Transplastomics: A Convergence of Genomics and Biotechnology

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Abstract

Transplastomics are developed predominantly for biotechnological applications since heterologous proteins can be expressed to high levels with *bona fide* structures and because of maternal inheritance of tailored traits in most of cultivated plants as rare gene leakage through pollens is experimentally witnessed. Further, advances in plastome sequencing and research have been exponential in the post-genomic era; hence, expressing multiple genes to develop biologically functional pharmaceuticals under strong promoters and translation control elements in operons is made possible. This chapter summarizes the developments from plastid genomics to gene expression and briefly describes how transplastome facilitates expression of vaccines, therapeutics, and plantibodies, in addition to tailoring agronomic traits in plants.

Keywords

Transplastomics • Plastome • Gene expression • Genomics • Biotechnology • Transplastomic biotechnology • Agronomic traits • Health traits

The Chloroplast Genome

Chloroplasts develop either from proplastids in meristematic tissues or from other differentiated plastids, such as chromoplasts, amyloplasts, and

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leucoplasts, on exposure to light. In a fully developed leaf cell, there may be as many as 100 chloroplasts, each with about 100 copies of the plastid genome, giving in total ~10,000 copies of the plastid genome per cell. Although, the plastid genome is very small with respect to the nuclear genome, it makes about 10–20 % of the total cellular DNA content because only two copies of nuclear genome exist in a diploid plant cell while it contains thousands of copies of plastid genome, thus extraordinarily increasing the ploidy level of the plastid genome: 50,000 copies of plastid DNA in a wheat leaf and 10,000 copies of plastid DNA in a single pea leaf (Bendich 1987). The chloroplast genome is a unique and double-stranded circular molecule of DNA which varies in size from 120 to 220 kb depending on the plant species (Palmer 1991; Sugiura et al. 1986; Sugiura 1992). The observed difference in size between different plastid DNA molecules is mainly due to the length of the inverted repeats. Two copies of a large inverted repeat (Palmer and Thompson 1982) divide the genome into four segments: the repeats, a small single-copy region, and a large single-copy region.

The complete nucleotide sequences of plastid DNA have been reported for a number of organisms, disclosing an enormous amount of functional and evolutionary information. The gene order in the tobacco chloroplast genome is perhaps the representative of land plants (Sugiura 1995). The chloroplast genomes encode about 30 transfer RNAs; 23S, 16S, 5S, and 4.5S ribosomal RNAs; about 21 ribosomal proteins; and 4 subunits of RNA polymerase. These RNAs together with ribosomal proteins enable the plastid to synthesize its own proteins. The chloroplast genome also encodes 30 proteins required for proper assembly of thylakoid complexes (Sugiura 1992) and the large subunit of Rubisco which catalyzes the first reaction in the pathway of carbon fixation. In addition, a set of eleven genes referred to as chlororespiratory genes, which resemble the genes for the respiratory chain NADH dehydrogenases in mitochondria, is found in the chloroplast genome. The genes *clpP* and *accD* encoding a putative subunit of an ATP-dependent protease and a subunit of acetyl-CoA carboxylase, respectively, have been identified by homology to *E. coli* proteins (Gray et al. 1990; Li and Coronan 1992). Genes required for light-independent conversion of protochlorophyllide to chlorophyllide have been identified in the chloroplast genome of black pine (Wakasugi et al. 1994; Khan 2013). In addition, all chloroplast genomes contain open reading frames (ORFs), some of which are conserved between species (Shimada and Sugiura 1991). The conserved ORFs have been called *ycfs*; *y* stands for hypothetical, *c* for chloroplast, and *f* for open reading frame. In recent years, most of these *ycfs* have been functionally analyzed through reverse genetics approaches.

The Chloroplast Genomics

Research on chloroplast genomics can be carried out at complete genome sequencing and functional genomics levels. The sequencing of the chloroplast genome of tobacco was commenced in the 1970s and completed in 1986. Based on complete nucleotide sequence information of tobacco plastome, initially 82 different genes from the tobacco chloroplast genome were identified. However, during the past 15 years, 31 additional genes have been identified, with only two genes per year (Wakasugi et al. 2001). This illustrates clearly that the identification of novel genes is an extremely strenuous task, requiring creative experimental approaches.

Complete sequences of two chloroplast genomes of liverworts *Marchantia polymorpha* and the angiosperm plant *Nicotiana tabacum* were the first targets for chloroplast functional genomics (Ohyama et al. 1986; Shinozaki et al. 1986). Striking similarities of chloroplast genomes with bacterial genomes (Schwarz and Kossel 1980) illustrate the prokaryotic origin of chloroplasts. Hence, based on sequence homology of chloroplast genes with bacterial genes, many plastid open reading frames were assigned with tentative functions. Nevertheless, the functions of those potential plastid genes that lack significant homology with known prokaryotic genes remained indescribable. For detailed functional analyses of plastid genes, reverse genetics

analysis is considered to be the most powerful tool in chloroplast functional genomics.

Since chloroplasts have acquired active homologous recombination and copy correction mechanisms (Cerutti et al. 1992), hence designing gene deletion or insertion or mutation cassettes and targeting the chloroplast genome exploiting chloroplast transformation approaches, the remaining *ycfs*/genes have been identified (Kuroda and Maliga 2002; Khan et al. 2007). Hence, the transformation technologies for chloroplasts (Boynton et al. 1988; Svab et al. 1990; Svab and Maliga 1993) have paved the way for addressing functional aspects of plastid genes and open reading frames by reverse genetics. Two model plants are being extensively exploited for chloroplast transformation technologies for reverse genetics studies, the unicellular green alga *Chlamydomonas reinhardtii* (Boynton et al. 1988) and *Nicotiana tabacum* (Svab et al. 1990; Svab and Maliga 1993). Reverse genetics technique works on two principles: (1) introduction of point mutations by site-directed mutagenesis of plastid genes and (2) inactivation of plastid-encoded genes by insertional or deletional mutagenesis ("gene knockout"). Using standard reverse genetics approaches based on homologous recombination, a number of genes and open reading frames have been targeted for their functions, including PSI, PSII, ndh, rpo, Ori, accD genes, and ycfs. However, some other approaches have also been employed that utilize copy correction and Cre/Lox systems to analyze plastid genes of unknown functions (Khan et al. 2007; Kuroda and Maliga 2003).

