Chapter 10
The Chloroplasts as Platform for Recombinant Proteins Production

Nunzia Scotti, Michele Bellucci and Teodoro Cardi

Abstract Chloroplasts are a useful platform for the expression of recombinant proteins in higher plants. Transgenes can be introduced into the plastid genome (plastome) either by PEG transformation of plant protoplasts, or, more commonly, by the biolistic method, using leaves or suspension cells. Transgenes are integrated by double recombination events between flanking sequences in the vector and homologous sequences in the plastome. The genetic engineering of the plastome allows high-level foreign protein expression, site-specific gene integration, expression of multiple genes as operons, marker gene excision, and transgene containment. Since the first example of stable plastid transformation in higher plants, methods for DNA introduction, marker genes and selection strategies, vector types, and methods for marker excision have been improved. Although the plastids of some species remain difficult to transform, positive results have been shown for about 20 species. In this chapter, we summarize the basic structural and expression features of the plastid genome of higher plants, and discuss the development of a number of innovative enabling technologies for plastome transformation, the most recent and significant biotechnological applications, and the future perspectives of this technology.

N. Scotti · T. Cardi
CNR-IGV, National Research Council of Italy, Institute of Plant Genetics, Res. Div. Portici, via Università 133, 80055 Portici, NA, Italy

M. Bellucci
CNR-IGV, National Research Council of Italy, Institute of Plant Genetics, Res. Div. Perugia, Via Madonna Alta 130, 06128 Perugia, PG, Italy

T. Cardi (✉)
Consiglio per la Ricerca e la Sperimentazione in Agricoltura Centro di Ricerca per l'Orticoltura, (CRA-ORT), via Cavalleggeri 25, 84098 Pontecagnano, SA, Italy
e-mail: teodoro.cardi@entecra.it

A.-M. Duchêne (ed.), *Translation in Mitochondria and Other Organelles*,
DOI: 10.1007/978-3-642-39426-3_10,

List of Abbreviations

aadA	aminoglycoside 3-adenylyltransferase
bar	phosphinothricin acetyl transferase gene
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
NEP	Nucleus-encoded RNA polymerase
PEG	PolyEthylene glycol
PEP	Plastid-encoded RNA polymerase
PPR	Pentatricopeptide repeat
PTMs	Post-translation modifications
RPOTmp	Nucleus-encoded RNA polymerase localized in mitochondria and plastids
RPOTp	Nucleus-encoded RNA polymerase localized in plastids
UTRs	Untranslated regions
ROS	Reactive oxygen species
TSP	Total soluble protein

10.1 Introduction

Since the development of methods for the genetic transformation of higher plants in the early 1980s, a wide number of transgenes have been used in genetic engineering approaches to study basic biological processes or modify agronomic and physiological traits. First generation of commercially available genetically engineered crops is largely confined to insect and herbicide resistance (Jones 2011). Nevertheless, many other agronomic/physiological applications are in the pipeline (Collinge et al. 2010; Cominelli and Tonelli 2010; Ceasar and Ignacimuthu 2012; Reguera et al. 2012). Last generation transgenic plants have been also proposed as "green biofactories" for the accumulation of recombinant products (Rojas et al. 2010; Egelkrout et al. 2012).

In most transgenic plants obtained so far, transgenes are introduced into the nuclear genome of plant cells by either the well-established *Agrobacterium*-mediated transformation or direct delivery methods, as the biolistics or the PEG/electroporation-mediated transformation approaches (Altpeter et al. 2005; Craig et al. 2005; Meyers et al. 2010). In many cases, however, some concerns have been raised, calling for the development of innovative technologies. Such concerns include: the optimization of expression level and stability of transgenes and recombinant proteins (Singer et al. 2012), the containment of transgenes (Husken et al. 2010), the possibility to transfer or stack multiple genes in order to engineer complex metabolic pathways (Naqvi et al. 2010; Que et al. 2010), the development of methods for "clean" and precise integration of transgenes in the host genome (Husaini et al. 2011; Wang et al. 2011), the identification of alternative marker genes for the selection of transformed cells (Manimaran et al. 2011;

Rosellini 2012), the feasibility of correct posttranslation modifications (PTMs) in recombinant proteins (Webster and Thomas 2012), and the use of genes only from sexually compatible sources (Orzaez et al. 2010).

Alternatively to the transformation of the nuclear genome, transgenes can be introduced into the plastid genome (plastome). Following gene delivery by PEG transformation of plant protoplasts, or, more commonly, by the biolistic method, applied to leaf tissues or suspension cells, transgenes are integrated by double recombination events between flanking sequences in the vector and homologous sequences in the plastome (Meyers et al. 2010). After transgene integration into one plastid genome, repeated cycles of cell divisions and shoot regeneration are usually required to reach homoplasmy (Fig. 10.1). In comparison with the transformation of the nuclear genome, the genetic engineering of the plastome shows some attractive advantages, partly responding to concerns mentioned above and including high-level foreign protein expression, site-specific gene integration, the possibility to transfer and express multiple genes arranged in native or synthetic operons, and marker gene excision and transgene containment because of maternal inheritance of plastids in most crops (Cardi et al. 2010; Meyers et al. 2010; Maliga and Bock 2011). After the first demonstration in tobacco (Svab et al. 1990), the technology has now been

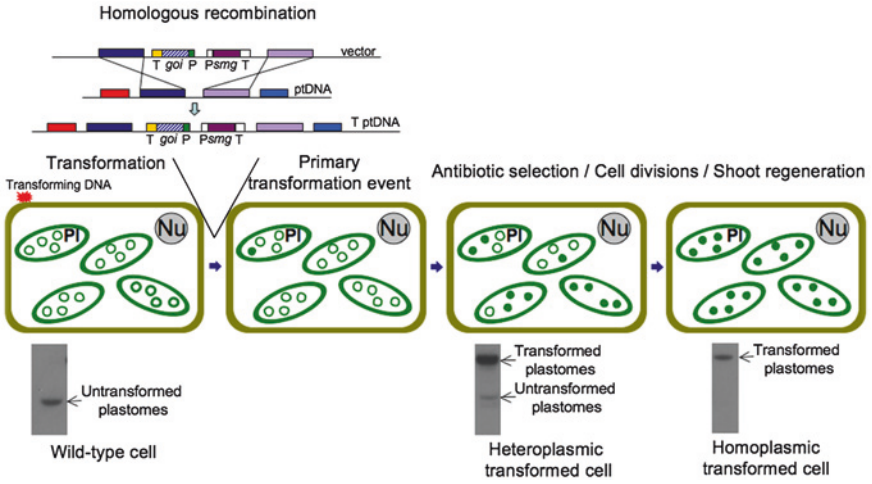

Fig. 10.1 Schematic representation of chloroplast transformation in higher plants. From *left* to *right*, a wild-type cell with untransformed plastomes (*empty circles*); the primary transformation event with a cell containing only one transformed plastome (*filled circle*); a heteroplasmic cell containing both transformed and untransformed plastomes; a homoplasmic cell with uniformly transformed plastomes. The double recombination events between vector and ptDNA sequences leading to transformed ptDNA are also shown. The Southern blots below the cells report results of restriction-digested DNA (obtained from wild type or regenerated plants) hybridized with a probe homologous to the plastome regions flanking the inserted DNA fragment. *Nu* nucleus, *Pl* plastid, *P* promoter, *T* terminator, *T ptDNA* transformed ptDNA, *goi*, gene of interest, *smg*, selectable marker gene

proved in a relatively large number of plant species belonging to several families, and for different purposes (Cardi et al. 2010; Maliga and Bock 2011; Maliga 2012). In this chapter, after reporting some basic structural and expression features of the plastid genome of higher plants, we discuss the development of a number of innovative enabling technologies for plastome transformation, aiming to overcome some inherent limitations of the procedure, and the most recent applications.

10.2 Genetic Transformation of the Plastome

The structural and expression features of higher plant plastid genomes have been recently reviewed (Bock 2007; Barkan 2011; Cardi et al. 2012; De Marchis et al. 2012; Jansen and Ruhlman 2012). Hereafter, only the principal aspects relevant to recombinant protein expression by plastid transformation are summarized.

10.2.1 Structural Features of the Plastome

The genetic system of the plant cell is organized as a network of compartments hosting the nuclear DNA (nucleus), the mitochondrial DNA or chondriome (mitochondria), and the plastid DNA or plastome (plastids). Intercompartmental exchange of genetic information among these organelles during plant evolution decisively contributed to the structure of the overall plant genome (Maier and Schmitz-Linneweber 2004). Plastids arose ~1.5 billion years ago by the endosymbiotic acquisition of a cyanobacterium and the process of transferring cyanobacterial genes to nucleus and mitochondria, which is still active both intracellularly and between species, originated the current plastomes (Jansen et al. 2011). Plastome displays structural plasticity because the genome molecules can be arranged in various circular or linear forms, either as monomers or multimers, which are organized as DNA-protein aggregates similar to bacteria nucleoids (Bendich 2004; Day and Madesis 2007; Majeran et al. 2012). A plant cell can harbor tens of plastids, each with several nucleoids, and many identical copies of the plastome can be packed in one nucleoid. Therefore, plastids have a high degree of polyploidy and plastome copy number varies with plant development and tissue type (Bock 2007). Plastid polyploidy, together with a gene-conversion mechanism, seems to be responsible for the maintenance of the low rates of mutation which characterizes the plastome. Plastid DNA (ptDNA) is largely inherited maternally, even though paternal and biparental inheritances have been observed in some cases (Bock 2007). However, even in species with strict maternal inheritance, a small percentage of paternal transmission has been described (Thyssen et al. 2012). Up to now, about 300 records exist for Eukaryota plastid genomes (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=2759&opt=plastid), including many crop plant species.

