches like Cre-lox system which will address the public acceptance of the new transplastomic crops [14]. Thus, the plastid biotechnology for crop plants, though a novel tool for crop improvement, has several challenges which needs to be addressed before realising its true potential in improving crop plants for agronomic and industrial applications.

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