During functional genomics era, the mechanism of chloroplast gene expression has been the most interesting target to manage, but recently it is revealed that chloroplast gene expression is much more complex than previously thought, because there are multiple classes of promoters and RNA polymerases, multiple RNA processing steps (RNA cleavage/trimming, *cis/trans* splicing, RNA editing, and RNA stability), and multiple mechanisms for translational initiation (reviewed in Sugiura et al. 1998). In vitro systems supporting accurate transcription (Kapoor and Sugiura 1999), translation (Hirose and Sugiura 1996, 1997), and RNA editing (Hirose and Sugiura 2001) are now available from tobacco chloroplasts. These systems together with tobacco chloroplast transformation techniques (Svab and Maliga 1993; Khan and Maliga 1999) will provide powerful tools to elucidate further gene expression processes and nuclear factors responsible for chloroplast genome expression.

The Chloroplast Gene Expression

The chloroplast gene expression has many similarities to the gene expression in prokaryotes (Igloi and Kössel 1992; Gruissem and Tonkyn 1993). Two separate RNA polymerases, the plastid-encoded plastid RNA polymerase (PEP) and the nuclear-encoded plastid RNA polymerase (NEP), are responsible for transcribing the plastid genes. The photosynthesis genes are transcribed by an RNA polymerase containing plastid-encoded subunits homologous to α, β, and β' subunits of *E. coli* RNA polymerase (Shinozaki et al. 1986; Sijben-Muller et al. 1986; Ruf and Kossel 1988; Purton and Gray 1989). PEP promoters are similar to eubacterial δ^{70}-type promoters: the core is comprised of two hexameric sequences corresponding to the eubacterial −35 (TTGACA) and −10 (TATAAT) promoter elements. The hexamer are spaced 17–19 nucleotides apart and transcription initiation 5–7 nucleotides downstream of the −10 box sequence (as recorded in Gruissem and Tonkyn 1993).

The activity of RNA polymerase was characterized in its soluble and DNA-bound forms (Igloi and Kössel 1992; Gruissem and Tonkyn 1993), and the expression of the *rpo* genes was confirmed by detection of corresponding subunits in highly purified enzyme preparations from maize chloroplasts (Hu and Bogorad 1990; Hu et al. 1991) and by Western blotting of extracts from spinach chloroplasts (Briat et al. 1987). Evidence for the expression of the *rpo* genes in the form of the corresponding RNA (Hudson et al. 1988; Ruf and Kossel 1988) and of specific proteins in soluble chloroplast extracts (Ruf and Kossel 1988; Purton and Gray 1989)

confirmed that the core subunits of a chloroplast RNA polymerase are encoded in the chloroplast genome. Further, the δ ^{70}-like factors required for promoter recognition (Tiller et al. 1991) are encoded in the nucleus (Tanaka et al. 1997).

The other polymerase, the NEP is related to the mitochondria and phage-type T3/T7 RNA polymerases (Lerbs-Mache 1993). PEP is derived from the RNA polymerase of the ancestral bacterium. It is assumed that the phage-type plastid RNA polymerase evolved by duplication of the nuclear gene encoding the mitochondrial enzyme and retargeting of the gene product to plastid (Hedtke et al. 1997). Several plastid promoters have been shown to direct the transcription of genes in prokaryotic cells (Thompson and Mosig 1988). Transcript levels from these promoters were decreased by cycloheximide, a cytoplasmic protein synthesis inhibitor, providing further evidence for a non-consensus-type plastid promoter (Kapoor et al. 1997). More recently, it has been shown that many plastid genes and operons have at least one promoter each for *E. coli*-like RNA polymerase and nuclear-encoded plastid RNA polymerase (Hajdukiewics et al. 1997).

Transcription of plastid genes by one or both RNA polymerases reflects their function. PEP transcribes Photosystem I and II genes; therefore, it plays an important role in chloroplast gene expression. In the absence of the PEP, non-photosynthetic proplastids are still maintained indicating that essential housekeeping genes are transcribed by the NEP. Indeed, most non-photosynthetic genes have promoters for both RNA polymerases. Only a few genes are known to be transcribed exclusively from an NEP promoter, *accD*, encoding a subunit of the acetyl-CoA carboxylase in dicots (Hajdukiewics et al. 1997). It is assumed that the phage-type plastid RNA polymerase evolved from the mitochondria enzyme (Hedtke et al. 1997) and the transcription of PEP genes by the NEP was probably a critical step in the nucleus indirectly taking control of the transcription of plastid genes, thereby fully integrating plastids in multicellular plants (Shiina et al. 2005).

Reproducible chloroplast transformation approaches have facilitated the study of chloroplast gene expression elements including promoters and UTRs (Untranslated Regions) analyzed by fusing with reporter genes (Monde et al. 2000; Khan and Maliga 1999). Heterologous translation-enhancing sequences, for example, bacteriophage T7 gene *10* leader sequence known to promote high-level protein accumulation in bacteria (Studier et al. 1990), have been fused with reporter genes, and Western blot analyses revealed the accumulation of ~16 to 18 % of total soluble proteins (Kuroda and Maliga 2001; Khan and Maliga 1999). Further studies have confirmed this where translational fusion of the 14 N-terminal amino acids of the chloroplast *rbcL* and *atpB* genes to a reporter sequence resulted in different levels of reporter protein accumulation, but they are not attributable to differences in transcript abundance. Furthermore, silent mutations in the fused N-terminal coding sequences were found to decrease reporter protein accumulation without influencing RNA level (Kuroda and Maliga 2001).

The Transplastomics in Outline

Chloroplast transformation henceforth will be referred to as a plastid transformation since chloroplasts are developed from other plastid types, as described earlier. The chloroplast transformation is carried out either by targeting plastids in dividing cells or mature cells of fully expanded leaves. Plastid transformation vectors are derivatives of *E. coli* plasmids with cloned plastid DNA sequences that flank both sides of a selectable marker gene and gene/s of interest with cloning sites. The flanking sequences serve as targeting regions that catalyze the integration of the marker gene and the gene of interest into the plastid genome at a predetermined site by two events of homologous recombination. Since the backbone of the vector that carries *E. coli* DNA sequences does not carry any plastid replication origin, hence it is subsequently lost (Maliga 2004).

