



Green Biotechnology: A Brief Update on Plastid Genome Engineering

4

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Abstract

Plant genetic engineering has become an inevitable tool in the molecular breeding of crops. Significant progress has been made in the generation of novel plastid transformation vectors and optimized transformation protocols. There are several advantages of plastid genome engineering over conventional nuclear transformation. Some of the advantages include multigene engineering by expression of bio-synthetic pathway genes as operons, extremely high-level expression of protein accumulation, lack of transgene silencing, etc. Transgene containment owing to maternal inheritance is another important advantage of plastid genome engineering. Chloroplast genome modification usually results in alteration of several thousand plastid genome copies in a cell. Several therapeutic proteins, edible vaccines, antimicrobial peptides, and industrially important enzymes have been successfully expressed in chloroplasts so far. Here, we critically recapitulate the latest developments in plastid genome engineering. Latest advancements in plastid genome sequencing are briefed. In addition, advancement of extending the tool-box for plastid engineering for selected applications in the area of molecular farming and production of industrially important enzyme is briefed.

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4.1 Introduction

The expected global population by 2050 will be approximately 9.1 billion (High-Level Expert Forum, FAO, October 2009; <http://www.fao.org>). To nourish the ever-increasing population, food production has to be increased simultaneously. Use of latest techniques in plant genetic engineering could help to increase the production of food crops. A range of different expression platforms have been used for heterologous production of foreign proteins having pharmaceutical, industrial, and agricultural applications (Demain and Vaishnav 2009). Among different production platforms, plants have been used as highly economical and scalable production systems for the expression of recombinant proteins, enzymes, and valuable metabolites. Plastid transformation technology has attracted strong interest for different applications in plant biology owing to several advantages compared to conventional nuclear transformation (Adem et al. 2017). The high ploidy number of chloroplast genome and compartmentalization of proteins allow high levels of foreign protein accumulation. Recombinant proteins are reported to accumulate up to several folds in total leaf soluble protein in plastids (Daniell et al. 2009a, b; Oey et al. 2009; Bock 2015). Plastid genomes are inherited through the maternal parent and thus provide a strong level of biological containment thus avoiding several ethical concerns. Integration of transgene proceeds via homologous recombination and is therefore highly precise and predictable (Verma and Daniell 2007). Another important feature of plastid transformation is that genetic machinery in chloroplast is devoid of gene silencing and other epigenetic mechanisms that interfere with stable transgene expression (Wani et al. 2010; Bock 2014). Recent advancement in plastid transformation is reviewed in (Kumar et al. 2017). In this chapter, we highlight the impact of chloroplast genome engineering on various plant biology applications. Advancement of chloroplast engineering for improving agronomic traits and plastids as platform for production high-value proteins for biofuel production is discussed.

4.2 Recent Developments in Plastid Genome Sequencing

Plant chloroplast genome (plastome) is a short double-stranded circular DNA of ~100–250 kb in size. Plastome of land plants is highly conserved with two ~25 kb inverted repeat (IR) regions that are separated from the rest of the genome into large single copy (LSC) and small single-copy regions (SSC) (Zhang et al. 2018a, b). In general, plastome includes approximately 130 genes encoding transcripts involved in carbon fixation (photosynthesis), transcription, and translation. Although plastid genome is highly conserved in plants, genome size varies across different species (Table 4.1) (Daniell et al. 2016).