The plastid genome architecture of embryophytes appears to be highly conserved (Wicke et al. 2011). The plastome of land plants typically is 120–160 kb and has a tetrapartite structure, with two copies of a large inverted repeat (IR) separating a large and a small single copy region, containing 100–120 unique genes (Bock 2007). Some plants, like legumes, contain no IRs (Shaver et al. 2008; Magee et al. 2010). The plastid-encoded genes can be grouped into three main functional categories: genes encoding components of the genetic apparatus, photosynthesis-related genes, and other genes (Bock 2007). A detailed description of these genes in land plants is given in Wicke et al. (2011).

10.2.2 Expression Features of the Plastome

Variable plastid types are specialized in different metabolic pathways, which are very important for the whole plant cell physiology. It is predicted that in *Arabidopsis* plastids are present from 2,000 to 3,500 proteins, but only for about 1,200 there are clear evidences of plastid localization: less than 100 are plastid encoded, while the others are nucleus encoded (van Wijk and Baginsky 2011). Plastid gene expression is a very complex and unique system, regulated at many steps, mainly posttranscriptional ones, by both nuclear-encoded and plastid-encoded factors combining eukaryotic and prokaryotic features (Fig. 10.2, Barkan 2011; Cardi et al. 2012).

Plastid genes are organized as single genes or grouped in transcriptional units (operons), with common promoters and terminators, originating polycistronic transcripts. However, due to limited ability of 3′-UTR regions to terminate transcription (Cardi et al. 2012), in transplastomic plants, polycistronic transcripts can originate also in case of independent genes arranged in the same orientation (Fig. 10.3). Two types of RNA polymerases are responsible for plastid gene transcription: one is a bacterial type, multimeric plastid-encoded polymerase (PEP), the other is a phage type, monomeric nucleus-encoded polymerase (NEP), which in dicotyledonous plants is represented by two enzymes (RPOTmp and RPOTp). According to this classification, the promoters of plastid genes have been divided into NEP and PEP promoters, but there is not a clear distinction between NEP or PEP-transcribed genes, because many plastid genes have two or even more promoters and can be transcribed by both RNA polymerases (Liere and Börner 2007). In addition, six nucleus-encoded sigma factors (SIG1-6) regulate, according to environmental and developmental cues, transcription initiation mediated by PEP (Lerbs-Mache 2011; Malik Ghulam et al. 2012).

Plastid primary transcripts can undergo a maturation process involving RNA editing, intron splicing, intercistronic and mRNA termini processing, and mRNA stabilization and decay. Mature mRNAs are then translated on 70S ribosomes. All these steps of plastid gene expression require the participation of nuclear-encoded RNA-binding factors (Stern et al. 2010; Barkan 2011), most of which are pentatricopeptide repeat proteins (PPRs), characterized by a degenerate structural

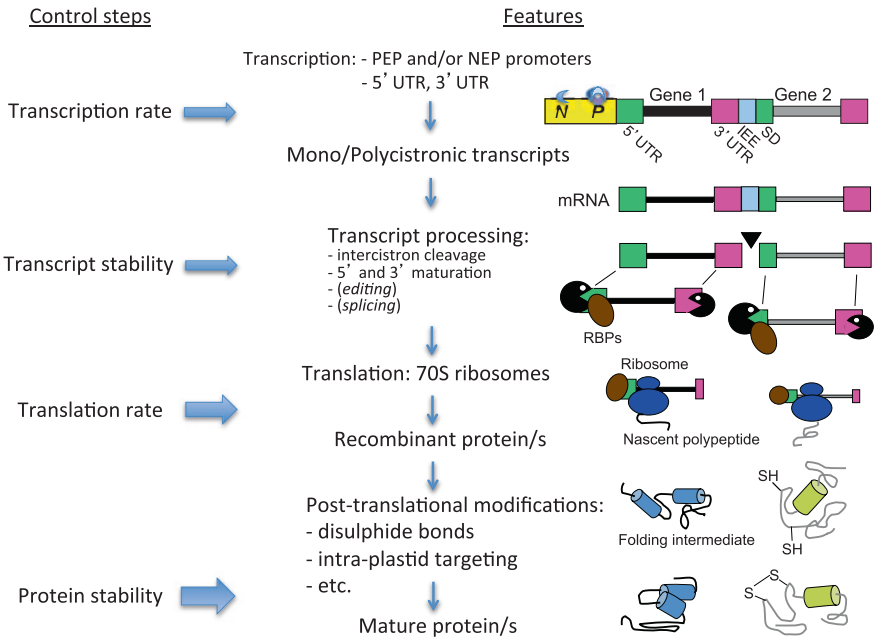

Fig. 10.2 Key control steps for the expression of recombinant proteins in transgenic plastids. The main control steps are reported on the *left* (*thicker arrows* suggest higher importance for the regulation of recombinant protein expression in plastids). The expression features reported in the figure are discussed in the text, paragraph 2.2. A dicistronic operon is represented in the figure and the *first* rectangle indicates the promoter region with NEP (N) and PEP (P) promoters recognized by nuclear-encoded monomeric and plastid-encoded multimeric RNA polymerases, respectively (number and type of promoters change in different genes). 5′-UTR and 3′-UTR are also indicated. The two genes are separated by an intercistronic expression element (IEE) which mediates intercistronic cleavage of dicistronic mRNA (Zhou et al. 2007). After the IEE, a Shine–Dalgarno (SD) sequence has been inserted to mediate translation initiation at the second cistron. However, heterologous operons inserted in the plastome have been also engineered without IEEs, because recombinant polycistrons can be efficiently translated without further processing (Quesada-Vargas et al. 2005). After intercistronic cleavage by an endonuclease (*black triangle*), the two monocistronic transcripts are processed by 5′ and 3′ exonucleases (pacmans) together with RNA-binding proteins (RBPs) and 3′ stem-loop structures (not indicated). RNA editing and splicing are not represented in the figure because, at our knowledge, no biotechnological applications with transgene sequences requiring such transcript modifications have been reported so far

motif of 35 amino acids (Schmitz-Linneweber and Small 2008). In plastids of angiosperms, mRNA editing occurs at approximately 40 sites as a conversion of a cytidine into a uridine nucleotide (Cardi et al. 2012). Editing events are generally important for gene function, because they modify the coding sequence in order to create start codons or functional polypeptides. The plastid intron repertoire of land plants comprises approximately 21 introns (with the exception of one group I, all are group II introns) in 18 genes (Tillich and Krause 2010). Intron removal by splicing represents an additional regulatory step of plastid gene expression and

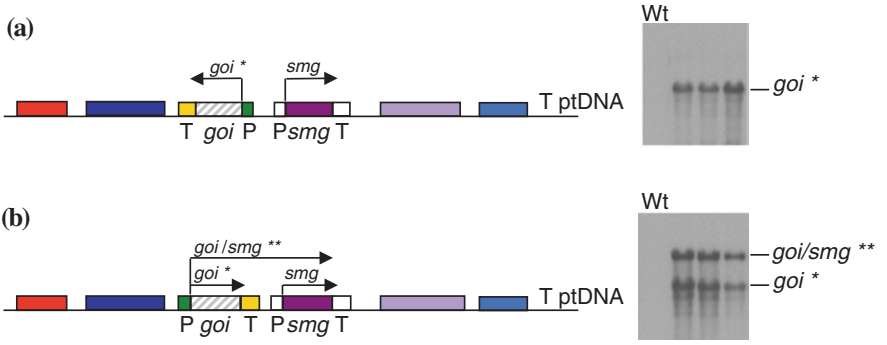

Fig. 10.3 Northern analysis showing the accumulation of mono (*goi*) and dicistronic (*goi/smg*) transcripts in plants transformed with the gene of interest (*goi*) and the selectable marker gene (*smg*) in the opposite (**a**) or same (**b**) orientation. Other boxes represent DNA flanking sequences involved in recombination-mediated transgene integration. *P* promoter, *T* terminator, *T ptDNA*, transformed ptDNA. The *goi* coding region was used as probe

a prerequisite for mRNA translation of these genes (Barkan 2011). Intercistronic processing is initiated by endonucleases, like for example RNase E (Walter et al. 2010). Mono or dicistronic translatable transcripts are then generated and maturated thanks to the concerted action of various 5′ and 3′ exonucleases with PPR-like proteins and 3′ stem-loop structures, which contribute to delimitate processed RNA termini by blocking exoribonucleases (Pfalz et al. 2009). RNA stability and decay seems to be mediated by the same ribonucleases and RNA-binding proteins described for intercistronic RNA processing (Schuster and Stern 2009; Prikryl et al. 2011), even though additional endoribonucleases and RNA-binding proteins have been characterized (Marchfelder and Binder 2004; Tillich et al. 2009).

Besides their role in RNA metabolism, PPR-like proteins also promote translation of plastid mRNAs, as recently demonstrated in maize for the ATP4 protein (Zoschke et al. 2012). To ensure efficient mRNA translation, ATP4 and other nucleus-encoded translational activators (Peled-Zehavi and Danon 2007) bind to 5′-untranslated regions (5′-UTRs), but the exact mechanism for translational activation is not completely clear yet. Most plastid mRNAs have a ribosome binding site containing a Shine–Dalgarno (SD) element, which in prokaryotes mediates ribosome recruitment. However, many plastid genes lack SD sequences; therefore, other mechanisms for translation initiation have been suggested (Marín-Navarro et al. 2007; Scharff et al. 2011). In addition, together with bacterial-type proteins, plastid ribosomes also have unique ribosomal proteins which, if down regulated, can decrease plastid translation efficiency (Tiller et al. 2012).