The genetic transformation of plastid genome requires (1) a method to deliver the DNA of the plastid transformation vector through the cell wall, the plasma membrane, and the double

membranes of the organelle, (2) a plastid-specific selectable marker gene to promote sorting of transformed and wild-type genome copies, and (3) a highly efficient tissue culture system. To date, the particle bombardment, a physical method of gene delivery, is widely used to engineer plastid genomes in a number of plant species (Daniell et al. 2002; Bock and Khan 2004; Khan et al. 2011). In the method, DNA is coated onto the surface of the inert metal particles, which are subsequently placed onto the surface of the macrocarriers. The macrocarriers are placed along with a stopping screen in the macrocarrier assembly. A rupture disk of appropriate pressure, normally of 1,100 psi, is used to develop the pressure that propels the particles down toward the particles carrying a macrocarrier. The particles hit the surface of the target leaf with a velocity optimized for high transformation efficiencies. The bombarded leaves are then placed in the dark. Leaves are cut into small pieces and placed on antibiotic-containing regeneration medium after 48 h of bombardments. Antibiotic-resistant shoots start appearing within 4–6 weeks of bombardment. Tiny shoots are excised from the bleached leaf sections and shifted to jars for proliferation; leaves from the proliferated shoots are either for genetic analysis or for further round of selection and regeneration to purify the transformed genome from wild-type genomes. During the process, sorting at genome, plastid, and cell levels is carried out, and the purified homoplasmic shoots for transgene are identified for further analyses as per designed experiments.

Genetic markers are either lethal or nonlethal markers used for primary selection of transformation events. These genetic markers provide resistance against spectinomycin, streptomycin, and kanamycin which inhibit protein synthesis on prokaryotic-type plastid ribosomes. These drugs inhibit greening, faster proliferation, and shoot formation in tobacco culture, which are the selection parameters to identify transplastomic clones on a selective medium. Spectinomycin selection is nonlethal that is widely used in selecting the transformed cells. However, kanamycin selection is a lethal selection that has also been used for the selection of transplastomic lines (Carrer et al. 1993). The *aadA* that encodes aminoglycoside 3′′-adenylyltransferase and confers resistance to spectinomycin or streptomycin (Goldschmidt-Clermont 1991; Svab et al. 1990), the *neo* gene that encodes neomycin phosphotransferase II (Carrer et al. 1993) and confers resistance to kanamycin, and the *aphA-6* gene that encodes aminoglycoside phosphotransferase and confers resistance to kanamycin and amikamycin (Bateman et al. 2000) are commonly used selectable marker genes.

In addition to selectable markers, reporter genes also contribute to the development of the technology by serving as tools for visual monitoring of transgene expression in transformed cells, tissues, and organisms. A number of genes have been used to study gene expression in plants, e.g., the genes encoding β-glucuronidase, GUS (*uid*A) and β-galactosidase (*lac*Z), chloramphenicol acetyltransferase *(cat)* and neomycin phosphotransferase (*nptII*), nopaline synthase (*nos*), octopine synthase (*ocs)*, and luciferase (*luc)*, as reporter genes. Of these, *uidA* has been expressed transiently (Seki et al. 1995) and stably in tobacco chloroplasts (Staub and Maliga 1994). However, histochemical detection of GUS in chloroplasts requires prolonged incubation because the chloroplast envelope membranes act as a selective barrier to substrate penetration into the chloroplasts. The use of nontoxic marker to identify transgenic cells after transformation is an effective procedure for discerning transformed cell/organs and removing untransformed or nonexpressing cells, tissues, or organs. Several chloroplast promoters have been shown to direct the transcription initiation of reporter genes in prokaryotic cells (Thompson and Mosig 1988). The green fluorescent protein *(gfp)* of the jellyfish, *Aequorea victoria*, has been introduced as a reporter gene in plants (Khan and Maliga 1999). The *gfp* provides an easily scored genetic marker in plants and major uses in monitoring gene expression, protein localization, and screening of transformation events at high resolution. It allows the direct imaging of the fluorescent gene product in living cells without the need for prolonged and lethal histochemical staining procedures (Chalfie et al. 1994). The chromophore forms

autocatalytically in the presence of oxygen and fluoresces green (508 nm) on absorption of blue or UV light of 395 nm. This protein has successfully been expressed in *E. coli* and chloroplasts of tobacco, potato, and rice (Khan and Maliga 1999) using chloroplast-specific expression signals. In a previous study, it was observed that a bacterial promoter was able to express reporter gene *gfp* successfully (Khan 2001). The development of a gene encoding bifunctional proteins can minimize the use of different set of promoters and terminators and may result in plastid DNA fragment deletion through homologous recombination due to homology with plastid DNA and physical separation of genes. Such a gene will facilitate both the selection and visual screening of recipient cells; therefore, a bifunctional protein was engineered through translational fusion of aa*d*A and *gfp* genes called FLARE-S. This bifunctional protein facilitates plastid transformation to rice in addition to tobacco, where plastid transformation is not associated with a readily identifiable phenotype (Khan and Maliga 1999).

The Transplastomic Biotechnology

After the plastid transformation was achieved in *Chlamydomonas reinhardtii* (Boynton et al. 1988) in 1988, a stable chloroplast transformation was achieved in tobacco using *aadA*, a gene of bacterial origin, which encodes aminoglycoside 3″-adenylyltransferase and confers resistance to spectinomycin and streptomycin (Svab and Maliga 1993) and a visual reporter gene encoding green fluorescent protein from jellyfish (Khan 1997), which facilitated the extension of plastid transformation to nongreen plastids with transient (Hibbered et al. 1998) and stable expression (Khan and Maliga 1999) of the gene. During the period, chloroplast transformation was also carried out to study the function of plastid genes and to express genes coding for industrially valuable enzymes, biomaterials, biopharmaceutical proteins, antibodies, antibiotics, vaccine antigens, and genes that confer important agronomic traits (Bock 2001; Daniell et al. 2002; Khan 2012). Now plastid transformation has been established in number of plant species, as detailed in Table 1. Salient examples of biotechnological applications of the technology are described in the following sections of the chapter.