Table 4.1 List of important plants with annotated plastome

Sl no	Name of plant	Common name	Genome size (Mb)	References
01	<i>Anomum compactum</i>	Cardamom	0.163	Wu et al. (2018)
02	<i>Ananas comosus</i>	Pineapple	0.159	Nashima et al. (2015)
03	<i>Arabidopsis thaliana</i>	Thale cress	0.154	Sato et al. (1999)
04	<i>Artemisia annua</i>	Sweet wormwood	0.150	Shen et al. (2017)
05	<i>Brassica napus</i>	Canola	0.152	Hu et al. (2011)
06	<i>Camellia sinensis</i>	Tea	0.157	Dong et al. (2018)
07	<i>Cannabis sativa</i>	Marijuana/hemp	0.153	Oh et al. (2016) and Vergara et al. (2016)
08	<i>Capsicum annum</i>	Pepper	0.156	Raveendar et al. (2015)
09	<i>Catharanthus roseus</i>	Periwinkle	0.154	Ku et al. 2013
10	<i>Cicer arietinum</i>	Chickpea	0.125	Jansen et al. (2008)
11	<i>Citrus sinensis</i>	Orange	0.160	Bausher et al. (2006)
12	<i>Cocos nucifera</i>	Coconut	0.154	Huang et al. (2013)
13	<i>Coffea arabica</i>	Coffee	0.155	Samson et al. (2007)
14	<i>Cucumis sativus</i>	Wild cucumber	0.155	Liu et al. (2016)
15	<i>Cuscuta reflexa</i>	Giant dodder	0.121	Funk et al. (2007)
16	<i>Cyamopsis tetragonoloba</i>	Clusterbean	0.152	Kaila et al. (2017)
17	<i>Daucus carota</i>	Carrot	0.155	Ruhlman et al. (2006)
18	<i>Drimys granatenis</i>	–	0.160	Cai et al. (2006)
19	<i>Eucalyptus globulus</i>	Tasmanian bluegum	0.160	Steane (2005)
20	<i>Glycine max</i>	Soybean	0.152	Saski et al. (2005)
21	<i>Hevea brasiliensis</i>	Rubber	0.161	Tangphatsornruang et al. (2011)
22	<i>Hordeum vulgare</i>	Barley	0.135	Middleton et al. (2014)
23	<i>Jasminum nudiflorum</i>	Winter jasmine	0.165	Lee et al. (2007)
24	<i>Lactuca sativa</i>	Lettuce	0.152	Timme et al. (2007)
25	<i>Lilium longiflorum</i>	Tiger lily	0.152	Kim et al. (2017)
26	<i>Lotus japonicas</i>	Wild legume	0.150	Kato et al. (2000)
27	<i>Malus hupehensis</i>	Wild apple	0.160	Zhang et al. (2018a, b)
28	<i>Manihot esculenta</i>	Cassava	0.161	Daniell et al. (2008)
29	<i>Medicago truncatula</i>	Barrel medic	0.124	Gurdon and Maliga (2014)
30	<i>Musa acuminata</i>	Banana	0.169	Martin et al. (2013)
31	<i>Musa balbisiana</i>	Wild banana	0.169	Niu et al. 2018
32	<i>Nicotiana tabacum</i>	Tobacco	0.155	Shinozaki et al. 1986
33	<i>Nymphaea alba</i>	Water lily	0.159	Goremykin et al. 2004
34	<i>Oryza minuta</i>	Wild rice	0.135	Asaf et al. 2017

(continued)

Table 4.1 (continued)

Sl no	Name of plant	Common name	Genome size (Mb)	References
35	<i>Oryza sativa</i>	Rice	0.134	Yu et al. 2017
36	<i>Panax ginseng</i>	Ginseng	0.155	Nguyen et al. 2018
37	<i>Pelargonium x hortorum</i>	Geranium	0.217	Chumley et al. 2006
38	<i>Piper coenoclatum</i>	–	0.160	Cai et al. 2006
39	<i>Rosmarinus officinalis</i>	Rosemary	0.152	Chen and Hua (2018)
40	<i>Saccharum</i> spp.	Sugarcane	0.141	Hoang et al. 2015
41	<i>Salvia miltiorrhiza</i>	Red sage	0.151	Qian et al. (2013)
42	<i>Secale cereale</i>	Rye	0.135	Middleton et al. (2014)
43	<i>Solanum lycopersicum</i>	Tomato	0.155	Wu (2016)
44	<i>Solanum tuberosum</i>	Potato	0.155	Chung et al. (2006)
45	<i>Sorghum bicolor</i>	Sorghum	0.140	Saski et al. (2007)
46	<i>Spinacia oleracea</i>	Spinach	0.150	Schmitz-Linneweber et al. (2001)
47	<i>Triticum aestivum</i>	Common wheat	0.135	Middleton et al. (2014)
48	<i>Vigna radiata</i>	Mungbean	0.151	Tangphatsornruang et al. (2010)
49	<i>Vitis vinifera</i>	Grapes	0.160	Jansen et al. (2006)
50	<i>Zea mays</i>	Maize	0.140	Maier et al. (1995)
51	<i>Zingiber officinale</i>	Ginger	0.162	Vaughn et al. (2014)

As mentioned above, plastome lacks recombination and exhibits a uniparental (maternal) inheritance. Conventionally, plastome sequencing was based on cloning of plastid DNA fragments to generate DNA libraries followed by long-range PCR. Alternatively a large set of DNA primers were used to amplify and sequence overlapping DNA fragments in plastid genome. With the rapid advancement in next-generation sequencing technology, it is becoming increasingly faster and cheaper to sequence and assemble plastomes. Although different sequencing platforms are available, Illumina and PacBio systems are widely used for sequencing of plastid genome and subsequent assembly (Chen et al. 2015; Lin et al. 2015; Jackman et al. 2016). However, several groups have reported reads generated through PacBio are often low (English et al. 2012). This was later improved by use of latest sequencing chemistry together with a hierarchical genome assembly process algorithm (Daniell et al. 2016).