Even if each step in plastid gene expression is responsive to developmental and environmental conditions and seems to give a significant contribution to the overall regulation of genes located in the plastome, expression of plastid genes is primarily regulated during translation of the corresponding mRNAs (Zerges 2004; Manuell et al. 2007). Moreover, several studies conducted with transgenes inserted

into the plastome suggest that posttranslational factors are extremely important for the expression of plastid genes as well (Oey et al. 2009a). Protein stability is a key determinant of protein accumulation in plastids and many aspects influencing protein stability, such as the identity of the N-terminal amino acids, protein co- or post-translational modifications and suborganelle localization, have been recently reviewed (De Marchis et al. 2012).

10.2.3 Enabling Technologies and Limiting Factors

Despite that plastid genetic engineering is today a well-established technology, the plastome of only a relatively small number of plant species and the green alga *Chlamydomonas* can be routinely engineered. Tobacco has been the first species to be transformed, using a mutant 16S rRNA gene conferring resistance to spectinomycin for selection (Svab et al. 1990). Although the use of mutated plastid genes conferring antibiotic resistance was successful in generating transplastomic plants (Dix and Kavanagh 1995; Craig et al. 2008), the real breakthrough in plastome transformation was the utilization of the selectable marker gene *aadA* (Day and Goldschmidt-Clermont 2011). Even low doses of the *aadA* enzyme detoxify spectinomycin and streptomycin; therefore, the few chloroplasts incorporating this marker gene after transformation can be selectively enriched in tissue culture until the obtainment of homoplastomic plants. Nowadays, chloroplast transformation reproducible protocols for tobacco (*Nicotiana tabacum*) and dycotyledonous species like potato, tomato, lettuce, and several others are available (Fig. 10.4), but not for monocot plants (Maliga and Bock 2011; Maliga 2012). Indeed, the main problem with cereals is that the most common antibiotics used for selection of transplastomic plants are not effective with these species. Kanamicin and streptomycin are not able to inhibit callus growth in the dark, while spectinomycin is useless due to the endogenous resistance of many cereal species to it. Therefore, a protocol based on chloramphenicol selection was developed in tobacco using the chloramphenicol acetyltransferase gene *cat* as an alternative selectable marker gene (Li et al. 2011) that could be hopefully next applied to cereal plastome transformation. Other studies tried to increase the efficacy of the existing marker genes. A recent modified version of the *aadA* gene, being both a selectable marker in tissue culture and a visual marker in transplastomic plants, was described by Tungsuchat-Huang et al. (2011). An alternative selection system has been recently described in tobacco, with the gene encoding D-amino acid oxidase used as plastid marker gene for positive/negative secondary selection once resistant plants are obtained using *aadA*-based spectinomycin selection (Gisby et al. 2012). Moreover, the anthranilate synthase α subunit has been also successfully employed as selectable marker gene (Barone et al. 2009). Marker genes can be removed after selection by: the flanking-repeat-mediated excision based on the native homologous recombination machinery, the use of site-specific foreign recombinases, the

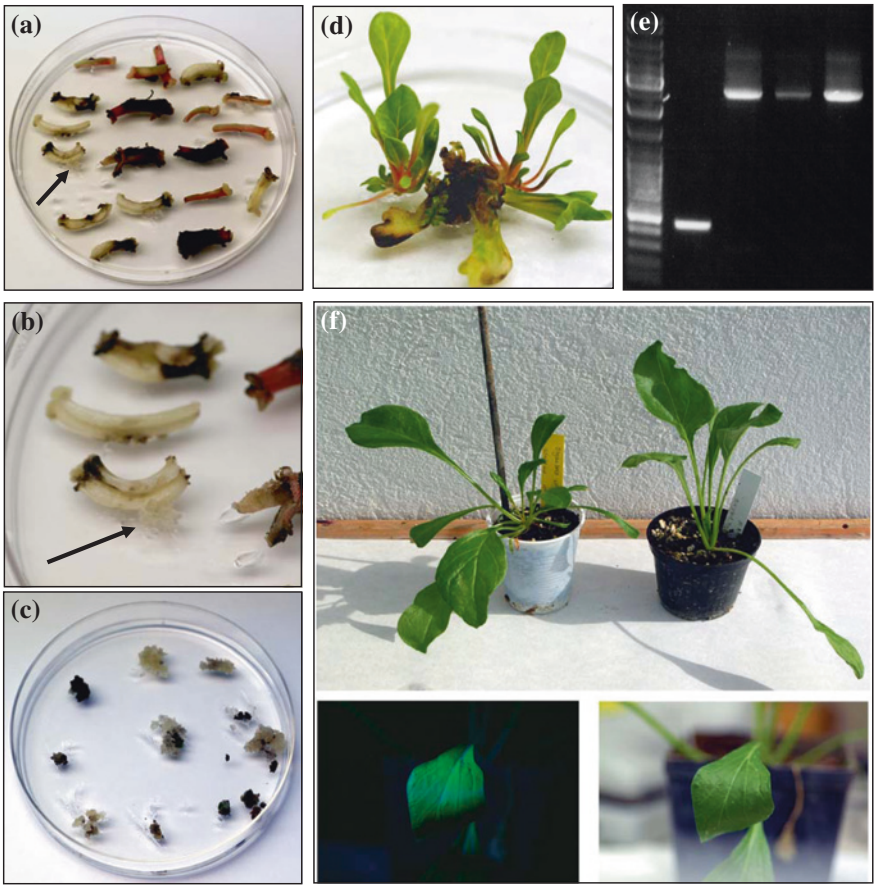

Fig. 10.4 Plastid transformation in sugar beet (De Marchis et al. 2009). **a** Leaf petioles bombarded with gold particles coated with a plastid transformation vector expressing GFP and placed in a regeneration medium containing spectinomycin. **b** A regenerable callus (indicated with an *arrow*). **c** Selected calli placed in a medium without spectinomycin to regenerate. **d** Putative transformed regenerated shoots. **e** PCR analysis with a primer pair located outside the vector transgene sequence. From *left* to *right*: DNA molecular size marker, wild-type sugar beet DNA, DNA of three transformed plants. **f** Transplastomic sugar beet plants expressing GFP. Fluorescence (*left*) and bright-field (*right*) images from leaves of a transplastomic plant are shown

co-transformation and segregation of marker-free plastid genomes, the transient co-integration of the marker gene (reviewed in Day and Goldschmidt-Clermont 2011).

Transgenes can be targeted to any region of the plastome by providing targeting/flanking regions, typically 1–2 kb long, to obtain efficient transgene integration by recombination between plastid and vector DNA sequences (Fig. 10.1). However, the choice of the integration site may have a great importance in

terms of transgene expression. The integration into a transcriptionally active site increases the level of the transcribed mRNA and that of the corresponding protein (De Cosa et al. 2001; Quesada-Vargas et al. 2005; Krichevsky et al. 2010), as well as the insertion into the plastome inverted repeat (IR) regions doubles the number of transgene copies per genome. The most used insertion sites include the regions between the *trnI-trnA*, *trnN-trnR*, *rccL-accD*, and *rrn16-rps7/12* genes.

Because of the high conservation of plastid genomes between most land plants, the first chloroplast transformation strategies utilized either homologous or heterologous flanking regions. However, the transformation efficiency using heterologous flanking regions was considerably lower in comparison to that with species-specific sequences. Indeed, even if the plastid genome is highly conserved between species with respect to protein coding regions and ribosomal RNAs, many differences can be present in the intergenic regions which are the common targets sites for homologous recombination (Saski et al. 2007). Therefore, a substantial increment in plastid transformation efficiency can be obtained with species-specific transformation vectors, as demonstrated by the decrease in the efficiency of tobacco chloroplast transformation when lettuce chloroplast targeting sequences were used to integrate transgenes into the *trn*I/*trn*A region of the tobacco plastome (Ruhlman et al. 2010). Similar results were obtained for potato and other species (Valkov et al. 2011, and references therein).

Several transgenes could be efficiently co-expressed in transgenic plastids arranging them in natural or synthetic operons (Quesada-Vargas et al. 2005; Krichevsky et al. 2010), and intercistronic elements facilitating the expression of monocistronic mRNAs from operons have been identified (Zhou et al. 2007). Many studies conducted with chimeric gene fusions have identified combinations of promoters, 5′-UTRs and 3′-UTRs, which can be used to achieve a high level of recombinant protein expression in chloroplasts, regulating transcript stability and translatability. Excellent results can be obtained in leaves with the promoter of the plastid rRNA operon (*Prrn*) or the *psbA* promoter, in combination with the 5′-UTR of gene 10 of the bacteriophage T7 (*T7g10*), or other 5′-UTRs from highly expressed chloroplast genes like *rbcL*, and the *E. coli rrnB* 3′ sequence as terminator (Maliga 2002; Herz et al. 2005; Tangphatsornruang et al. 2011). However, there is the need to find additional nonplastid regulatory sequences to be used in vectors designed to express multiple recombinant genes, in order to avoid unintended recombination events. Yang et al. (2013) investigated the efficacy of two additional bacteriophage 5′-UTRs (*T7g1.3* and *T4g23*) demonstrating that they can regulate *aadA* expression in chloroplasts. Conversely, transgene expression in nongreen plastids is very low, mainly due to the absence of data about gene expression in such plastid types. Recently, studies on the regulation of plastome gene expression in nongreen tissues started to fill this gap, showing a general downregulation of expression (Kahlau and Bock 2008; Valkov et al. 2009). As a consequence, expression elements that could increase transgene expression in the amyloplasts of tobacco roots and potato tubers have been identified (Valkov et al. 2011; Zhang et al. 2012). The combination of a strong plastid promoter (*psbA* promoter) with a strong *T7g10*-derived 5′-UTR

that are not prone to severe developmental downregulation during fruit development has been shown to reach high-level gene expression in tomato chromoplasts, triggering GFP accumulation up to 1 % of the total protein of the fruit (Caroca et al. 2013).