Transplastomics Conferring Resistance Traits

A number of crystal toxin proteins of *Bacillus thuringiensis* have been expressed and commercialized through nuclear genomes of plants in the recent years, owing to their advantages over traditional chemical insecticides. However, low levels of expression of toxin proteins have shown concerns of developing resistance in feeding insects against those toxins. Such evolving levels of resistance development in insects may be addressed by expressing those proteins from chloroplast genomes, known to express transproteins to high levels due to polyploidy of the genome (Daniell et al. 2002). The chloroplast genome was exploited for its biotechnological applications for the first time when a native *Bacillus* gene was expressed to an unprecedented level from tobacco chloroplasts (McBride et al. 1995), and the plants were extremely toxic to larvae of *Heliothis virescens*, *Helicoverpa zea*, and *Spodoptera exigua* as demonstrated by the insect feeding experiments; there was no need of codon optimization of toxin genes since they are of prokaryotic origin (Kota et al. 1999; De Cosa et al. 2001). In these experiments, Daniell and colleagues reported transgene expression to the level of 45 % of the total soluble protein (TSP) when the transgene, *cry2Aa2*, was expressed in an operon along with two open reading frames, as was natively expressed in the *Bacillus*, which potentially encodes a chaperonin protein, capable of correct folding of the toxin protein that ultimately leads toward crystallization of the toxin protein inside the chloroplasts (De Cosa et al. 2001). Hence, exceedingly difficult to control insects (10-day old cotton bollworm, armyworm) were killed 100 % when fed on transgenic tobacco leaves, extensively reviewed elsewhere (Daniell et al. 2002). Encouraged from such experiments on model plant tobacco,

Table 1 Targeting sequences from plastid genomes and the transplastomic plants

Crop plants	Targeted tissues	Targeting sites	References
Tobacco	Leaves	*rbcL-accD*	Svab and Maliga (1993)
Arabidopsis	Leaves	*trnV-rps12/7*	Sikdar et al. (1998)
Rice	Embryogenic cells	*trnV-rps12/7*	Khan and Maliga (1999)
Potato	Leaves	*rbcL-accD*	Sidorov et al. (1999)
		trnV-3,rps12	
Tomatoes	Leaves	*trnfM-trnG*	Ruf et al. (2001)
Lesquerella	Leaves	*trnV-rps12/7*	Skarjinskaia et al. (2003)
Oilseed rape	Cotyledon petioles	*rps7-ndhB*	Hou et al. (2003)
Cotton	Embryogenic calli	*trnI-trnA*	Kumar et al. (2004b)
Petunia	Leaves	*rbcL-accD*	Zubkot et al. (2004)
Carrot	Embryogenic cells	*trnI-trnA*	Kumar et al. (2004a)
Soybean	Embryogenic tissues	*trnV-rps12/7*	Dufourmantel et al. (2004)
Lettuce	Protoplasts	*trnI-trnA*	Lelivelt et al. (2005)
Cauliflower	Protoplasts	*rbcL-accD*	Nugenta et al. (2006)
Poplar	Calli	*trnI-trnA*	Okumura et al. (2006)
Cabbage	Leaves	*rrn16S-rrn23S*	Liu et al. (2007)
Sugar beet	Leaf petioles	*rrn16-rps12*	De Marchis et al. (2009)
Eggplant	Green stem segments	*trnV-3′rps12*	Singh et al. (2010)
Alfalfa	Leaves and calli	*trnI-trnA*	Wei et al. (2011)
Sugarcane	Embryogenic calli	*trnI-trnA*	Mustafa (2011)
Wheat	Immature embryos and immature inflorescences	*atpB-rbcL*	Cui et al. (2011)

Dufourmantel et al. (2005) expressed *cry1Ab* gene from soybean chloroplasts under the control of complete promoter of 16S ribosomal RNA gene with few mutations, fused with a ribosome binding site (RBS) from the bacteriophage t7 gene 10 leader (Ye et al. 2001) and the 3′ untranslated region of *rbcL* gene from tobacco. Transgenic soybean plants showed strong insecticidal activity in velvet bean caterpillars.

When it comes to developing transgenic plants to fight against pathogens, the progress made to date is promising for antifungal activity (Cary et al. 2000); nevertheless, bacteria manage to adapt to plant defense mechanisms. Therefore, it is needed to express such molecules from plant genomes that could bind to the bacterial surface and cause their lysis. One of the molecules' AMP (antimicrobial peptide) is an amphipathic alpha-helix that can bind to negatively charged phospholipids from outer membranes of bacteria and fungi. In a concentration-dependent fashion, these molecules aggregate to form pores in the membrane and cause microbial lysis. Considering the concentration-dependent action of this peptide, it was expressed in tobacco chloroplasts reportedly to levels of 21.5–43 % of the total soluble protein (De Gray et al. 2001) and retained biological activity against *Pseudomonas syringae* and other pathogens.

Weeds are a serious threat to crop production, competing for food and light. One of the accepted strategies to manage weeds is the expression of herbicide-resistant genes from plant genomes since crops are susceptible to broad-spectrum herbicides. Glyphosate is a widely used broad-spectrum herbicide that acts as a potent inhibitor of the plant aromatic amino acid biosynthetic pathway by competitively inhibiting the key enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and does not distinguish crops from weeds, thereby restricting its use. EPSPS is a plastid-targeting nuclear-encoded gene in plants; hence, the feasibility of expressing the gene from plastid genome was explored (Daniell

et al. 1998). In the attempt, a wild-type EPSPS gene from petunia was expressed in tobacco, and the transgenic plants were reported to be resistant to glyphosate 10-fold higher than the lethal concentration. In another study, *Agrobacterium* EPSPS gene (C4) was expressed from tobacco chloroplasts, and the plants exhibited tolerance to field dose of glyphosate (Ye et al. 2001). Pal Maliga and colleagues explored the possibility of expressing a bacterial gene, *bar*, encoding the herbicide inactivating phosphinothricin acetyltransferase (PAT) enzyme from tobacco chloroplasts to confer tolerance to glufosinate (Lutz et al. 2001). The transgenic plants expressing the enzyme to the level of 7 % of the total soluble protein conferred field level tolerance to phosphinothricin (PPT), the active ingredient in the Liberty (Lutz et al. 2001); nevertheless, attempts to develop herbicide resistance genes as selectable markers for chloroplast transformation have failed. Reasons are not known why direct selection of chloroplast transformation events on PPT-containing medium has failed since direct selection for PPT-resistant nuclear transformants in maize (Spencer et al. 1990), wheat (Vasil et al. 1992), rice (Cao et al. 1992), barley (Wan and Lemaux 1994), and sugarcane (Khan et al. 2011) has been reported.