Since the advancement in sequencing technology, several research groups across the world sequenced plastid genomes of different plants (Table 4.1). Till date, ~650 plant species have their plastid genome sequence publically available in GenBank database. Plastome of two rice varieties (*Oryza sativa indica* and *O. sativa japonica*) were sequenced, and authors reported that divergence of plastome of both varieties occurred approximately 86,000 to 200,000 years ago (Tang et al. 2004). Tong et al. (2016) reported variation in chloroplast genome of rice ecotypes from Asia and Africa. Sequencing of plastid genomes of wild rice *Oryza australiensis* and *Zizania latifolia* gave insights about evolution of rice varieties (Wu and Ge 2016; Zhang

et al. 2016). Plastomes of wheat, rye, barley, and other *Triticeae* species were sequenced using Roche/454 technology. Sequence alignments revealed exchange of genetic material by translocation of segment of plastome to the nuclear genome specific to rye/wheat lineage (Middleton et al. 2014). Kim et al. (2014) reported AT-rich plastid genome in orchid *Cypripedium japonicum* and proposed importance of AT residues in effective splicing of the plastid genome. Plastome analysis of banana *Musa acuminata* revealed IR/SSC expansion occurred independently multiple times in monocots during course of evolution (Martin et al. 2013).

In addition to cereals, plastome of vegetables and horticultural crops has been sequenced (reviewed in Rogalski et al. 2015). Comparisons of chloroplast genome structure between tomato (*Solanum lycopersicum*) and a wild diploid potato species (*Solanum bulbocastanum*) revealed that, at gene order, these genomes are identical, and this conservation extends even to more distantly related genera (like tobacco and *Atropa*) (Daniell et al. 2006). Analysis of carrot plastid genome revealed presence of several dispersed direct and inverted repeats scattered throughout coding and noncoding regions (Ruhlman et al. 2006). A comparative plastome analysis of grapes from different geographical locations revealed insights in origin and molecular basis of evolution of *Vitis* (Pipia et al. 2017).

Parasitic plants are interesting host to understand the evolutionary adaptation and ~1% of all known angiosperm species being parasitic plants (Westwood et al. 2010). Plastid genome analysis of several parasitic plants revealed evolutionary reduction in genome size is associated with gene loss (Funk et al. 2007; Gruzdev et al. 2016). It has been proposed that DNA in plastid loci could be horizontally acquired from its host as a result of parasitism (Molina et al. 2014). This is further supported by that finding that plasmodesmatal continuity between partners allows movement of genetic material (Birschwilks et al. 2006; Roney et al. 2007; Talianova and Janousek 2011). Thus comparative plastome analysis revealed information about simplification of plastid gene expression machinery in parasitic plants. The availability of the complete plastid genome sequence for several plant species facilitated generation of novel plastid expression vectors for efficient foreign gene expression in plants through utilization of endogenous flanking sequences and regulatory elements.

4.3 Plastid Bioreactors for Molecular Farming

Advancement in genetic engineering has revolutionized the use of therapeutically and pharmaceutically valuable proteins in a variety of clinical treatments (Grevich and Daniell 2005). In industrial scale, an ideal expression platform should produce safe and biologically active protein (with appropriate post-translational modification) at the lowest cost. Chloroplast engineering offers great promise for both agriculture and pharmaceutical industries, for production of recombinant proteins of interest in plants is emerging as an economical and safe alternative to bacterial, yeast, or animal expression platform (Daniell 2006). Several proteins have been produced in transplastomic plants in the last two decades (Tables 4.2 and 4.3).

Table 4.2 Selected list of vaccine antigens and therapeutic proteins produced in plants by chloroplast genome engineering