A high number of studies on heterologous gene expression in plant plastids demonstrated that codon optimization of transgene sequences is not essential for efficient translation rate (Maliga and Bock 2011). This, together with the possibility to efficiently express bacterial single genes or operons, theoretically allows an easy genetic manipulation of a plethora of genes belonging to different kingdoms for expression in plant plastids. However, there are cases in which codon optimized genes of viral (Madesis et al. 2010), bacterial (Bohmert-Tatarev et al. 2011), or human (Gisby et al. 2011) origin significantly improved transgene expression in comparison with noncodon-optimized sequences.

The N-terminal sequence of recombinant proteins expressed in the chloroplasts is a key factor for both mRNA stability/translatability (Kuroda and Maliga 2001) and protein stability (Ye et al. 2001). Elghabi et al. (2011) fused N-terminal segments of highly expressed proteins in plastids to the transgene coding region, stabilizing the cyanovirin-N mRNA. Unfortunately, there are no precise rules for the best performing sequences and empiric attempts have to be made (Scotti et al. 2009; Gray et al. 2011). However, the existence of an N-end rule-like pathway with stabilizing and destabilizing N-residues has been postulated also for plastids, even though the identity of the penultimate amino acid is not the sole responsible for plastid protein stability (Apel et al. 2010). That the N-terminal part has a significant role for recombinant protein stability has been also demonstrated with the addition of extra amino acids at the 5′ end of the rotavirus VP6 protein (Inka Borchers et al. 2012). Most PTMs in plastids occur on the N-terminal part of proteins, and there are an increasing number of evidences that PTMs play a strategic role in the regulation of protein turnover (Adam et al. 2011; Bienvenut et al. 2011). In addition, many PTMs are involved in protein folding which is necessary to reach the polypeptide tertiary or quaternary stable structure. An important aspect for stability of recombinant proteins inserted into the plastome can be their subplastidial localization. Indeed, proteins can be accumulated in the stroma, the thylakoid lumen, the envelope, or the thylakoid membranes. For example, the thylakoid lumen seems to be a more adequate environment than the stroma for the accumulation of proteins that require formation of disulfide bonds (Bally et al. 2008; Lentz et al. 2012). In some cases, PTMs can be also important for protein functionality. Besides multimerization, N-terminal methionine excision and disulfide bond formation, plastids allow protein lipidation (Glenz et al. 2006), but not glycosylation, preventing the production of vaccine antigens that require glycosylation for their function and other glycoproteins.

Once accumulated in plastids, recombinant proteins must be extracted and purified for further uses but no many detailed purification methods from plastids have been published. Recently, glutathione-S-transferase and maltose-binding protein have been expressed in transplastomic plants and purified by affinity chromatography,

demonstrating that they can be used as affinity tags for the rapid purification of recombinant proteins localized in the chloroplast (Ahmad et al. 2012a).

The expression of native expression elements seems to originate mutant phenotypes in some transplastomic plants, suggesting as likely cause the already reported competition for gene-specific transcription and/or translation factors (Kuroda and Maliga 2002). In other studies, the occurrence of mutant phenotypes characterized by chlorotic phenotype and growth retardation seems to be due to interference of the recombinant proteins with the plastid metabolism instead of competition for expression factors (Rigano et al. 2012). Therefore, the use of systems for inducible gene expression is desirable in such transplastomic plants (Verhounig et al. 2010; Lössl and Waheed 2011). Temporary immersion bioreactors have been also proposed as an alternative method for the production of proteins toxic for plants (Michoux et al. 2013).

10.3 Modification of Agronomic and Physiological Traits by Plastid Transformation

To date, numerous agronomic and physiological traits have been modified by plastid transformation as summarized in Table 10.1 and Fig. 10.5 and reported in recent reviews (Hasunuma et al. 2009, 2010; Rogalski and Carrer 2011; Scotti et al. 2011; Hanson et al. 2013). In this chapter, we focus on the most recent and/or significant examples.

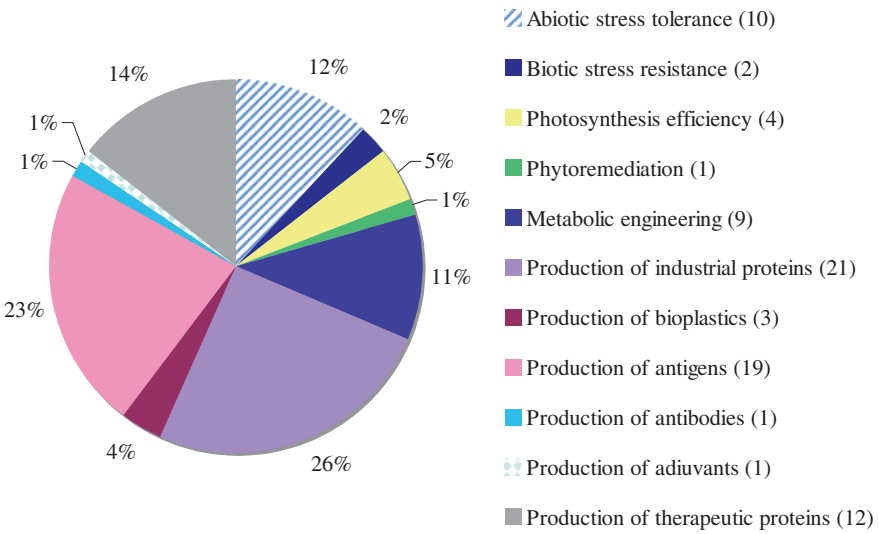

Fig. 10.5 Pie chart summarizing the transgenes engineered by plastid transformation from January 2010 to 2013

Table 10.1 Agronomic and physiological traits engineered by plastid transformation (January 2010–2013)

Trait	Enzyme/Transgene[a]	Source of transgene	Species	Yield[b]	Reference
Stress tolerance					
	Transglutaminase (*TGZ*)	Maize	Tobacco	1.1 mg g^{-1} FW	Ortigosa et al. (2010a)
	Flavodoxin (Fld)	*Anabaena* sp.	Transgenic tobacco with silenced Ferredoxin (Fd)	NR	Blanco et al. (2011)
	β-glucosidase (*Bgl-1*)	*T. reesei*	Tobacco	44.4 units g^{-1}	Jin et al. (2011)
	dehydroascorbate reductase (*DHAR*)	Rice	Tobacco	0.75 % TSP	Le Martret et al. (2011)
	glutathione-S-transferase (*GST*)	*E. coli*	Tobacco	0.75 % TSP	Le Martret et al. (2011)
	DHAR-GR	Rice-*E. coli*	Tobacco	0.75 % TSP	Le Martret et al. (2011)
	GST-GR	*E. coli*	Tobacco	0.75 % TSP	Le Martret et al. (2011)
	Mitochondrial superoxide dismutase (*MnSOD*)	*N. plumbaginifolia*	Tobacco	NR	Poage et al. (2011)
	Glutathione reductase (*GR*)	*E. coli*	Tobacco	NR	Poage et al. (2011)
	Plastid terminal oxidase 1 (*Cr-PTOX1*)	*C. reinhardtii*	Tobacco	NR	Ahmad et al. (2012b)
	Flavodoxin (*Fld*)	*Anabaena* sp.	Tobacco	11 μmol m^{-2}	Ceccoli et al. (2012)
	Agglutinin (*PTA*)	*P. ternata*	Tobacco	9.2 % TSP	Jin et al. (2012)
Photosynthesis efficiency					
	Mutated β-subunit of ATP synthase (*atpB*)	Tobacco	Tobacco	NR	Rott et al. (2011)
	rbcL-rbcS hybrid enzyme (*LLS2*)	Tobacco-Tomato	Tobacco	NR	Zhang et al. (2011)
	rbcL-rbcS hybrid enzyme (*LLS4*)	Tobacco-Tomato	Tobacco	NR	Zhang et al. (2011)
	Hypothetical chloroplast reading frame no. 4 (*ycf4*)	Tobacco	Tobacco	NR	Krech et al. (2012)
Phytoremediation					
	Metallothionein (*mt1*)	Mouse	Tobacco	NR	Ruiz et al. (2011)

(continued)

Table 10.1 (continued)

Trait	Enzyme/Transgene[a]	Source of transgene	Species	Yield[b]	Reference
Metabolic engineering					
	Operon containing six genes of the cytoplasmic mevalonate pathway (*MEV6.1*)	Synthetic	Tobacco	NR	Kumar et al. (2012)
	Tocopherol cyclase (*TC*)	Arabidopsis	Tobacco Lettuce	NR	Yabuta et al. (2013)
	γ-Tocopherol methyltransferase (*γ-TMT*)	Arabidopsis	Tobacco	NR	Yabuta et al. (2013)
	TC-γ-TMT	Arabidopsis	Tobacco	NR	Yabuta et al. (2013)

[a]*DHAR-GR* fusion between dehydroascorbate reductase and glutathione reductase (GR), *GST-GR* fusion between glutathione-S-transferase and glutathione reductase (GR), *LLS2* fused Rubisco containing tobacco small subunit (*rbcS*) and mutated (Q437R) tomato large subunit (*rbcL*), *LLS4* fused Rubisco containing tobacco small subunit (*rbcS*) and mutated (Y226F, A230T, S279T and Q437R) tomato large subunit (*rbcL*), *MEV6.1* synthetic operon containing genes encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), mevalonate diphosphate decarboxylase (MDD), acetoacetyl CoA thiolase (AACT), C-terminal truncated 3-hydroxy-3methylglutaryl-coenzyme A reductase (HMGRt), *TC-γ-TMT* fusion between tocopherol cyclase and γ-tocopherol methyltransferase.

[b] yield according to original reference; TSP = total soluble protein; NR = not reported; FW = fresh weight

10.3.1 Stress Tolerance

Early experiments aiming to improve by plastid transformation salt/drought or cold tolerance relied on genes for the biosynthesis of the osmoprotectant glycine betaine or on Δ9 fatty acid desaturase genes, respectively (Kumar et al. 2004; Craig et al. 2008; Zhang et al. 2008). The study of Craig et al. (2008) represents the first example of transplastomic plants expressing an agronomically relevant gene produced with the ''binding-type'' vectors, in which chloroplast antibiotic insensitive point mutations are used to select transformants in place of heterologous marker genes.