Transplastomics Conferring Medicinal Traits

The human serum albumin was the first recombinant plant-derived protein expressed from transgenic tobacco and potato plants in 1990 (Sijmons et al. 1990). Since the transgenic crops were allowed to cultivate for open field trials in 1992, the U S Department of Agriculture approved planting of recombinant protein containing transgenic crops in every state. In the proof of concept, several therapeutic proteins, including growth hormones, cytokines, antibodies, recombinant enzymes, and human and veterinary vaccines, have been expressed from plants (Twyman et al. 2003).

Resistance conferring proteins have been expressed successfully to exceptionally high levels from chloroplast genome of tobacco, representing an unprecedented opportunity to manufacture affordable modern medicines and make these available cost-effectively at the world level. In a proof of concept, fragment C of the tetanus toxin (TetC), which is already known to be a good antigen against *Clostridium tetani*, the causal agent of tetanus, was expressed from the tobacco chloroplast genome to levels of 10–25 % of the total soluble protein (Tregoning et al. 2003), using varied gene contents. Similarly, promising progress has been made with developing a chloroplast-based vaccine for anthrax. Anthrax is a zoonotic disease transmitted from animals to human and is caused by *Bacillus anthracis*, a gram-positive spore-forming organism. The virulent strain of *Bacillus anthracis* carries plasmids: pX01 and pX02. Of these plasmids, pX01 harbors *pagA*, *lef*, and *cya* genes that encode protective antigen (PA), lethal factor (LF), and edema factor (EF), respectively. None of these proteins are toxic when administered individually to cells or animals. However, PA in combination with EF, known as edema toxin, causes edema. Similarly, PA in combination with LF forms LT, the lethal toxin, extensively reviewed elsewhere (Collier and Young 2003). The term "protective antigen" is derived because of this protein's ability to elicit a protective immune response against anthrax. Considering the importance of the subject, Daniell and colleagues expressed PA in transgenic tobacco by inserting the *pagA* gene into the chloroplast genome. Chloroplast integration of the *pagA* gene was confirmed by PCR as well as by doing Southern blotting. Mature leaves grown under continuous illumination contained PA up to 14.2 % of the total soluble protein. The efficacy of the plant-derived PA was compared with that of PA derived from *B. anthracis* in both in vitro and in vivo studies (Koya et al. 2005), and the chloroplast-derived PA was found equally effective to PA derived from *B. anthracis*. Posttranslational modifications are vital for several proteins to be immunogenic; one of the examples is the outer surface lipoprotein A (OspA) from the pathogenic bacterium *Borrelia burgdorferi*, which has been used as a vaccine

against Lyme disease. Chloroplast-based OspA protein together with an adjuvant was subcutaneously injected in mice that induced protective antibodies at levels that should be sufficient to protect the animals from *B. burgdorferi* (Glenz et al. 2006). Cholera toxin B (CTB) acts as a strong mucosal adjuvant, and its nontoxic B subunit when fused with antigens for mucosal immunization has immunostimulatory effects (Freytag and Clements 2005). It is therefore encouraging that CTB can be expressed to high levels in tobacco chloroplasts both alone (Daniell et al. 2001; Takahashi et al. 2009) and as a fusion protein (Glenz et al. 2006).

Several chloroplast-derived biopharmaceutical proteins have been reported. A protein-based polymer, GVGVP, that has medical uses such as wound coverings, artificial pericardia, and programmed drug delivery, was stably expressed in chloroplasts of tobacco (Guda et al. 2000). Similarly, human somatotropin (hST), a secretory protein, was expressed from chloroplasts in a soluble and biologically active form (Staub et al. 2000). The key use of hST is in the cure of hypopituitary dwarfism in children; additional indications are treatment of Turner syndrome, chronic renal failure, and human immunodeficiency virus wasting syndrome. Another important therapeutic protein that comprises approximately 60 % of the protein in blood serum is HSA, prescribed in multigram quantities to restore blood volume in trauma and other clinical conditions. Early attempts at expressing HSA have achieved inadequately low levels of HSA (Human Somatotropin, 0.2 % of tsp) in nuclear transgenic plants (Farran et al. 2002). Nevertheless, in chloroplast transgenic plants, the expressed protein was harvested to the levels of 11.2 % of the total soluble protein (Fernandez-San Millan et al. 2003). Similarly, attempts have been made to express interferon alpha 5 and 2 genes from chloroplasts, but the expression levels were found suboptimal (Nurjis and Khan 2011; Khan and Nurjis 2012), suggesting to reengineer the 5′ ends of the genes.

Oral delivery of pharmaceuticals expressed from chloroplasts required extension of the technology edible plants. Recently, chloroplast transformation has been developed in potato (Sidorov et al. 1999), tomato (Ruf et al. 2001), and carrot (Kumar et al. 2004a; Usman and Khan, unpublished). A green fluorescent protein was expressed from plastids of all three plants; however, betaine aldehyde dehydrogenase (BADH) was also expressed from carrot plastids (Kumar et al. 2004a). Thus, transgenes could be expressed from fruit chromoplasts, carrot roots, and chromoplast-containing plant tissues when routine plastid transformation protocols become available.