Sl no	Name of enzyme	Gene source	Host	References
01	Anti-HIV microbicide griffithsin	<i>Griffithsia</i> sp	Tobacco	Hoelscher et al. (2018)
02	Human factor VIII (FVIII)	Human	Tobacco	Kwon et al. (2018)
03	Tetravalent EDIII antigen (EDIII-1-4)	Dengue virus	Lettuce	van Eerde et al. 2018
04	CTB- factor IX	Human	Lettuce	Herzog et al. (2017)
05	CTB-VP1	Poliovirus	Tobacco	Chan et al. (2016)
06	Capsid protein (VP1)	Poliovirus	Lettuce	Daniell et al. (2016)
07	Envelope protein domain III-based antigens	Dengue virus	Tobacco	Gottschamel et al. (2016)
08	gp120 and gp41	HIV	Tobacco	Rubio-Infante et al. (2015)
09	Protective antigen	<i>Bacillus anthracis</i>	Tobacco	Gorantala et al. (2014)
10	HPV-16 L1	Human papillomavirus	Tobacco	Hassan et al. (2014)
11	E7 antigen	Human papillomavirus	Tobacco	Morgenfeld et al. (2014)
12	ESAT-6 (6 kDa early secretory antigenic target), Mtb72F (a fusion polyprotein from two TB antigens, Mtb32 and Mtb39), and LipY (a cell wall protein)	<i>Mycobacterium tuberculosis</i>	Lettuce/tobacco	Lakshmi et al. (2013)
13	E protein (junction site of domains I and II)	Dengue virus	Lettuce	Maldaner et al. (2013)
14	Human granulocyte colony-stimulating factor (hG-CSF)	Human	Lettuce	Sharifi Tabar et al. (2013)
15	CTB-proinsulin	Human	Tobacco	Boyhan and Daniell (2011)
16	Premembrane (prM) and truncated envelope (E) protein	Dengue virus	Lettuce	Kanagaraj et al. (2011)
17	Malarial vaccine antigens apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1) fused with cholera toxin-B subunit	<i>Plasmodium falciparum</i>	Lettuce/tobacco	Davoodi-Semiromi et al. (2010)
18	Insulin-like growth factor	Human	Tobacco	Daniell et al. (2009b)
19	Envelope protein A27L	Vaccinia virus	Tobacco	Rigano et al. (2009)
20	Pr55 ^{gag}	HIV-1	Tobacco	Scotti et al. (2009)

(continued)

Table 4.2 (continued)

Sl no	Name of enzyme	Gene source	Host	References
21	p24	HIV-1	Tobacco	McCabe et al. (2008)
22	p24-Nef	HIV-1	Tobacco/ tomato	Zhou et al. (2008)
23	Viral capsid antigen	Epstein-Barr virus	Tobacco	Lee et al. (2006)
24	SARS-CoV spike protein	Synthetic gene corresponding to homologue protein in SARS virus	Lettuce/ tobacco	Li et al. (2006)
25	E2 protein	Hepatitis E virus	Tobacco	Zhou et al. (2006)

Table 4.3 List of industrially important enzymes/biomaterials produced by plastome engineering

Sl no	Name of enzyme	Gene source	Host	References
01	Polyhydroxybutyrate	<i>Ralstonia eutropha</i>	Tomato	Mozes-Koch et al. (2017)
02	β -Glucosidase, xylanase, and endoglucanase	<i>Trichoderma reesei</i>	Tobacco	Castiglia et al. (2016)
03	Cellulases and polygalacturonase	<i>Chaetomium globosum</i> / <i>Paenibacillus</i> sp./ <i>Phanerochaete chrysosporium</i>	Tobacco	Longoni et al. (2015)
04	Xylanase	<i>Bacillus</i> sp.	Tobacco	Pantaleoni et al. (2014)
05	Cellulase	<i>Thermotoga maritima</i>	Tobacco	Jung et al. (2013)
06	β -1,4-endoglucanase	<i>Pyrococcus horikoshii</i>	Tobacco	Nakahira et al. (2013)
07	Cutinase and swollenin	<i>Fusarium solani</i> / <i>T. reesei</i>	Tobacco	Verma et al. (2013)
08	Agglutinin	<i>Pinellia ternata</i>	Tobacco	Jin et al. (2012)
09	β -Mannanase	<i>T. reesei</i>	Tobacco	Agrawal et al. (2011)
10	Cellulase	<i>Thermobifida fusca</i>	Tobacco	Petersen and Bock (2011)
11	Cellulase	<i>Thermobifida fusca</i>	Tobacco	Gray et al. (2009)
12	Chitinase	<i>Brassica juncea</i>	Tobacco	Guan et al. (2008)
13	Choline monooxygenase	<i>Beta vulgaris</i>	Tobacco	Zhang et al. (2008)
14	Cellulase	<i>Acidothermus cellulolyticus</i>	Tobacco	Jin et al. (2003)
15	Bioelastic protein-based polymers (PBP)	PBP gene (synthetic)	Tobacco	Guda et al. (2000)

Targeted transformation to plastid genome was thought to be highly challenging in plants as a typical leaf contains more than 2000 copies of the plastid genome with more than 100 chloroplasts (reviewed in Kumar et al. 2017). Nevertheless, the success achieved in green algae *Chlamydomonas* was also achieved in model plant tobacco (Svab et al. 1990; Svab and Maliga 1993). Since then, these two platforms remained the models for plastid transformation for production of several therapeutic proteins and industrially important enzymes. Nevertheless, progress of plastid transformation in agriculturally relevant crops like cereals and monocots is in its infancy (Maliga and Bock 2011; Rigano et al. 2012; Bock 2014). The availability of sequences of plastome, generation of novel expression vectors and development of plastid transformation protocols in crop plants extended use of chloroplast engineering in economically important species.