More recently, different enzymes with anti-oxidant activities were expressed to enhance the tolerance of transplastomic tobacco plants to abiotic stresses. Genes encoding the dehydroascorbate reductase (DHAR) from rice, the glutathione-S-transferase (GST) and glutathione reductase (GR) from *E. coli* were expressed alone or as DHAR:GR or GST:GR combinations (Le Martret et al. 2011). In all transplastomic lines, the expression of the inserted genes gave an increment of specific enzyme activity and an alteration of anti-oxidant metabolism. Progeny of transplastomic plants, subjected to environmental stresses, proved to be less sensitive to low temperatures and salt stress, whereas no advantages were observed for heavy metal stress. A higher increase in tolerance to cold stress were detected in transplastomic plants expressing DHAR:GR or GST:GR gene combinations. In another study (Poage et al. 2011), the prospect for enhancing ROS scavenging and stress tolerance was pursued expressing a mitochondrial manganese superoxide dismutase (*MnSOD*) from *N. plumbaginifolia* and glutathione reductase (GR) from *E. coli* in the inverted regions of tobacco chloroplast genome. An increase of specific enzyme activity (three- and sixfold for *MnSOD* and GR, respectively) was revealed in all transplastomic plants. *MnSOD* transplastomic plants showed an enhanced tolerance to methyl-viologen and UV-B radiation, whilst GR plants an improved tolerance to the same radiation and heavy metal. No effect on photosynthetic capacity was observed in both transplastomic plants.

The expression level of proteins like ferredoxin decreases under environmental stresses in photosynthetic organisms. In cyanobacteria, this phenomenon is compensated by induction of flavodoxin, a flavoprotein with the same function not present in higher plants. In order to investigate the effect of flavodoxin on photosynthesis and stress tolerance in higher plants, Ceccoli et al. (2012) expressed a flavodoxin (Fld) of *Anabaena* sp. in the plastid genome of tobacco and compared its protective effect to tobacco nuclear transgenic lines overexpressing the same gene in the chloroplasts. Transplastomic lines expressed the flavodoxin at about 11 μmol m^{-2} of leaf tissue, fourfold more than the transgenic lines showing the highest amounts of flavodoxin. Comparative analysis between transgenic and transplastomic plants displayed a flavodoxin dose-dependent increase on photosynthetic performance and tolerance to the exposure to methyl viologen followed by a drop in high-expressing lines. The optimal photosynthetic performance and stress tolerance were observed at flavodoxin levels comparable to those of endogenous ferredoxin (about 3 μmol m^{-2}). In this case, the high increase obtained by plastid transformation resulted detrimental to plant fitness. Similarly, Ortigosa et al. (2010a) demonstrated that the overexpression

of maize plastidial transglutaminase (chlTGZ) in the plastid genome of tobacco induced an oxidative stress in transplastomic plants. In particular, they evaluated several aspects such as fluorescence parameters, chloroplast ultrastructure, and oxidative and antioxidative metabolism. These analyses revealed different alterations of chloroplast ultrastructure and physiology that increased with leaf age.

As far as biotic stresses are concerned, one of the earliest biotechnological applications of plastid transformation reported the expression of the *Bacillus thuringiensis crylA(c)* gene in tobacco (McBride et al. 1995). Subsequently, De Cosa et al. (2001) produced transplastomic tobacco plants expressing the *B. thuringiensis* toxin (*cry2Aa2*) operon, at the level of about 46 % of total soluble protein (TSP), engineering two small open reading frames encoding for a chaperonin that facilitated the correct folding of Cry2Aa2 in stable crystals. Insect bioassay carried out with cotton bollworm and beet armyworm demonstrated that both insects were killed after consuming transplastomic leaves. The *B. thuringiensis* toxin (*cry1Ab*) was also expressed in the chloroplast genomes of soybean and cabbage (Dufourmantel et al. 2005; Liu et al. 2008), resulting in high protein yield and complete insect mortality in both cases.

Recently, the β-glucosidase gene (Bgl-1) was expressed in tobacco transplastomic plants (Jin et al. 2012). Bgl-1 transplastomic plants showed earlier flowering and increased biomass, height and leaf area compared to untransformed plants; these effects were associated with an increase of different plant hormones. Further, in the same plants, an increase in sugar esters located within the globular trichomes functioned as biopesticide against whiteflies and aphids.

10.3.2 Herbicide Resistance

The first attempt to improve herbicide tolerance by plastid transformation was pursued by Daniell et al. (1998) that introduced a mutated form of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from petunia in tobacco chloroplasts. The transplastomic plants obtained were tolerant to low concentration of glyphosate (5 mM) corresponding to 8 oz acre^{-1} Roundup, not enough for weed control, for which about 64 oz acre^{-1} Roundup are normally required. Better results (about 10 % TSP, corresponding to 128 oz acre^{-1} Roundup) were obtained by Ye et al. (2001) with the *Agrobacterium epsps* gene fused to the first 14 amino acids of GFP protein.

Although all forms of *bar* genes, encoding the enzyme phosphinothricin acetyl transferase (PAT) inactivating the herbicide glufosinate (phosphinothricin), were expressed at high level in transplastomic plants and these were tolerant to field-level of glufosinate, the gene did not work well as marker for direct selection of transplastomic clones (Lutz et al. 2001). Tobacco transplastomic plants expressing different mutated versions of the *als* gene from *A. thaliana* were able to grow in the presence of various herbicides (pyrimidinylcarboxylate, imidazolinone, and sulfonylurea/pyrimidinylcarboxylate), proving the possibility of using these plants on the rotation of three or more herbicide combinations (Shimizu et al. 2008).

10.3.3 Photosynthesis Efficiency

Improvement of photosynthesis efficiency would have a very high impact on agriculture, and thus many efforts are underway to engineer photosynthesis via nuclear or plastid transformation, as recently reviewed by Hanson et al. (2013).

Since the efficiency of photosynthesis is dependent on the performance of the ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), attempts have been made to engineer this enzyme in order to enhance its catalytic turnover rate and increase its specificity for CO_2 (Whitney et al. 2009; Zhang et al. 2011). The former authors tried to manipulate Rubisco via chloroplast engineering, linking the small (S, encoded by nuclear genome) and large (L, encoded by plastid genome) subunits of tobacco by a flexible 40-amino acid tether (S40L). This strategy allowed to replace native *rbcL* in tobacco plastids with the synthetic S40L fusion gene. They found that the fusion S40L protein was able to assemble into catalytic oligomers in tobacco plastids, with higher affinities for CO_2 and O_2. Zhang et al. (2011) engineered Rubisco creating two lines of transplastomic tobacco carrying a hybrid enzyme. LLS2 plants contained tobacco small subunits (SS) and mutated large subunit (LS) with one substitution, whereas LLS4 plants displayed a tobacco SS and a tomato mutated LS bearing four substitutions. Compared to wild-type and LLS2 plants, LLS4 plants showed lower chlorophyll and Rubisco content, lower photosynthesis rates and biomass during early stages of development, but were able to reach maturity. The enzyme assay detected a carboxylase activity in both plants similar to wild type, demonstrating that hybrid enzymes were able to assemble into functional Rubisco.

Plastid transformation was also used to identify the role of plastid open reading frames in the assembly and stability of photosystem I (Krech et al. 2012), or to study the functional significance of the ATP synthase adjustment in response to the metabolic demand (Rott et al. 2011). In particular, the latter authors demonstrated that the repression of ATP synthase complex, pursued by an antisense approach on essential nuclear *atpC* gene and by the introduction of different point mutations into the translation initiation codon of the plastid *atpB* gene via plastid transformation, led to a restriction of photosynthetic electron transport due to decreased rates of plastoquinol reoxidation at the cytochrome b_6f complex, and a reduction of the quantum efficiency of CO_2 fixation due to an increased steady-state proton motive force resulting in overacidification of the thylakoid lumen.

10.3.4 Male Sterility

The cytoplasmic male sterility (CMS) phenotype is a maternally inherited trait, important to produce commercial F_1 hybrids (Schnable and Wise 1998). To date, only a manuscript described the engineering of this trait through plastid transformation by expression of the *phaA* gene encoding a β-ketothiolase (Ruiz and

Daniell 2005). Since a previous study (Lössl et al. 2003) reported that the expression of the entire polyhydroxybutyrate (PHB) operon (*phaA*, *phaB*, *phaC*) in the chloroplast genome of tobacco produced severe pleiotropic effects (stunted phenotype and male sterility) in transplastomic plants, Ruiz and Daniell (2005) investigated the specific role of *phaA* gene and evaluated its effect under a specific photoperiod or continuos illumination, using as regulatory sequences the *psbA* promoter and 5′-UTR. The transplastomic plants overexpressing β-ketothiolase, whose enzymatic activity was in the range of 14.08–14.71 units mg^{-1} plant protein during regular photoperiod, were normal except for the male sterile phenotype. In particular, electron microscopy analyses showed a collapsed morphology of pollen grains and an accelerated pattern of anther development resulting in aberrant tissue development. Further, they observed a reversibility of the male sterile phenotype (production of viable pollen and seeds) under continuous light.

