Technical Advances in Chloroplast Biotechnology

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Abstract

Chloroplasts are highly organized cellular organelles after master organelle nucleus. They not only play a central role in photosynthesis but are also involved in several crucial cellular activities. Advancements in molecular biology and transgenic technology have further groomed importance of the organelle, and they are the most ideal ones for the expression of transgene. No doubt, limitations are there, but still research is advancing to resolve those. Certain valuable traits have been engineered for improved agronomic performance of crop plants. Industrial enzymes and therapeutic proteins have been expressed using plastid transformation system. Synthetic biology has been explored to play a key role in engineering metabolic pathways. Further, producing dsRNA in a plant's chloroplast rather than in its cellular cytoplasm is more effective way to address desired traits. In this chapter, we highlight technological advancements in chloroplast biotechnology and its implication to develop biosafe engineered plants.

Keywords: chloroplast biotechnology, value-added crops, RNAi, trouble-rescue organelles, plastid functional genomics

1. Introduction

Food security is a long-lasting challenge for the growing world and is becoming more alarming in the developing countries where one out of every nine people is malnourished. So-called processing (polishing, milling, and pearling) of the cereals makes them even poorer in micronutrients [1]. Climate change is another challenge that poses continuous stress on the crop productivity. Sharply decreasing arable soils and use of heavy inputs to get high crop yield are further deteriorating our environment and quality of available food. All this demands availability of improved crop cultivars having ability to perform in the changing climate scenario and even with balanced dose of micronutrients. Gene revolution is the only hope for second green revolution to attain these ideal crop cultivars [2]. Since the commercialization of transgenic crops in 1994, the area under GM crops is sharply increasing and has now increased to 180 million hectares. This includes crops for improved agronomic traits (herbicide tolerance and insect resistance, salinity and drought tolerance, and efficient nutrient utilization), enhanced level of micronutrients, and for the expression of therapeutic proteins and industrially important enzymes. At the same time, emotional or obsolete arguments are there to oppose the use of GM (genetically modified) products. Opponents of GM crops have straightforwardly rejected genetically modified products and have produced questionable scientific data to ban their commercialization. Plastid biotechnology has emerged as a competent field of research having potential to address all of the questions raised by the opponents of GM crops [3]. This chapter highlights significance of plastid transgenic technology to develop valuable crop plants. Further, technological advancements have been discussed to get an update about the recent research to resolve existing bottlenecks in the development and commercialization of transplastomic plants.

2. Chloroplast biotechnology—an overview

Transgenic technology is the technology of the day to develop crop plants with desired traits but crucial traits need to be engineered through plastid genome instead of nuclear genome [4]. It is an amazing organelle where more than 120 genes from various sources have been integrated and expressed. This organellar genome has well been explored for a wide variety of applications including crops with elevated level of resistance against biotic (insects, bacterial, viral, and fungal diseases) and abiotic stresses (salinity, drought, and cold); phytoremediation of toxic metals, cytoplasmic male sterility [5]; and production of biopharmaceuticals, vaccine antigens, industrial enzymes, biomaterials, and biofuel [6]. Hyperexpression of recombinant protein in plant expression system is only possible through plastid transformation. The high ploidy number of the plastid genome results in higher level of protein expression, and up to 70% total soluble protein is reported to be produced in tobacco [7]. Moreover, hyperexpression of therapeutic proteins and vaccine antigens in chloroplasts (leaves), leucoplasts (roots), or chromoplasts (fruits) makes it ideal organelle for the oral delivery of vaccine antigens against tetanus, cholera, anthrax, canine parvovirus, and plague [8]. Other salient advantages include possibility of multigene engineering, absence of gene silencing, position effect, epigenetic, complete absence of pleiotropic effects due to subcellular compartmentalization, and transgene containment due to maternal inheritance of plastids in most of the crops [9].