Human papillomavirus (HPV) is the causative agent of cervical cancer, and HPV-E7 antigen is one of the major candidates for therapeutic vaccine production. For heterologous production of E7 in tobacco plastids, the expression of E7 as a translational fusion to β -glucuronidase enzyme was attempted. In addition, redirection of E7 into thylakoid lumen was also tried. The use of β -glucuronidase as a fusion protein turned out to be a successful strategy for improving E7 accumulation, and recombinant proteins accumulated ~40 times relative to unfused E7 (Morgenfeld et al. 2014). A high-risk HPV-16 candidate therapeutic vaccine (LALF32–51-E7) was developed by plastid targeting and resulted in 27-fold higher expression compared to cytosolic targeting in *Nicotiana benthamiana*. The authors proposed plastids-based production could be a more affordable therapeutic vaccine for HPV-16 (Yanez et al. 2018). Basic fibroblast growth factor (bFGF) accelerates cell proliferation and differentiation and hence possesses wide clinical applications. A codon-optimized bFGF gene was transformed to tobacco chloroplasts, and recombinant protein accumulation was observed (Wang et al. 2015). Serum antibodies developed in hemophilia B patients against coagulation factor IX (FIX) is highly challenging to eliminate due to nephrotic syndrome after continued infusion. Su et al. (2015) fused FIX with a transmucosal carrier (CTB) in lettuce chloroplast, and recombinant proteins accumulated up to ~1 mg/g. Moreover it was also demonstrated that feeding transgenic lettuce to hemophilia B mice delivered CTB-FIX effectively and not only induced LAP(+) regulatory T-cells but also suppressed IgE formation, anaphylaxis against FIX.

Toxoplasma gondii is an obligate intracellular parasite that causes toxoplasmosis. SAG1 is the main surface antigen in *T. gondii* and proposed as a promising vaccine candidate to produce anti-*T. gondii* vaccine. Transplastomic tobacco expressing SAG1 accumulated ~0.1–0.2 μ g protein. Further, transplastomic plants expressing a 90-kDa heat shock protein of *Leishmania infantum* (LiHsp83) fused to SAG1 resulted in antigen accumulation (up to 500-fold). Subsequent oral immunization of fusion protein elicited increase in levels of SAG1-specific antibodies (Albarracín et al. 2015). Similarly, Del et al. (2012) expressed *T. gondii* GRA4 antigen in tobacco chloroplast, and immunization elicited mucosal immune response resulting in production of specific IgA, interferon (IFN- γ), and interleukin (IL-4 and IL-10). Interleukin-2 (IL-2) is a T lymphocyte-derived cytokine. Tobacco expressing human

interleukin-2 (targeted to plastids) induced in vitro proliferation of IL-2-dependent murine T lymphocytes (Zhang et al. 2014).

There are approximately 36.9 million people living worldwide with acquired immunodeficiency syndrome (AIDS) in 2017 (UNAIDS). About 5000 new infections are being reported every day. Although human immunodeficiency virus (HIV) causes one of the most deadly infectious diseases, attempts to develop an effective vaccine remain unsuccessful till date. C4V3 is a protein known to induce systemic and mucosal immune responses during HIV infection. Rubio-Infante et al. (2012) expressed a synthetic gene encoding a C4V3 in tobacco plastids. The authors demonstrated that plant-derived C4V3 elicited both systemic and mucosal antibody responses in BALB/c mice. Further, CD4+ T cell proliferation responses were also reported. The authors strongly proposed plant chloroplasts as biofactories to produce HIV candidate vaccines. Human transforming growth factor- β 3 (TGF β 3) has high therapeutic value and is used to reduce scarring during wound healing. A synthetic gene with codons optimized for plastid expression resulted in accumulation of the 13-kDa TGF β 3 polypeptide by 75-fold in tobacco (Gisby et al. 2011).