10.3.5 Phytoremediation

Phytoremediation of mercury and organomercurial compounds via plastid transformation was pursued using two approaches. The first one was based on the integration of bacterial native operon containing the *merA* and *merB* genes, coding for mercuric ion reductase and organomercurial lyase, respectively, into the tobacco chloroplast genome. When grown in soil containing up to 400 μM phenylmercuric acetate (PMA), transplastomic plants showed a higher tolerance to PMA than wild-type plants (Ruiz et al. 2003). Further, they were used to investigate the uptake and translocation of different forms of mercury from roots to shoots, and their volatilization, demonstrating that they accumulated both organic and inorganic mercury forms to concentrations much higher than those usually found in the soil (Hussein et al. 2007). In transplastomic plants, the organic mercury was uptaken and translocated more efficiently than the inorganic form. The efficient translocation from roots to shoots seems to facilitate the reduction of toxic ionic mercury (Hg^{2+}) into less toxic and volatile elemental mercury (Hg^0) (Hussein et al. 2007). Although this approach demonstrated an improved phytoremediation capability in transplastomic plants, the volatile Hg^0 form can be released back into the environment where it accumulates and potentially converted again into highly toxic forms. For this reason, an alternative approach based on the expression of chelator molecules, such as metallothioneins, was developed (Ruiz et al. 2011). Transplastomic plants expressing the mouse metallothionein gene (*mt1*) were resistant up to 20 μM mercury without any phenotypic effect, and accumulated high concentrations of mercury in all tissues. In particular, the high concentration of mercury accumulated in leaves of transplastomic plants (up to 106 ng) is indicative of an active phytoremediation and translocation of mercury.

10.3.6 Metabolic Engineering

Based on the fact that several biosynthetic pathways occur in plastids, some studies demonstrated the potential of plastome engineering for the nutritional enhancement of vegetables. Recently, Yabuta et al. (2013) improved the quality and quantity of vitamin E (tocopherol), an important anti-oxidant, through the expression of different genes involved in this pathway in the chloroplast genomes of tobacco and lettuce. In particular, they observed that the overexpression of tocopherol cyclase (TC) gene in both plant species induced an increase of total tocopherol level and vitamin E activity of about 2.2- and 1.3-fold in tobacco and lettuce, respectively, compared to wild-type plants. Carotenoid biosynthesis was manipulated in tomato in order to increase the content of provitamin A (β-carotene). In a first study (Wurbs et al. 2007), two microbial lycopene β-cyclase and a β-cyclase/phytoene synthase fusion gene from the fungus *Phycomyces blakesleeanus* were integrated in the chloroplast genome of tomato. HPLC analyses demonstrated that transplastomic tomato fruits accumulated a 4-fold higher provitamin A content compared to wild-type plants. A subsequent manuscript (Apel and Bock 2009) described the enhancement of provitamin A content in tomato fruits through the expression of lycopene β-cyclase from a higher plant (*Narcissus pseudonarcissus*, daffodil). In comparison with the previous study, a higher efficient conversion of lycopene to β-carotene was obtained, resulting in an accumulation of provitamin A of about 1 mg g^{-1} dry weight. In addition, the expression of lycopene β-cyclase from daffodil induced an increase of the total fruit carotenoid content and an alteration of carotenoid composition in leaves.

Another interesting example of the effectiveness of plastid transformation technology for the manipulation of metabolic pathways is the production of astaxanthin (Hasunuma et al. 2008), a carotenoid synthesized by some bacteria and fungi with wide industrial applications due to its various biological functions. This goal was pursued by the expression of plant codon optimized genes of marine bacterium *Brevundimonas* sp. encoding the ß-carotene ketolase and ß-carotene hydroxylase. Transplastomic plants expressing both genes accumulated astaxanthin at a level higher than 0.5 % (dry weight), corresponding to approximately at 70 % of total carotenoids, and synthesized also the novel carotenoid 4-ketoantheraxanthin. Further, they were characterized by a dramatic change of color in leaves and stems (reddish brown), and stigmas and corolla (pink), instead of their normal green color.

The possibility to manipulate by plastid transformation the lipid pathway has been recently discussed by Rogalski and Carrer (2011). However, besides the report of Craig et al. (2008) cited above, only another study investigated the feasibility to alter the fatty acid content by overexpression of the *accD* gene in the plastome (Madoka et al. 2002). Resultant transplastomic plants showed a significantly higher fatty acid content in leaves, extended leaf longevity and a 2-fold increase of seed yield over the controls.

10.4 Production of Recombinant Molecules in Plants by Plastid Transformation

The diffusion of the 'Molecular Farming' concept has given a significant boost to explore the suitability of chloroplast genetic engineering to produce recombinant molecules. Herein, we summarize the most successful and recent examples for each subgroup of recombinant molecules (Table 10.2 and Fig. 10.5).

10.4.1 Industrial Proteins

Most recent examples of industrial proteins produced by plastid transformation belong to enzymes involved in the hydrolysis of lignocellulosic biomass to produce fermentable sugar. Verma et al. (2010a) expressed in tobacco chloroplasts an enzyme cocktail based on genes from bacteria and fungi, and compared their yields and enzyme activities with those obtained in *E. coli*. The chloroplast-derived enzymes had higher temperature stability, wider pH range and higher enzyme activities compared to *E. coli*-derived enzymes. Further, the chloroplast-derived crude extracts of enzyme cocktails were able to release a higher amounts of glucose from filter papers, pine wood, or citrus peel than commercial enzyme cocktails. Petersen and Bock (2011) focused on the production of four enzymes from the thermophilic bacterium *Thermobifida fusca*, demonstrating also in this case that such enzymes can be successfully expressed in the chloroplast genome. By contrast with Verma et al. (2010a), the high protein yields (between 5 and 40 % of plant total soluble protein) produced by plastid transformation resulted in pigment-deficient phenotypes. Enzyme activity assay carried out on crude extracts of transplastomic plants demonstrated that all enzymes were highly active and hydrolyzed their synthetic substrates in a dose-dependent manner. On the contrary, enzyme assay carried out on natural substrates (wheat straw) showed that without a standard thermochemical treatment of plain straw it was not possible to observe a sugar release. The expression of a GH10 xylanase gene (*Xyl10B*) from *Thermotoga maritima* in the tobacco chloroplast genome gave an enzyme yield up to 15 % TSP without any phenotypic effects (Kim et al. 2011). The enzyme assay carried out with crude extracts of Xyl10B-transplastomic plants showed an exceptional catalytic activity and enabled the complete hydrolysis of natural substrates to fermentable sugars with the help of α-glucuronidase accessory enzyme. Successful results were also obtained with the expression of β-mannase gene (*man1*) from *Trichoderma reesei* (Agrawal et al. 2011).

Other important industrial polypeptides recently produced via plastid transformation include a laccase and a monellin variant. Davarpanah et al. (2012) produced transplastomic tobacco plants to develop commercial level of a fungal laccase, an enzyme with potential applications as waste detoxification and in textile industry. Although an accumulation up to 2 % of total protein was reached,

Table 10.2 Recombinant molecules produced by plastid transformation (January 2010–2013)

Sub-group	Molecule/Transgene	Source of transgene	Species	Yield[a]	Reference
Industrial proteins					
	Endoglucanase (*celD*)	*C. thermocellum*	Tobacco	4930 units g^{-1} FW	Verma et al. (2010a)
	Exoglucanase (*celO*)	*C. thermocellum*	Tobacco	NR	Verma et al. (2010a)
	Lipase (*lipY*)	*M. tuberculosis*	Tobacco	NR	Verma et al. (2010a)
	Pectate liases (*pelA, pelB* and *pelD*)	*F. solani*	Tobacco	32 units g^{-1} FW	Verma et al. (2010a)
	Cutinase	*F. solani*	Tobacco	NR	Verma et al. (2010a)
	Swollenin (*swo1*)	*T. reesei*	Tobacco	NR	Verma et al. (2010a)
	Xylanase (*xyn2*)	*T. reesei*	Tobacco	NR	Verma et al. (2010a)
	Acetyl xylan esterase (*axe1*)	*T. reesei*	Tobacco	NR	Verma et al. (2010a)
	ß-glucosidase (*bgl1*)	*T. reesei*	Tobacco	NR	Verma et al. (2010a)
	ß-mannase (*man1*)	*T. reesei*	Tobacco	25 units g^{-1} FW	Agrawal et al. (2011)
	ß-glucosidase (*bglC*)	*T. fusca*	Tobacco	12 % TSP	Gray et al. (2011)
	GH10 xylanase (*Xyl10B*)	*T. maritima*	Tobacco	15 % TSP	Kim et al. (2011)
	ß-glucosidase (*bgl1C*)	*T. fusca*	Tobacco	>5 % TSP	Petersen and Bock (2011)
	Exoglucanase (*cel6B*)	*T. fusca*	Tobacco	5 % TSP	Petersen and Bock (2011)
	Endoglucanase (*cel9A*)	*T. fusca*	Tobacco	40 % TSP	Petersen and Bock (2011)
	Xyloglucanase (*xeg74*)	*T. fusca*	Tobacco	<40 % TSP	Petersen and Bock (2011)
	Laccase	*P. ostreatus*	Tobacco	2 % TP	Davarpanah et al. (2012)
	Monellin variant (*MNEI*)	synthetic	Tobacco	5 % TSP	Lee et al. (2012)
	β-glucuronidase (*GUS*)	pBI121 vector	Potato	41 % TSP	Segretin et al. (2012)
Bioplastics					
	Polyhydroxybutyrate (*phaA*, *phaB* and *phaC*)	*Acinetobacter* sp.	Tobacco	18.8 % DW	Bohmert-Tatarev et al. (2011)
Antigens					
	Cholera toxin-B subunit/apical membrane antigen-1 (*CTB-AMA1*)	*V. cholera-P. falciparum*	Tobacco Lettuce	13.2 % TSP 7.3 % TSP	Davoodi-Semiromi et al. (2010)
	Cholera toxin-B subunit/merozoite surface protein-1 (*CTB-MSP1*)	*V. cholera-P. falciparum*	Tobacco Lettuce	10.1 % TSP 6.1 % TSP	Davoodi-Semiromi et al. (2010)

(continued)

Table 10.2 (continued)