Plastid transformation was first established in unicellular green algae (*Chlamydomonas reinhardtii*) followed by model tobacco plant. It has now been well established in economically valuable crops (rice, brassica, cotton, carrot, spinach, lettuce, etc.) and even in woody plant like popular. Small circular plastid genome (plastome) facilitates targeted engineering, which has been exploited not only for basic research but also for the applied research [10]. Most of the genes present in plastome have been characterized through functional genomics. Organellar transcription and translation have been thoroughly elucidated to understand transcriptional and translational machinery of the plastids. Even the proteins involved in cross-talk between chloroplast and nucleus have been worked out. Further, plastid transformation is the most ideal technology to develop marker-free transgenic plants where antibiotic resistance genes, used for the selection of putative transformants, are not acceptable by the ultimate consumer. Different techniques have been developed to produce marker-free plants in order to facilitate the acceptance of transplastomic crops. In spite of so many advantages of plastid transformation technology, there are still difficulties impeding expansion of this technology to economically valuable crops particularly monocots. These include lack of species-specific regulatory sequences, inefficient selection system, metabolic burden in case of hyperexpression and unavailability of green plastids in monocots.

3. Making better crops through chloroplast engineering

It is predicted that sharply increasing population necessitates an increase in crop yield at 30% per annum. In this scenario, chloroplast biotechnology is the most ideal approach to develop crop plants with improved photosynthetic performance, enhanced nutritional value, improved agronomic traits, and producing valuable fatty acids. Plastid transformation was first established in flowering plants almost 30 years ago. Though it has been extended to other crop plants, most of the studies have been conducted in tobacco, which is nonfood nonfeed crop. This demands further efforts by the scientific community to engineer plastid genome of valuable crop plants for desired traits, leading to increased quality and quantity of food.

Most of the efforts to increase crop productivity had been made to improve photosynthetic performance of the plants. RuBisCO (the core enzyme of photosynthesis), large subunit, is encoded by chloroplasts, whereas small subunit is encoded by nuclease, which is then imported to chloroplast. Efforts have been made to engineer RuBisCO large subunit, small subunit, or both. Lin [11] attempted to express complete RuBisCO protein in tobacco from *Synechococcus elongatus* by disrupting the host native enzyme. CO2 fixation rate and carboxylase activity of the RuBisCO were increased, especially at higher concentrations of CO_2. Hence, photosynthetic performance can be improved by introducing more competent complete photosynthetic system into a plant. Raising concentration of CO_2 in plastids is another possibility strategy, to improve photosynthetic carbon fixation and crop productivity. Cyanobacterial bicarbonate transporter was expressed in tobacco plastid genome, but any considerable improvement in photosynthetic performance was not observed. Expression of fructose-1,6-sedoheptulose-1,7-bisphosphatase in lettuce and tobacco chloroplasts appeared to increase productivity of engineered plants. Likewise, chloroplast-encoded chlB gene from *Pinus thunbergii* was found to promote root growth and early chlorophyll pigment development in tobacco [12]. Hence, research is in progress to engineer C3 plants to C4 by manipulating RuBisCO large subunit and photorespiratory pathway for enhanced biomass production [13].

Insect resistant crops had successfully been grown in the field since 1994. Resistance development against Bt crops is an emerging concern, which needs to be addressed through high-dose strategy and gene pyramiding. Another possibility to develop insect-resistant transplastomic plants is the upregulation of their pathogen defense mechanisms. Expression of β-glucosidase in tobacco plastome showed not only growth of the plants but also more resistance against insect pests [14]. A novel non-Bt-type insect resistance strategy has been evaluated by expressing dsRNA, targeting an essential insect gene in transplastomic plants. Disruption of target gene by RNA interference resulted in 100% mortality in adult beetles and in the larvae within 5 days of feeding [15]. Expression of agglutinin gene (pta) in leaf chloroplasts resulted in broad spectrum resistance against lepidopteran insects, aphids, and viral and bacterial pathogens [16]. A gene stack comprising CeCPI (sporamin, taro cystatin) gene from sweet potato and chitinase from *Paecilomyces javanicus* was introduced into tobacco, and resultant plants showed resistance not only against various pests and diseases but also against salinity, osmotic stress, and oxidative stress [17]. Expression of osmoprotectant (yeast trehalose phosphate synthase) in tobacco plastids resulted in 20-fold higher trehalose accumulation; as a result, plants were tolerant to drought and osmotic stress [18]. The overexpression of *mdar* transgene in tobacco plastids and the fusion of such chloroplasts to Petunia cells were suggested to possibly protect the plants against oxidative stress. Oxidative stress tolerance was also enhanced in transplastomic tobacco plants expressing flavodoxin (fld) from cyanobacteria. Transplastomic plants overexpressing panD not only appeared to produce 30–40% higher biomass but also appeared to be more tolerant to increased heat stress. Similarly, expression of arabitol dehydrogenase (*ArDH)* in tobacco chloroplasts enabled them to survive even at 400 mM NaCl [19]. Chilling tolerance as well as growth was observed to be increased in tropical forage *Stylosanthes guianensis* expressing chloroplast protein 12 [20]. Recently, plastid transformation has been reported in a valuable vegetable *Momordica charantia* [21], marine microalga *Nannochloropsis oceanica* [22], and *Cyanidioschyzon merolae* [23], a red alga having ability to survive in high sulfur acidic hot spring environments. This may open new horizons in understanding stress adaptability and role of transplastomic technology in developing stress-tolerant plants.