Bacillus anthracis is the causative agent of anthrax, and vaccines are limited as most of them are potentially reactogenic (Gorantala et al. 2011). Gorantala and coworkers expressed domain IV of protective antigen gene [PA(dIV)] from *B. anthracis* in tobacco plastids leading to more than 5% of total soluble protein accumulation. Further, mice challenged with *B. anthracis* and immunized with adjuvanted plant PA(dIV) exhibited 60% and 40% protection upon intraperitoneal and oral immunizations, respectively. Expression of plague F1-V fusion antigen in chloroplasts resulted in ~15% of the total soluble protein. Mice were immunized with F1-V extracts and subsequently exposed to an inhaled aerosolized *Yersinia pestis*. It was interesting to note that 88% of the oral F1-V mice survived aerosolized *Y. pestis*, while all control mice died within 3 days (Arlen et al. 2008). Soria-Guerra et al. (2009) expressed a fusion DPT protein encoding immune-protective epitopes of *Corynebacterium diphtheriae*, *Bordetella pertussis*, and *Clostridium tetani* in tobacco chloroplasts. Transplastomic lines accumulated recombinant proteins, and mice orally immunized with leaf extract accumulated IgG and secretory antibodies specific to DPT toxin in serum and mucosal tissues.

Human thioredoxin 1 (hTrx1) is a stress-responsive protein that functions as an antioxidant during oxidative stress. Lettuce expressing *hTrx1* in chloroplast accumulated upto 1% total soluble protein and recombinant protein protected mouse insulinoma line 6 cells from peroxide-induced damage (Lim et al. 2011). Daniell et al. (2009a, b) reported expression of insulin-like growth factor-1 (IGF-1) in transgenic tobacco chloroplasts, and IGF-1 accumulation reached close to 11 total soluble proteins in transplastomic lines. Transplastomic lettuce and tobacco lines expressing cholera toxin B subunit-human proinsulin (CTB-Pins) fusion protein accumulated up to ~16% and 2.5%, respectively, in tobacco and lettuce. Oral administration of CTB-Pins extract exhibited decreased infiltration of cells characteristic of lymphocytes (insulinitis). Further, increased expression of IL-4 and IL-10 was observed in the pancreas of CTB-Pins-treated mice (Ruhlman et al. 2007).

4.4 Plastid as a Biofactory for Industrially Important Enzymes, Metabolites, and Enzyme Cocktails for Biofuel Production

Overproduction of industrially important enzymes through conventional nuclear transformation has met with little success as many reports suggest that recombinant enzymes can negatively affect transgenic plant growth and development. A gene encoding thermostable xylanase enzyme from *Bacillus* sp. when overexpressed in tobacco resulted in accumulation of xylanase up to 6% of the total soluble protein (Leelavathi et al. 2003). Similarly, Kim et al. (2011) demonstrated GH10 xylanase Xyl10B from *Thermotoga maritima* expressed in plastids exhibited high stability and catalytic activities. Further, endoglucanases like endo- β -1,4-xylanase and β -glucosidase expressed in tobacco plastids accumulated more than 75% of total soluble proteins. Subsequent bioconversion experiments confirmed that plastid-derived enzymes were able to hydrolyze industrially pretreated giant reed biomass (Castiglia et al. 2016). *Pyrococcus horikoshii* hyperthermostable archaeal β -1,4-endoglucanase expressed in tobacco plastids produced high levels of active enzymes that were even recovered from dry tissues (Nakahira et al. 2013). Another study reported transplastomic plants expressing four different thermostable cell wall-degrading enzymes from *Thermobifida fusca* accumulated up to 40% of total soluble protein. However, transplastomic lines exhibited pigment-deficient phenotypes (Petersen and Bock 2011). Klinger et al. (2015) proposed that enzymes of prokaryotic origin are efficiently expressed in plants than in a bacterial production platform. Espinoza-Sánchez et al. (2015) expressed pectin lyase and manganese peroxidase in tobacco plastids and recombinant enzymes exhibited improved enzyme activity. In another study, it was demonstrated that thermostable cellulases (*Cel6A* and *Cel6B*) from *Thermobifida fusca* expressed in tobacco chloroplasts were able to hydrolyze crystalline cellulose (Yu et al. 2007).