Sub-group	Molecule/Transgene	Source of transgene	Species	Yield[a]	Reference
	Capsid protein VP1 *(VP-ß-GUS)*	Food and Mouth Disease Virus (FMDV)	Tobacco	51 % TSP	Lentz et al. (2010)
	Core polypeptide of HCV (*core*^{syn})	synthetic	Tobacco	0.1 % TP	Madesis et al. (2010)
	Peptide 2L21from VP2 protein (*2L21-TD*)	Canine parvovirus (CPV)	Tobacco	6 % TSP	Ortigosa et al. (2010b)
	Protective antigen (*CTB-PA*)	*B. anthracis*	Tobacco Lettuce	29.6 % TSP 22.4 % TSP	Ruhlman et al. (2010)
	Major F4ac fimbrial subunit protein (*faeG*)	*E. coli* strain C_{83907}	Tobacco	0.15 % TSP	Shen et al. (2010)
	Domain IV protective antigen [*PA(dIV)*]	*B. anthracis*	Tobacco	5.3 % TSP	Gorantala et al. (2011)
	Dengue-3 serotype Polyprotein (*DENV3prM/E*)	Dengue	Lettuce	NR	Kanagaraj et al. (2011)
	Viral protein 8 (*VP8*)	Bovine Rotavirus strain C486	Tobacco	600 $\mu g\ g^{-1}$ FW	Lentz et al. (2011)
	Fragment C of tetanus toxin (*TetC*)	*C. tetani*	Tobacco	8 % TSP	Michoux et al. (2011)
	Mutated L1 (*L12xCysM*)	Human Papilloma Virus (HPV-16)	Tobacco	1.5 % TSP	Waheed et al. (2011b)
	Mutated L1 (*LTB-L12xCysM*)	Human Papilloma Virus (HPV-16)	Tobacco	2 % TSP	Waheed et al. (2011a)
	GRA4 antigen (*chlGRA4*)	*T. gondii*	Tobacco	0.2 % TP	Del L Yácono et al. (2012)
	viral protein 6 (*VP6*)	Rotavirus	Tobacco	15 % TSP	Inka Borchers et al. (2012)
	Outer surface protein A (*OspA*)	*B. burgdorferi*	Tobacco	7.6 % TSP	Michoux et al. (2013)
	Polypeptide containing V3 loop and the C4 domain from HIV gp120 (*C4V3*)	synthetic	Tobacco	25 $\mu g\ g^{-1}$ FW	Rubio-Infante et al. (2012)
	Plastoglobulin 35-HIV/p24 (*PGL35–HIVp24*)	Arabidopsis-HIV	Tobacco	1 % TP	Shanmugabalaji et al. (2013)
	Plastoglobulin 35-HCV core protein (*PGL35–HCVcore)*	Arabidopsis-HCV	Tobacco	NR	Shanmugabalaji et al. (2013)

(continued)

Table 10.2 (continued)

Sub-group	Molecule/Transgene	Source of transgene	Species	Yield[a]	Reference
Adjuvants					
	Extra domain A from fibronectin (*EDA*)	Mouse	Tobacco	2 % TP	Farran et al. (2010)
Antibodies					
	Fragments from camelid single-chain antibodies (*VHH*, *GUS-E-VHH*, *pep-VHH*)	Camelid	Tobacco	3 % TSP	Lentz et al. (2012)
Therapeutic proteins					
	Proinsulin (*CTB-Pins*)	Human	Tobacco Lettuce	72 % TP 12.3 % TP	Ruhlman et al. (2010)
	Coagulation factor IX (*CTB-FFIX*)	Human	Tobacco	3.8 % TSP	Verma et al. (2010b)
	ß-site of amyloid precursor protein cleaving enzyme (*BACE*)	Human	Tobacco	2 % TSP	Youm et al. (2010)
	A, B, C peptides of proinsulin (*CTB-PFx3*)	Human	Tobacco Lettuce	47 % TP 53 % TP	Boyhan and Daniell (2011)
	Cyanovirin-N (*CV-N*)	*N. ellipsosporum*	Tobacco	0.3 % TSP	Elghabi et al. (2011)
	Human transforming growth factor-ß3 (*TGFß3*)	synthetic	Tobacco	12 % TP	Gisby et al. (2011)
	Retrocyclin 101 (*RC101-GFP*)	Human	Tobacco	38 % TSP	Lee et al. (2011)
	Protegrin-1 (*PG1-GFP*)	Porcine	Tobacco	26 % TSP	Lee et al. (2011)
	Thioredoxin m-human serum albumin (*Trx m-HAS*)	Plastid-Human	Tobacco	26 % TSP	Sanz-Barrio et al. (2011)
	Thioredoxin f-human serum albumin (*Trx f-HAS*)	Plastid-Human	Tobacco	22 % TSP	Sanz-Barrio et al. (2011)
	Interferon α 5 (IFNA 5)	synthetic	Tobacco	4.4 pg g^{-1} FW	Khan and Nurjis (2012)
	Exendin-4 (*CTB-EX4*)	synthetic	Tobacco	14.3 % TP	Kwon et al. (2013)

yield according to original reference; *FW* fresh weight, *NR* not reported, *TSP* total soluble protein, *TP* total protein; *DW* dry weight

no laccase enzyme activity was detected. In addition, the transplastomic plants showed a retarded growth and a pale-green phenotype. The sweet protein monellin is a natural protein derived from berries of *Dioscoreophyllum cumminsii* (tropical rainforest vine) that has potential uses as noncarbohydrate sweetener for individuals that must control their sugar intake, but it is unstable at high temperatures and acidic pH. Hence, Lee et al. (2012) evaluated plastid transformation to produce more stable monellin variants. The ELISA assay detected a yield for each variant of about 50–60 μg/mg protein comparable to those obtained from the natural source.

10.4.2 Bioplastics

Several attempts have been done to produce polyhydroxyalkanoates (PHAs), a family of biodegradable and renewable plastics, by plastid transformation. Some studies were performed with vectors including a minimal transgene expression cassettes that gave very low polymer accumulation levels (Nakashita et al. 2001; Arai et al. 2004).

The *Ralstonia eutropha phb* operon (three bacterial enzymes) was expressed in tobacco plastids using two different approaches. In a first study, Lössl et al. (2003) used the promoter and 5′-UTR of the plastid *psbA* gene. Transplastomic plants showed a PHB accumulation level very variable with the maximum yield (up to 1.7 % dry weight in leaves) at the early stages of in vitro culture. Because the highest yield achieved was associated with a growth reduction, an inducible system to regulate the transcription of the *phb* operon in tobacco plastids was subsequently developed (Lössl et al. 2005). This approach was based on a nuclear located, ethanol-inducible T7 RNA polymerase which was targeted to plastids harboring the *phb* operon under the control of T7 regulatory sequences. Double transformed plants were sprayed with a 5 % ethanol solution and 4 weeks after induction, the PHB synthesis was evaluated by gas chromatography. After induction, the highest PHB content in transformed plants was 1,383 ppm in dry weight, whereas the background level in uninduced transformed plants was 171 ppm.

In order to optimize expression and reduce the chance of unwanted rearrangements between sequences in the expression cassette and host genome, Bohmert-Tatarev et al. (2011) selected PHB biosynthetic gene sequences (*phaA*, *phaB*, and *phaC*) from two bacteria (*Acinetobacter* sp. and *Bacillus megaterium*) with GC content and codon usage similar to that of tobacco plastome, regulatory elements with limited homology to the host plastome and short spacer elements of plastidial origin upstream of each transgene. The three genes of interest plus the marker gene were cloned downstream of the *psbA* coding sequence exploiting promoter and UTRs of the same plastid gene ("operon extension strategy"). Transplastomic plants were capable of producing up to 18.8 % dry weight PHB in leaves. Furthermore, in contrast to previous results (Lössl et al. 2003), they were fertile and produced progeny with a high PHB content.

10.4.3 Antigens, Antibodies, and Adjuvants

During the past few years, exciting progress has been made with plastid-based production of pharmaceuticals and in particular with vaccine subunits, as recently summarized in several reviews (Cardi et al. 2010; Lössl and Waheed 2011; Maliga and Bock 2011; Scotti et al. 2012). Issues particularly important for this class of molecules are the achievement of high protein yields and stability, because the stimulation of the immune system, especially mucosal immunity, requires high doses of a stable antigen.

Recently, Inka Borchers et al. (2012) increased the accumulation and stability of rotavirus VP6 protein altering its 5′-UTR and 5′ end of the coding region. Compared to previous results (Birch-Machin et al. 2004), the inclusion of the 5′-UTR from T7g10 and of 15 nucleotides at the 5′ end of the *VP6* coding region increased its expression level up to 15 % of total leaf protein. Further, they observed that these sequences stabilized the protein accumulation in both young and old leaves, and that the plastid-based VP6 proteins assembled into trimeric forms similarly to rotavirus capsids (Inka Borchers et al. 2012). For other viral antigens, it has been observed that the use of a 5′-UTR plus additional nucleotides at the 5′ end of coding region (HPV-16 L1 or HIV-1 $Pr55^{gag}$) or the production of a fusion protein by adding partial or complete protein sequences at the 3′ end of coding sequences (2L21 peptide from canine parvovirus or epitope of VP1 protein of the foot and mouth disease virus) can increase protein yields up to 51 % TSP (Lenzi et al. 2008; Scotti et al. 2009; Lentz et al. 2010; Ortigosa et al. 2010b). On the other hand, Rigano et al. (2009) demonstrated that the only use of the T7g10 5′-UTR was sufficient to accumulate the envelope protein (A27L) of vaccinia virus up to 18 % TSP.