4. Chloroplasts as trouble-rescue organelles

Chloroplasts not only are the central hubs for photosynthesis but also have evolved as fundamental trouble-rescue organelles. Recent studies have revealed that chloroplasts play a key role in switching plants from vegetative mode to defense mode. In addition to intraorganellar functions, they also play crucial role in the regulation of extraorganellar processes such as plant stress response, apoptosis, and immunity. Both of the cellular organelles (chloroplast and mitochondria) evoke their own particular Ca^{2+} signals [24], have their own Ca^{2+} binding proteins, and Ca^{2+} sensors, which are expected to play a significant role in Ca^{2+} signaling within the plant cell [25]. As a result, they have capacity to sequester and serve as sink for Ca^{2+}, which plays a key role in physiological and environmental responses of eukaryotic cells.

Chloroplasts are important intracellular calcium (Ca^{2+}) stores and may accumulate up to 15 mM or even higher. Most of the plastidic Ca^{2+} resides within the stroma or thylakoid membranes through interaction with calcium-binding proteins [26]. The concentration of free calcium was found to be very low when determined by targeting apoaequorin to the stroma of tobacco chloroplasts [27]. Hence, stroma is not the major sequester of Ca^{2+} in chloroplasts. This helped to elucidate that chloroplasts have their own active transporters on the envelope membranes, which help them to accumulate high concentrations of Ca^{2+} within the thylakoid membranes or some other unidentified Ca^{2+} stores. Identification of CAS (high capacity Ca^{2+}-binding protein) in the thylakoid membranes of *Arabidopsis thaliana* revealed out that active calcium uptake machinery is present on the membranes; so, the thylakoid membrane may be the major sequester of Ca^{2+} in chloroplasts [28]. It was further elucidated that activity of these transporters is regulated by light or photosynthesis, so chloroplasts take up calcium during the day and store it in the lumen or some identified sequestering sites. During the night, Ca^{2+} is released from the store houses for long-lasting, dark-induced Ca^{2+} signals; hence, sensing of photoperiod and light/dark transition seems to be regulated by Ca^{2+} signaling. In addition to light, Ca^{2+} signaling is also influenced by other abiotic stimuli (salinity, cold) and hence plays some crucial role in stress tolerance as well.