Plastome has been successfully engineered to produce important biomaterials. Bioelastic protein-based polymers (PBP) have huge industrial applications. These polymers are often used in soft tissue augmentation and regeneration. A synthetic PBP was targeted to both nucleus and plastids. It was confirmed that PBP transcripts accumulated up to 100-fold higher in transplastomic lines compared to nuclear transgenic plants (Guda et al. 2000). Despite the diversion of major metabolic pathway intermediate, metabolic engineering using chloroplast genomes led to production of several bioplastics. Bacterial genes encoding the polyhydroxybutyrate (PHB) pathway encoding enzymes were expressed in tobacco plastome. Transplastomic lines produced ~18.8% dry weight PHB (Bohmert-Tatarev et al. 2011). Another study reported expression of *Ralstonia eutropha* polyhydroxybutyrate operon in plastids and transplastomic lines produced biodegradable PHB (Mozes-Koch et al. 2017). Engineering chloroplast genome resulted in advancement of different biotechnological applications including production of thermostable industrial enzymes, biomaterials, and immunologicals. Nonetheless, how many of these will be approved for commercial level production and will reach market is still a matter of debate.

One of the important applications of plastid transformation is for the expression of enzyme for biofuel production. With the growing interest in biofuels, enzyme cocktails that can digest lignocellulosic biomass into fermentable sugars have gained much attention. A major breakthrough in biofuel production was the report from Verma et al. (2010) about chloroplast-derived enzyme cocktails for the production of fermentable sugars from different sources of lignocellulosic materials. The authors reported that cost of plastid-derived endoglucanase was estimated to be ~3000-fold lower than for the same commercially available recombinant enzymes (Verma et al. 2010). Verma et al. (2013) demonstrated treatment of cotton fiber with plastid produced cutinase exhibited enlarged segments and the intertwined inner fibers due to activity of cutinase. In addition, recombinant protein showed improved esterase and lipase activity in addition to its cutinase activity. Endo- β -mannanase enzyme cocktail mixture derived from chloroplast exhibited 20% more glucose equivalents formation from pinewood compared to cocktail without mannanase (Agrawal et al. 2011). Verma et al. (2010) reported plastid-derived enzyme cocktails yielded more than 3625% glucose from substrates like citrus peel and pine wood than commercially available enzyme cocktails. Transplastomic plants accumulating cell wall-degrading enzymes will be an alternate and cheap renewable source of enzymes for the production of cellulosic ethanol in the near future.

4.5 Updates on Plastid Transformation toward Improving Agricultural Traits

Chloroplast genome engineering has led to stable integration and expression of transgenes to confer valuable agronomic traits (Daniell et al. 2002; Maliga and Block 2011; and Jin and Daniell 2015). Poage et al. (2011) demonstrated expression of mitochondrial superoxide dismutase and *E.coli* glutathione reductase through plastid transformation in tobacco. The transformed lines exhibited increased radical scavenging activity thereby increasing tolerance to heavy metal stress and UV-B radiation. Expression of genes involved in secondary metabolites production has been demonstrated through plastid transformation. Tocochromanol pathway is introduced to plastids as single gene constructs into tobacco and tomato plants which resulted in tenfold higher accumulation of tocochromanol compared to controls (Lu et al. 2013). Plastomic transformation of acetolactate synthase (ALS) gene which catalyzes the first step in branched chain amino acid biosynthesis into the *Arabidopsis thaliana* conferred resistance against herbicides such as pyrimidinylcarboxylate and imidazolinon (Shimizu et al. 2008). Overexpression of cry9Aa2 (insecticidal protein) in chloroplasts of tobacco plants conferred resistance against *Phthorimaea operculella* (potato tuber moth), but this higher expression of protein delayed plant growth and development (Chakrabarti et al. 2006). Similarly, expression of cry1Ac protein through chloroplast transformation in rice resulted in enhanced resistance against common pests of rice skipper and caterpillar (Kim et al. 2009). In another study, transformation of *Pseudomonas pyrocinia* chloroperoxidase when expressed in tobacco plastids not only resulted in accumulation of recombinant protein but also improved

resistance toward *Alternaria Alternata*, *Aspergillus flavus*, *Fusarium* sp., and *Verticillium* sp. (Ruhlman et al. 2014).

Fathi Roudsari et al. (2009) generated improved glyphosate-resistant tobacco plants through biolistic transformation of chloroplasts by introducing a mutated herbicide-tolerant gene coding for EPSP synthase. p-Hydroxybenzoic acid (pHBA) is the major monomer in liquid crystal polymers. *E. coli ubiC* encoding chorismate pyruvate-lyase (CPL) was transformed to tobacco plastome. Total CPL accumulated up to 35% of total soluble protein and was 250 times higher compared to nuclear integration events. Authors reported that CPL integration to chloroplast genome provides a proof of concept of the high-flux potential of shikimate pathway for chorismate biosynthesis and also as a cost-effective route to pHBA production (Viitanen et al. 2004).