Perspective

Complete chloroplast genome sequencing made it possible to compare the chloroplast genome sequences with that of bacteria. Based on sequence homology of chloroplast ycfs/genes with bacterial genes, plastid genes except of divergent sequences were assigned functions. Nevertheless, these genes were analyzed using reverse genetics approaches when the chloroplast transformation was developed. Additionally, chloroplast transformation made it possible to express foreign genes for biotechnological applications in the sectors of agriculture, industry, environment, and health. Contrary to dicotyledonous plants, the plastid transformation in monocots is at its early development because of a number of reasons, explained in details elsewhere (Khan 2012).

References

Bateman A, Birney E, Durbin R, Eddy SR, Howe KL, Sonhammer ELL (2000) The Pfam protein families database. Nucleic Acids Res 28:263–266

Bendich AJ (1987) Why do chloroplasts and mitochondria contain so many copies of their genome? BioEssays 6:279–282

Bock R (2001) Transgenic chloroplasts in basic research and plant biotechnology. J Mol Biol 312:425–438

Bock R, Khan MS (2004) Taming plastids for a green future. Trends Biotechnol 22:311–318

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988)

Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. Science 240:1534–1538

Briat J-F, Bisanz-Seyer C, Laulhere J-P, Lerbs S, Lescure A-M, Mache R (1987) The RNA polymerase from chloroplasts and its use for in vitro transcription of plastid genes. Plant Physiol 25:273

Cao J, Duan XL, McElroy D, Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep 11:586–591

Carrer H, Hockenberry TN, Svab Z, Maliga P (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. Mol Gen Genet 241:49–56

Cary JW, Rajasekaran K, Jayens JM, Cleveland TE (2000) Transgenic expression of a gene coding a synthetic antimicrobial peptide results in inhibition of fungal growth in vitro and in planta. Plant Sci 154:171–181

Cerutti H, Osman M, Grandoni P, Jagendorf AT (1992) A homolog of *Escherichia coliRecA* protein in plastids of higher plants. Proc Natl Acad Sci U S A 89:8068–8072

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805

Collier RJ, Young JA (2003) Anthrax toxin. Annu Rev Cell Dev Biol 19:45–70

Cui C, Song F, Tan Y, Zhou X, Zhao W, Ma F, Liu Y, Hussain J, Wang Y, Yang G, He G (2011) Stable chloroplast transformation of immature scutella and inflorescences in wheat (Triticum aestivum L.). Acta Biochim Biophys Sin 43:284–291

Daniell H, Datta R, Verma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol 16:345–348

Daniell H, Lee S-B, Panchal T, Wiebe PO (2001) Expression of cholera toxin B subunit oligomers in transgenic tobacco chloroplasts. J Mol Biol 311:1001–1009

Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci 7:84–91

De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Hyper-expression of the Bt Cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19:71–74

De Gray G, Rajasekaran K, Smith F, Sanford J, Daniell H (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol 127:852–862

De Marchis F, Wang Y, Stevanato P, Arcioni S, Bellucci M (2009) Genetic transformation of the sugar beet plastome. Transgenic Res 18:17–30

Dufourmantel N, Pelissier B, Garcon F, Peltier G, Ferullo JM, Tissot G (2004) Generation of fertile transplastomic soybean. Plant Mol Biol 55:479–489

Dufourmantel N, Tissot G, Goutorbe F, Garcon F, Muhr C, Jansens S, Pelissier B, Peltier G, Dubald M (2005) Generation and analysis of soybean plastid transformants expressing *Bacillus thuringiensis* Cry1Ab protoxin. Plant Mol Biol 58:659–668

Farran I, Sanchez-Serrano JJ, Medina JF, Prieto J, Mingo-Castel AM (2002) Targeted expression of human serum albumin to potato tubers. Transgenic Res 11:337–346

Fernandez-San Millan A, Mingo-Castel A, Miller M, Daniell H (2003) A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol J 1:71–79

Freytag LC, Clements JD (2005) Mucosal adjuvants. Vaccine 23:1804–1813

Glenz K, Bouchon B, Stehle T, Wallich R, Simon MM, Warzecha H (2006) Production of a recombinant bacterial lipoprotein in higher plant chloroplasts. Nat Biotechnol 24:76–77

Goldschmidt-Clermont M (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of Chlamydomonas. Nucleic Acids Res 19:4083–4089

Gray JC, Hird SM, Dyer TA (1990) Nucleotide sequence of a wheat chloroplast gene encoding the proteolytic subunit of an ATP-dependent protease. Plant Mol Biol 15:947–954

Gruissem W, Tonkyn J (1993) Control mechanisms of plastid gene expression. Crit Rev Plant Sci 12:19–55

Guda C, Lee SB, Daniell H (2000) Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. Plant Cell Rep 19:257–262

Hajdukiewics P, Allison LA, Maliga P (1997) The two plastid RNA polymerases encoded by the nuclear and plastid compartments transcribe distinct groups of genes in tobacco plastids. EMBO J 16:4041–4048

Hedtke B, Borner T, Weihe A (1997) Mitochondrial and chloroplast phage-type RNA polymerases in Arabidopsis. Science 277:809–811

Hibbered JM, Linley PJ, Khan MS, Gray JC (1998) Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. Plant J 16:627–632

Hirose T, Sugiura M (1996) Cis-acting elements and trans-acting factors for accurate translation of chloroplast *psbA* mRNAs: development of an in vitro translation system from tobacco chloroplasts. EMBO J 15:1687–1695

Hirose T, Sugiura M (1997) Both RNA editing and RNA cleavage are required for translation of tobacco chloroplast ndhD mRNA: a possible regulatory mechanism for expression of a chloroplast operon consisting of functionally unrelated genes. EMBO J 16:6804–6811

Hirose T, Sugiura M (2001) Involvement of a site-specific *trans*-acting factor and a common RNA-binding protein in the editing of chloroplast mRNAs: develop-

ment of a chloroplast *in vitro* RNA editing system. EMBO J 20:1144–1152

Hou BK, Zhou YH, Wan LH, Zhang ZL, Shen GF, Chen ZH, Hu ZM (2003) Chloroplast transformation in oilseed rape. Transgenic Res 12:111–114