An active Ca^{2+} uptake machinery is present in chloroplast, which is regulated by transporters. Much research has not been conducted on these transporters; as a result, only few are known, whereas others are still to be elucidated. Two potential membrane transporters (Ca^{2+}-ATPase) in *Arabidopsis* are AtACA1 and AtHMA1. AtACA1 is an autoinhibited Ca^{2+} transporter, which is predicted to be targeted to the chloroplasts. It is specifically expressed in the root and is then localized to endoplasmic reticulum or tonoplast. AtHMA1 is a heavy metal P-type ATPase in the chloroplast envelope and plays an important role in calcium transportation [29]. Recently, another transporter AtGLR3.4 (glutamate receptor) has been explored to form Ca^{2+} permeable nonselective cation channels and is localized in the chloroplasts [30]. In addition, two MscS homologs, localized in the chloroplast, have also been evaluated to be essential for plastidic osmoregulation [31]. Since these transporters play a key role in the sequestration of calcium ions into the thylakoid lumen, Ca^{2+}/H^{+} antiporter also plays a significant role in Ca^{2+} uptake via thylakoid proton gradient. Pea thylakoid protein (PPF1) is another candidate calcium transporter at thylakoid membrane, which has been found to enhance Ca^{2+} currents when tested in human cultured cells. These findings demonstrate that Ca^{2+}-binding protein and Ca^{2+} transporters play a significant role in immune signal transduction pathway. Anyhow, most of the genes involved in these pathways are still to be elucidated.

5. Advances in plastid functional genomics

Plastids are known to get evolved from primitive cyanobacteria through a process known as endosymbiosis [32]. Although plastid genomes are much smaller as compared to their cyanobacterial progenitors, similarities in gene sequence as well as genome topology are evident. Just like cyanobacterial genome, plastid genomes are tightly packed with genes as a circular molecule [33]. *In vivo* genes of plastid may be present as a linear molecule or a complex branched form, and many copies of plastid genome can be harbor in each organelle. Size of plastid genomes varies from <100 to >1000 kbp (kilobase pair). The region of small single copy (SSC) and large single copy (LSC) are separated by two inverted repeats (IRs) present in the plastid genome (**Figure 1**). The thymine and adenine residues are often rich in plastid genome; a reductive evolution is also seen in those of mitochondrial genomes and bacterial endosymbionts [34]. Noncoding DNAs are abundantly present in some plastid genomes, while the others have self-splicing introns. The genome of some dinoflagellates spreads across a sea of minicircles; recently, multiple linear chromosomes formed a hairpin structure, which have been found in the plastid genome of certain green algae [35].