Astaxanthin is a rich antioxidant and occurs naturally in certain algae that underlie the red coloration of salmon and other organisms. Expression of different genes including β -carotene ketolase, β -carotene hydroxylase, and isopentenyl diphosphate isomerase from marine bacteria in lettuce plastome led to accumulation of astaxanthin fatty acid esters (Harada et al. 2014). Vitamin E (α , β , γ , and δ -tocopherols) are lipid-soluble antioxidants. Tocopherol biosynthetic machinery in plastids utilized precursors derived from two different metabolic pathways, homogentisic acid, an intermediate of shikimate pathway, and phytyldiphosphate, intermediate from methylerythritol phosphate pathway (Lushchak and Semchuk 2012). Expression of γ -tocopherol methyltransferase (γ -TMT) and tocopherol cyclase (TC) in tobacco and lettuce plastids resulted in improved α -tocopherol levels (Yabuta et al. 2013). Plastome expression of homogentisate phytyltransferase (HPT), TC, and γ -TMT confirmed HPT as the rate-limiting enzymatic step in tocopherol biosynthesis (Lu et al. 2013). Wurbs et al. (2007) demonstrated plastid expression of a bacterial lycopene β -cyclase in tomato-mediated conversion of lycopene to β -carotene and resulted in fourfold enhanced provitamin A content in fruits. Apel and Bock (2009) produced transplastomic tomato by overexpressing β -cyclase that resulted in an increase of up to 50% in provitamin A. Further, transplastomic plants accumulated ~50% increase in total carotenoid, and this could be possible by enhancing the flux through the pathway in chromoplasts. Overall, these findings highlight the potential of chloroplast engineering for production of high-value metabolites in plastid biofactories.

RNA interference (RNAi) technology was used for the first time to engineer the chloroplast genome by Jin et al. (2015). A lepidopteran chitin synthase (Chi), cytochrome P450 monooxygenase (CYP450), and V-ATPase were used as RNAi targets by the above group of researchers. In insects feeding assay in leaves of transplastomic lines, CYP450, Chi, and V-ATPase siRNAs, transcript levels were reduced to undetectable levels in insect midgut. In addition, net weight of the larvae and their growth and pupation were significantly reduced indicating success of technology. Zhang et al. (2015) introduced dsRNA via the chloroplast genome to target the insect β -actin gene and to subsequently elicit resistance against potato beetle. Transgenic potato lines producing dsRNAs targeted against the β -actin gene of the Colorado potato beetle, a deadlier agricultural pest, were successful. Chloroplast expression of long dsRNAs provided protection of potato lines without application of chemical

pesticides. A construct encoding ~200-nucleotide duplex-stemmed-hairpin (hp) RNAs, targeting the cholinesterase gene of *Helicoverpa armigera* (cotton bull worm), was targeted to both nuclear and plastid genome of *N. benthamiana*. Undiced, full-length hairpin RNAs (hpRNA) accumulated in *N. benthamiana* and conferred strong protection against *H. armigera* herbivory (Bally et al. 2016). Overall, successful expression of dsRNAs through plastome engineering opens the great possibility of using RNAi approaches to confer desired agronomic traits in plants.

4.6 Concluding Remarks and Future Focus

Engineering of plastid genome has taken a huge leap in plant biology owing to several advantages over nuclear genome engineering. In addition, recent availability of plastid genome sequence of many plants resulted in broadening the host range just from tobacco and lettuce to other crops like tomato, carrot, potato, cotton, soybean, etc. Despite the several advancements made in plastid transformation, there are still several major concerns that need to be addressed. One of the main disadvantages of plastome engineering is related to post-translational modification. Plastids lack the necessary machinery to glycosylate proteins, and absence of glycosylation is one of the major bottlenecks for the proper folding and functioning of glycoproteins like antibodies. Another major concern is stability of recombinant proteins in plastids. Chloroplast stroma contains several proteases that could degrade recombinant proteins produced. The expression of transgenes in non-green plastids is not as efficient as in green plastids (chloroplasts), and challenges related to poor gene expression in non-green plastid are still a major concern. Although the last two decades witnessed expression of a large number of vaccine antigens and therapeutic proteins in plastids (Table 4.2), hardly any chloroplast-made proteins completed clinical trials. This is due to the strict regulatory and IP issues related to the use of plant-made proteins for human consumption. Nevertheless, addressing some of the above concerns could lead to a new paradigm in plastome engineering and could contribute for the development of sustainable production of therapeutic proteins and industrial enzyme in plastids for human consumption and commercial exploitation, respectively.

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