Hu J, Bogorad L (1990) Maize chloroplast RNA polymerase: the 180-, 120-, and 38-kilodalton polypeptides are encoded in chloroplast genes. Proc Natl Acad Sci U S A 87:1531–1535

Hu J, Troxler RF, Bogorad L (1991) Maize chloroplast RNA polymerase: the 78-kilodalton polypeptide is encoded by the plastid rpoCl gene. Nucleic Acids Res 19:3431–3434

Hudson GS, Holton TA, Whitfeld PR, Bottomley W (1988) Spinach chloroplast rpoB/C genes encode three subunits of the chloroplast RNA polymerase. J Mol Biol 200:639–654

Igloi GL, Kössel H (1992) The transcriptional apparatus of chloroplast. Crit Rev Plant Sci 10:525–558

Kapoor S, Sugiura M (1999) Identification of two essential sequence elements in the non-consensus type II PatpB-290 plastid promoter by using plastid transcription extracts from cultured tobacco BY-2 cells. Plant Cell 11:1799–1810

Kapoor S, Suzuki JY, Sugiura M (1997) Identification and functional significance of a new class of non-consensus-type plastid promoters. Plant J 11:327–337

Khan MS (1997) Tobacco chloroplast transformation using microprojectile bombardment. PhD dissertation, University of Cambrige, UK

Khan MS (2001) Utilizing heterologous promoters to express green fluorescent protein from jellyfish in tobacco chloroplasts. Pak J Bot 33:43–52

Khan MS (2012) Plastid genome engineering in plants: present status and future trends. Mol Plant Breed 3:91–102

Khan MS (2013) Towards engineering dark-operative chlorophyll synthesis pathway in transgenic plastids. In: Barh D et al (eds) OMICS: applications in biomedical, agricultural and environmental sciences. Taylor & Francis, New York, pp 423–436

Khan MS, Maliga P (1999) Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nat Biotechnol 17:910–915

Khan MS, Nurjis F (2012) Synthesis and expression of recombinant interferon alpha-5 gene in tobacco chloroplasts, a non-edible plant. Mol Biol Rep 39:4391–4400

Khan MS, Hameed W, Nozoe M, Shiina T (2007) Disruption of the *psbA* gene by the copy correction mechanism reveals that the expression of plastid-encoded genes is regulated by photosynthesis activity. J Plant Res 120:421–430

Khan MS, Ali S, Iqbal J (2011) Developmental and photosynthetic regulation of Bacillus thuringiensis δ-endotoxin reveals that engineered sugarcane conferring resistance to ` dead heart` contains no toxins in cane juice. Mol Biol Rep 38:2359–2369

Kota M, Daniell H, Varma S, Garczynski SF, Gould F, Moar WJ (1999) Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc Natl Acad Sci U S A 96:1840–1845

Koya V, Moayeri M, Leppla SH, Daniell H (2005) Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect Immun 73:8266–8274

Kumar S, Dhingra A, Daniell H (2004a) Plastid-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol 136:2843–2854

Kumar S, Dhingra A, Daniell H (2004b) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol 56:203–216

Kuroda H, Maliga P (2001) Sequences downstream of the translation initiation codon are important determinants of the translation efficiency in chloroplasts. Plant Physiol 125:430–436

Kuroda H, Maliga P (2002) Overexpression of the clpP 5-untranslated region in a chimeric context causes a mutant phenotype. Suggesting competition for a clpP-specific RNA maturation factor in tobacco chloroplasts. Plant Physiol 129:1600–1606

Kuroda H, Maliga P (2003) The plastid clpP1 protease gene is essential for plant development. Nature 425:86–89

Lelivelt C, McCabe M, Newell C, DeSnoo C, Dun K, Birch-Machin I, Gray J, Mills K, Nugent J (2005) Stable plastid transformation in lettuce (Lactuca sativa L.). Plant Mol Biol 58:763–774

Lerbs-Mache S (1993) The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: Single-subunit enzyme or catalytic core of multimeric enzyme complexes? Proc Natl Acad Sci U S A 90:5509–5513

Li S-J, Coronan JE (1992) A putative zing finger protein encoded by a conserved chloroplast gene is very likely a subunit of a biotin-dependent carboxylase. Plant Mol Biol 20:759–761

Liu CW, Lin CC, Chen J, Tseng MJ (2007) Stable chloroplast transformation in cabbage (Brassica oleracea L. var. capitata L.) by particle bombardment. Plant Cell Rep 26:1733–1744

Lutz KA, Knapp J, Maliga P (2001) Expression of bar in the plastid genome confers herbicide resistance. Plant Physiol 125:1585–1590

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55:289–313

McBride KE, Svab Z, Schaaf DJ, Hoga PS, Stalker DM, Maliga P (1995) Amplification of a chimeric Bacillus gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology 13:362–365

Monde RM, Schuster G, Stern DB (2000) Processing and degradation of chloroplast mRNA. Biochimie 82:573–582

Mustafa G (2011) Development of plastid transformation in sugarcane. PhD dissertation, Quaid-i-Azam University, Islamabad

Nugenta GD, Coyne S, Nguyen TT, Kavanaghb TA, Dix PJ (2006) Nuclear and plastid transformation of Brassica oleracea var. botrytis(cauliflower) using PEG-mediated uptake of DNA into protoplasts. Plant Sci 170:135–142

Nurjis F, Khan MS (2011) Expression of recombinant interferon alpha-2a in tobacco chloroplasts using microprojectile bombardment. Afr J Biotechnol 10:17016–17022

Ohyama K, Fukuzawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono K, Shiki Y, Takeuchi M, Chang Z, Aota S, Inokuchi H, Ozeki H (1986) Chloroplast gene organization deduced from complete nucleotide sequence of liverwort Marchantia polymorpha chloroplast DNA. Nature 322:572–574

Okumura S, Sawada M, Park Y, Hayashi T, Shimamura M, Takase H, Tomizawa KI (2006) Transformation of poplar (Populus alba) plastids and expression of foreign proteins in tree chloroplasts. Transgenic Res 15:637–646

Palmer JD (1991) Plastid chromosomes: structure and evolution. In: Bogorad L, Vasil IK (eds) The molecular biology of plastids, vol 7A, Cell culture and somatic cell genetics of plants. Academic, New York, pp 5–53