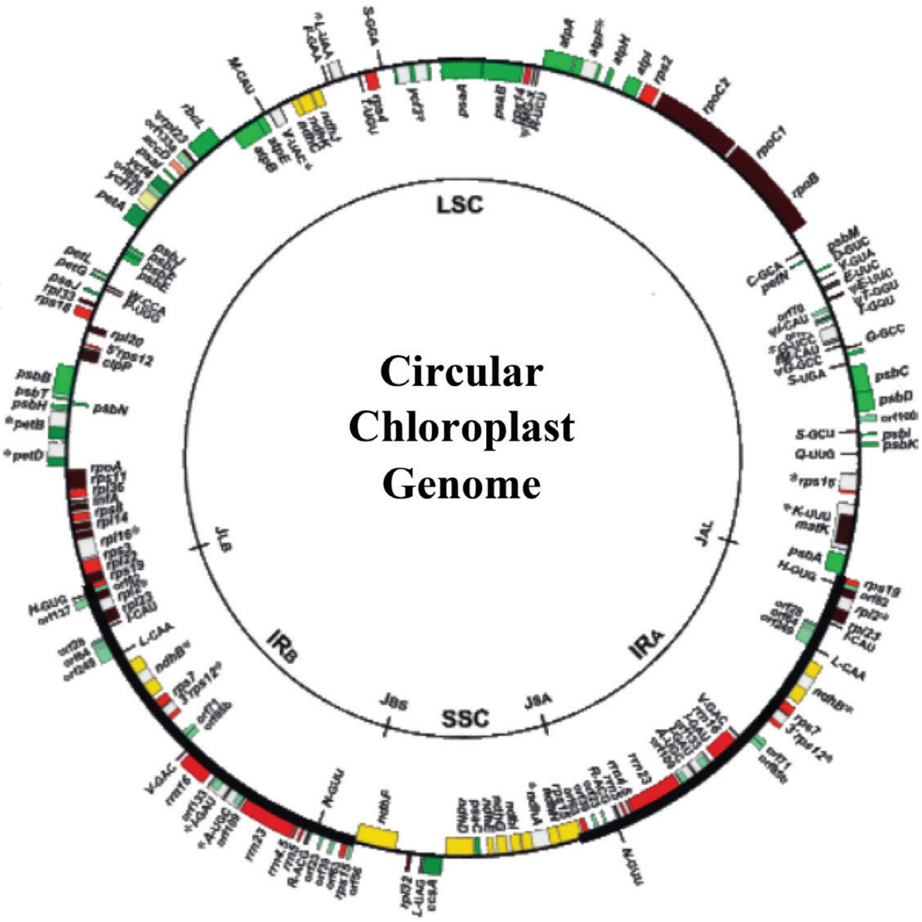

Figure 1. Circular map of chloroplast genome showing one large single copy (LSC), one small single copy (SSC), and two inverted repeats (IRa and IRb).

A huge portion of the cyanobacterium derivative genes required for plastid function now exist in the nucleus, having transferred through a process known as endosymbiotic gene transfer (EGT). Subsequently, most of the plastome proteins are introduced posttranslationally. Nevertheless, genomes of plastid normally encode some of their own processing machinery, including ribosomal proteins, ribosomal RNAs, bacterial RNAs polymerase, and tRNAs—however, land plants also have nuclear-encoded plastid RNA polymerases. Remarkably, genome of plastid also encodes many photosynthesis components, such as proteins of photosystem I and II (e.g., *psbA* gene of photosystem II coding for the D1 unit) as well as cytochrome *b6f*, which facilitates electron transfer between both photosystems I and II [36].

6. Role of synthetic biology in engineering plastid metabolic pathways

During past two decades, the synthetic biology approach has brought about several remarkable accomplishments regarding engineering of biological systems particularly microbes and yeast. However, such promising attributes of synthetic biology have not been explored for plastid genome engineering except few striking instances [37]. The future of the recombinant DNA technology is linked with advancement and implications of synthetic biology because development of novel biological systems is dependent on this area of research. Like traditional disciplines of engineering, synthetic biologists also use abstraction, standardization, and decoupling to design more efficient biological systems [38]. So, to design an organism of choice synthetic biology is of fundamental importance. Unique advantages of plastid transformation technology regarding metabolic pathway engineering make it more important than nuclear transformation technology. Hence, coalescing synthetic biology with plastid genome engineering can be more fruitful and valuable for the production of economical recombinant proteins [39]. However, understanding plastid genomics is equally important in order to harvest potential benefits of synthetic biology. The anterograde and retrograde signaling (nucleus to plastid and vice versa) of plastid proteins revealed that most of the protein complexes were chimeric and contained both plastid encode subunits as well as nucleus encoded subunits. Further, nucleus encoded proteins follow eukaryotic mode of expression, whereas plastid encoded proteins follow prokaryotic mode of expression, though plastid genome is quite smaller in size than nuclear genome (less than 10%) [40]. Since efforts have been made to develop synthetic plastid genome of minimum size for efficient transplantation into a cell without plastids or to replace native plastid genome with engineered operons coding for valuable proteins. This requires information about the most essential genes involved in the stability and integrity of the plastome. Owing to high cost of synthetic DNA, initially it was used only for the optimization of codon usage of transgene, but now it is affordable to synthesize complete vector or genome. This not only avoids intensive cloning work but also facilitates synthesis of multiple genes with desired regulatory sequences. Use of synthetic expression elements has helped to get appropriate expression of transgene in nongreen plastids including tubers and fruits [41]. Chloroplast being metabolic center of the cell is the most attractive organelle whose metabolic pathways need to be engineered. Further, it has ability to stack multiple synthetic operons. Major limitation in this context is size of the transgene as engineering metabolic pathways require engineering of the multiple genes involved in that particular pathway [42–43]. The identification of intercistronic expression elements (minimum sequence elements involved in the proper processing of polycistronic transcript into monocistronic) has helped to devise workable synthetic operons for the expression of multiple proteins involved in the biosynthesis of vitamin E [44], artemisinic acid [45], carotenoids [46], and dhurrin [47] or other metabolic pathways [48]. Likewise, synthetic operons can be helpful for the transformation of C3 photosynthetic pathway into C4, engineering of nitrogen fixation pathway, or molecular farming for the production of industrial enzymes and therapeutics [49].

7. Regulation of RNA editing in chloroplasts

An important process of gene regulation is RNA editing. This occurs at posttranscriptional level through nucleotide modification for many functional genes. RNA editing restores the conserved amino acid residues for functional proteins in plants. Changes in RNA sequence of functional gene occurs during RNA editing, through the molecular mechanisms [50].

Cytidine-to-uridine editing and adenosine-to-inosine editing are two types of RNA editing identified in *Arabidopsis thaliana* [51]. RNA editing is a rare process where RNA polymerase is involved in insertion, deletion, and base substitution of nucleotide within the transcript [52–55]. Many studies reported the evidence of RNA editing in tRNA, rRNA, and mRNA. However, RNA editing has also been reported in noncoding RNA, like microRNAs of eukaryotes. The RNA editing occurs in all DNA-containing organelles like nucleus, mitochondria, and plastids. In nucleus, chloroplast and mitochondria RNA editing occurs during the process of transcription and posttranscriptional modifications [56, 57]. Caseinolytic protease complex component (CLPC1) plays a crucial role in RNA homeostasis [58]. Anyhow, discrete changes in RNA before its translation into protein occur by RNA editing. Besides this, RNA editing is also a vibrant mechanism to produce functional and molecular diversity [59].

In chloroplast gene expression system, RNA editing is an important posttranscriptional modification. The use of pentatricopeptide repeat (PPR) protein family for RNA editing in chloroplast has been reported [51]. Mostly genes in chloroplast are cotranscribed and arranged in clusters. To control gene expression, posttranscriptional RNA editing is an essential step, and this step is also required for gene function [52]. It has been studied that C-to-U editing is the major type of RNA editing in chloroplasts. In chloroplast, etioplast, and amyloplast of maize, expression of almost 15 different genes has been affected by 27 C-to-U RNA editing sites. In chloroplast, RNA editing plays an important role to correct harmful mutations instead of producing protein diversity. Genomic DNA sequence is not changed by C-to-U editing because this editing changes the nucleotide sequence only within RNA molecule. RNA polymerase is used to produce RNA editing [60]. Insertion, deletion, and base substitution are events of RNA editing. That is why RNA editing can reverse harmful genomic mutations in consistent RNA transcript. In chloroplast, different sites are edited by C-to-U RNA editing enzymes as well [61]. Around 126 C-to-U editing events and 11 U-to-C editing events were identified in the chloroplast DNA of moth orchid (*P. aphrodite* subsp. Formosana). In leaf tissues, 110 editing events and in floral tissue, 106 editing events were identified. In non-protein-coding RNA such as introns, tRNA, and regulatory sequences, RNA editing occurred [62]. Besides C-to-U editing, which is mostly reported in chloroplast of plants, adenosine-to-inosine editing in plastid tRNA of *Arabidopsis thaliana* has also been characterized. Adenosine-to-inosine editing was recognized in the anticodon of the plastid tRNA-Arg (ACG). AtTadA gene expression is involved in adenosine-to-inosine editing in the chloroplast [51].

8. Conclusions and future directions

Chloroplasts are the most important solar-energy-capturing natural systems on earth. They not only capture it but also convert it into a form useful for all living organism on earth. Molecular oxygen is liberated as a by-product, which is a vital source for respiration of all aerobic organisms. Chloroplasts are believed to be evolved from prokaryotic ancestors through a process known as endosymbiosis. Chloroplast contains circular genome having compactly arranged genes, which are involved in not only photosynthesis but also many other vital biological processes. Keeping in view its utmost physiological importance, plant as well as algal plastome has been engineered for a number of agronomic as well as pharmaceutical traits [63, 64]. Advancements in molecular biology and transgenic technology have further groomed importance of the organelle, and they are the most ideal ones for the expression of transgene. Resolving current limitations including vector design, gene regulation control and DNA delivery may further improve this important field of biotechnology [65]. Synthetic biology is being explored in this regard, which is expected to play a major role in enhancing contribution of chloroplasts not only for sustainable food production but also for other important molecules in future.

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