Palmer JD, Thompson WF (1982) Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. Cell 29:537–550

Purton N, Gray JC (1989) The plastid rpoA gene encoding a protein homologous to the bacterial RNA polymerase alpha subunit is expressed in pea chloroplasts. Mol Gen Genet 217:77–84

Ruf M, Kossel H (1988) Structure and expression of the gene coding for the alpha-subunit of DNA-dependent RNA polymerase from the chloroplast gene of *Zea mays*. Nucleic Acids Res 16:5741–5755

Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat Biotechnol 19:870–875

Schwarz Z, Kossel H (1980) The primary structure of 16S rDNA from Zea mays chloroplast is homologous to E. coli 16S rRNA. Nature 283:739–742

Seki M, Shigemoto N, Sugita M, Sugiura M, Koop H-U, Irifune K, Morikawa H (1995) Transient expression of β-glucuronidase in plastids of various plant cells and tissues delivered by a pneumatic particle gun. J Plant Res 108:235–240

Shiina T, Tsunoyama Y, Nakahira Y, Khan MS (2005) RNA polymerases: promoters and transcription regulators in higher plant chloroplasts. Int Rev Cytol Cell Biol 244:1–68

Shimada H, Sugiura M (1991) Fine structural features of the chloroplast genome: comparison of the sequenced chloroplast genomes. Nucleic Acids Res 19:983–995

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kato A, Tohdoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence of the tobacco chloroplast genome; its gene organization and expression. EMBO J 5:2043–2049

Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PTJ, Staub JM, Nehra NS (1999) Stable chloroplast transformation in potato: Use of green fluorescent protein as a plastid marker. Plant J 19:209–216

Sijben-Muller G, Hallick RB, Alt J, Westhof P, Herrmann RG (1986) Spinach plastid genes coding for initiation factor IF-1, ribosomal protein S-11 and RNA polymerase a-subunit. Nucleic Acids Res 14:1029–1045

Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, Elzen VD, Peter JM, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. Bio/Technology 8:217–221

Sikdar SR, Serino G, Chaudhuri S, Maliga P (1998) Plastid transformation in Arabidopsis thaliana. Plant Cell Rep 18:20–24

Singh AK, Verma SS, Bansal KC (2010) Plastid transformation in eggplant (Solanum melongena L.). Transgenic Res 19:113–119

Skarjinskaia M, Svab Z, Maliga P (2003) Plastid transformation in Lesquerella fendleri, an oilseed brassicaceae. Transgenic Res 12:115–122

Spencer TM, Gordon-Kamm WJ, Daines RJ, Start WG, Lemaux PG (1990) Bialaphos selection of stable transformants from maize cell culture. Theor Appl Genet 79:625–631

Staub JM, Maliga P (1994) Translation of psbA mRNA is regulated by light via the 5'-untranslated region in tobacco plastids. Plant J 6:547–553

Staub JM, Garcia B, Graves J, Hajdukiewicz PTJ, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L et al (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat Biotechnol 18:333–338

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 185:60–89

Sugiura M (1992) The chloroplast genome. Plant Mol Biol 19:149–168

Sugiura M (1995) The chloroplast genome. Essays Biochem 30:49–57

Sugiura M, Shinozaki K, Zaita N, Kusuda M, Kumano M (1986) Clone bank of the tobacco (*Nicotiana tabacum*) chloroplast genome as a set of overlapping restriction endonuclease fragments: mapping of eleven ribosomal protein genes. Plant Sci 44:211–216

Sugiura M, Kusumegi T, Sugishita H, Murakami K, Ideue T, Hirose T (1998) Translational control of photosynthetic genes in tobacco plastids. In: Garab G (ed) Photosynthesis: mechanisms and effects, vol IV. Kluwer Academic, Dordrecht, pp 2943–2946

Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc Natl Acad Sci U S A 90:913–917

Svab Z, Hajduckiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci U S A 87:8526–8530

Takahashi I, Nochi T, Yuki Y, Kiyono H (2009) New horizon of mucosal immunity and vaccines. Curr Opin Immunol 21:352–358

Tanaka K, Tozawa Y, Mochizuki N, Shinozaki K, Nagatani A, Wakasa K, Takahashi H (1997) Characterization of three cDNA species encoding plastid RNA polymerase sigma factor heterogeneity in higher plant plastids. FEBS Lett 413:309–313

Thompson RJ, Mosig G (1988) Integration host factor (IHF) represses a Chlamydomonas chloroplast promoter in E. coli. Nucleic Acids Res 16:3313–3326

Tiller K, Eisermann A, Link G (1991) The chloroplast transcription apparatus from mustard. Evidence for three different transcription factors which resemble bacterial sigma factors. Eur J Biochem 198:93–99

Tregoning JS, Nixon P, Kuroda H, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, van Wijk KJ, Dougan G, Maliga P (2003) Expression of tetanus toxin fragment C in tobacco chloroplasts. Nucleic Acids Res 31:1174–1179

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578

Vasil V, Castillo A, Fromm M, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Biotechnology 10:667–674

Wakasugi T, Tsudzuki J, Ito S, Nakashima K, Tsudzuki T, Sugiura M (1994) Loss of all ndh genes as revealed by sequencing the entire chloroplast genome of black pine, Pinus thunbergii. Proc Natl Acad Sci U S A 91:9794–9798

Wakasugi T, Tsudzuki T, Sugiura M (2001) The genomics of land plant chloroplasts: gene content and alteration of genomic information by RNA editing. Photosynth Res 70:107–118

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104:37–48

Wei Z, Liu Y, Lin C, Wang Y, Cai Q, Dong Y, Xing S (2011) Transformation of alfalfa chloroplasts and expression of green fluorescent protein in a forage crop. Biotechnol Lett 33:2487–2494

Ye G-N, Hajdukiewics PTJ, Broyles D, Rodriguez D, Xu CE, Nehra NS, Staub JM (2001) Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. Plant J 25:261–270

Zubkot MK, Zubko EI, Zuilen KV, Meyer P, Day A (2004) Stable transformation of petunia plastids. Transgenic Res 13:523–530