In order to confer dual immunity against cholera and malaria, Davoodi-Semiromi et al. (2010) fused the cholera toxin-B subunit (CTB) to two malarial antigens, apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1), and expressed them in tobacco and lettuce chloroplasts. CTB-AMA1 and CTB-MSP1 were accumulated up to 13.2 % and 10.1 % TSP in transplastomic tobacco plants and up to 7.3 % and 6.1 % TSP in transplastomic lettuce plants, respectively. The tobacco plastid-based fusion proteins were used to immunize subcutaneously or orally nine groups of mice. Significant levels of specific antibodies were detected for both diseases. In addition, the analysis of several immunological markers suggests that immunity was conferred via the Tr1 cellular and Th2 humoral immune responses. Another multicomponent vaccine that elicited both systemic and mucosal immune responses was based on fusion protein containing epitopes against diphtheria, pertussis, and tetanus (DPT) expressed in tobacco plastome (Soria-Guerra et al. 2009).

Gorantala et al. (2011) developed a vaccine against anthrax based on domain IV of protective antigen [PA(dIV)] in tobacco chloroplasts, obtaining an expression level up to 5.3 % TSP with an AT rich sequence of the gene. Further, they compared the protective response of plant- and *E. coli*-derived [PA(dIV)] in

mice intraperitoneally or orally immunized with or without adjuvant. Although the highest antibody titers (>10^5) were detected in adjuvanted *E. coli* [PA(dIV)] groups, adjuvanted plastid-derived [PA(dIV)] also induced significant specific antibody titers (>10^4) in both intraperitoneal and oral immunizations. Challenge with *Bacillus anthracis* in mice intraperitoneally immunized with adjuvanted plastid-derived [PA(dIV)] conferred a lower protection (60 % vs. 100 %) than in mice immunized with adjuvanted *E. coli* [PA(dIV)].

Recently, Lentz et al. (2012) produced in tobacco chloroplasts a fragment from camelid single-chain antibodies, also known as nanobody or VHH, directed against rotavirus VP6 protein and able to neutralize rotavirus infection. They pursued three strategies: expression of the original VHH in the chloroplast stroma (VHH 3B2), a translational fusion between GUS protein and VHH (GUS-E-VHH), and the targeting of VHH to thylakoid lumen by adding a N-terminal signal peptide of the pectate lyase B of *Erwinia carotovora* (pep-VHH). Transplastomic plants expressing the nanobodies VHH 3B2 and pep-VHH were characterized by transgene instability, heteroplasmic genotype and loss of the transgene in the seeds, whereas GUS-E-VHH plants had a normal development and accumulated the nanobody in the stroma at 3 % TSP. However, the few pep-VHH homoplasmic lines obtained confirmed protein translocation into the thylakoid lumen, where the pep-VHH polypeptide was stable and its expression levels reached 2–3 % of the total soluble proteins.

To date, there is only one example of a plastid-derived adjuvant corresponding to the extra domain A (EDA) from fibronectin (Farran et al. 2010). Similarly to previous examples, the highest protein yield (2 % of total cellular protein) was obtained when the protein was translationally fused to the first 15 amino acids of the green fluorescent protein (GFP). The EDA protein was purified from tobacco leaves and used in biological assay in order to demonstrate that it retained its pro-inflammatory properties and hence that could be used as adjuvant.

10.4.4 Therapeutic Proteins

Recently, Khan and Nurjis (2012) expressed in tobacco a synthetic interferon alpha 5 gene, belonging to an important class of proteins used for the treatment of different malignancies and virologic diseases. ELISA assay carried out on total extracts of transplastomic plants demonstrated that this protein accumulated to very low yield (up to 4.4 pg/g fresh weight). Similarly, a low yield was obtained for cyanovirin-N (CV-N), a small protein (11 kDa) difficult to express in chloroplasts, able to inactivate at nanomolar concentrations all variants of HIV-1 (Elghabi et al. 2011). Various terminal fusions were tested to improve the production of CV-N in such organelle, and the highest protein yield (0.3 % TSP) was obtained with either N- or N- and C-terminal GFP fusions.

A much higher yield (12 % of total leaf protein) was obtained with a synthetic gene containing 33 % GC encoding for human transforming growth factor-β3

(TGFβ3) that was accumulated in insoluble aggregates (Gisby et al. 2011). Its insolubility facilitated initial purification and refolding in homodimeric chains linked by disulfide bonds. A biologic assay based on the ability of TGFβ3 to inhibit the proliferation of mink lung epithelial cells was carried out on refolded plastid-based protein and standard protein, and showed a similar dose-response curve between the two proteins.

Recently, various therapeutic agents, such as retrocyclin-101 (RC101) and protegrin-1 (PG1), proinsulin, and coagulation factor IX, were produced in transgenic chloroplasts (Verma et al. 2010b; Boyhan and Daniell 2011; Lee et al. 2011). In order to confer protein stability, all genes were fused to GFP or CTB sequences. The protein yields of RC101 and PG1 antimicrobial peptides, promising therapeutic agents against bacterial and/or viral infections, especially those caused by the HIV-1 or sexually transmitted bacteria, were estimated to be approximately 35 % and 25 % TSP, respectively. The antimicrobial activity of both proteins was confirmed by inoculation of potted plants with *E. carotovora*. Further, RC101 transplastomic plants were resistent to tobacco mosaic virus infections confirming also the antiviral activity (Lee et al. 2011). Hence, such peptides could have also a role against plant pathogens.

Some lysine-type antibiotics were recently developed in tobacco chloroplasts using two strategies. The highest yield (more than 70 % TSP) was obtained with the synthetic *plyGBS* gene (Oey et al. 2009a). The other two antibiotics (Cpl-1 and Pal), that proved to be unclonable into standard plastid expression cassettes, were expressed using an innovative strategy (called toxin shuttle), based on preventing lethal transgene transcription in *E. coli* by inducing premature transcription termination upstream of the transgene coding region using bacterial transcription terminators (Oey et al. 2009b). The bacterial terminators were flanked by *loxP* sites and could therefore be excised *in planta* by site-specific recombination after chloroplast transformation.

Since it is widely known that thioredoxins, small ubiquitous proteins, were able to enhance solubility and stability of recombinant proteins in microbial expression systems, Sanz-Barrio et al. (2011) evaluated the role of thioredoxins as modulators of the expression of the human serum albumin (HSA), which has been previously shown to form inclusion bodies in plastids (Fernandez-San Millan et al. 2003). For such a purpose, two strategies were assayed based on the fusion of thioredoxins m and f to HSA, or on co-expression with HSA on the same vector. Trx m-HSA and Trx f-HSA fusion lines accumulated, in inclusion bodies, the human serum albumin to 26 % TSP and 22 % TSP, respectively; whilst the HSA in co-expressed lines was mainly found as soluble protein with accumulation level of 1.5 and 3.1 % TSP for Trx m and Trx f, respectively. The differences observed in terms of protein accumulation were mainly due to higher HSA stability of the fused proteins.

The human proinsulin (A, B, and C peptides) was expressed in tobacco and lettuce chloroplasts and accumulated up to 47 % and 53 % of total leaf protein, respectively (Boyhan and Daniell 2011). Accumulation of this protein was stable also in senescent and dried lettuce leaves. Proinsulin was purified from tobacco

leaves up to 98 % of purity. Oral and injectable delivery of plastid-based proinsulin into mice showed reduction of glucose level in blood similar to that obtained with commercial processed insulin.

Current treatment of the hemophilia disorders based on intravenous infusion of recombinant or plasma-derived coagulation factors VIII or IX can induce anaphylactic reactions. In order to prevent these reactions, Verma et al. (2010b) tested a fusion protein CTB-coagulation factor IX (CTB-FFIX) produced by plastid transformation in a murine hemophilia B model. Oral delivery of plastid-based CTB-FFIX blocked the formation of inhibitory antibodies and eliminated fatal anaphylactic reactions.

An interesting therapeutic target of Alzheimer disease, the β-site of the amyloid precursor protein cleaving enzyme (BACE), was produced at 2 % of TSP in transgenic tobacco chloroplasts (Youm et al. 2010). Mice gavaged with extracts from transplastomic plants expressing the BACE enzyme showed a specific immune response.

10.5 Conclusions and Perspectives

The first example of stable plastid transformation in higher plants (tobacco) dates back to more than 20 years ago (Svab et al. 1990). Since then, the technology has been improved in many aspects, including the development of various methods for DNA introduction, marker genes and selection strategies, vector types, and methods for marker excision. Although the plastids of some species (e.g., the monocots) remain difficult to transform, a reproducible protocol is now available for about 20 species, belonging to 8 families (Maliga 2012). Besides to biotechnology, for which some applications have been discussed in the present chapter, the transformation of the plastome is also relevant to basic studies (Maliga 2004). In the last 3 years, more than 40 original articles showing a total production of about 80 proteins for applications in different fields have been published. Important challenges for the future remain the improvement of transformation protocols in species other than tobacco and related Solanaceae, the development of inducible expression vectors, the increase of the expression level in nongreen plastids and/or for difficult-to-accumulate proteins, and the optimization of recombinant protein purification protocols. For all these aspects, however, significant improvements have been made lately, as reported in this chapter and other recent reviews (Maliga and Bock 2011; Maliga 2012). “Aspirational goals” for plastid biotechnology have been recently discussed (Clarke and Daniell 2011). In the next future, only a small number of proteins will likely remain not expressible in transgenic plastids, such as those requiring glycosylation for their functionality.

To our knowledge, no transplastomic plants have been grown so far in the field for commercialization. Field trials, however, have been conducted with transplastomic petunia, soybean and tobacco to test degree of gene containment (greenbiotech.eu/?page_id = 501), herbicide tolerance (www.faqs.org/patents/app/20120023615),

and production of pharmaceuticals (Arlen et al. 2007), industrial enzymes (www.icongenetics.com/html/5935.htm) or bioplastic (www.metabolix.com/Products/Crop-based-Technologies/Research).

References

Adam Z, Frottin F, Espagne C, Meinnel T, Giglione C (2011) Interplay between N-terminal methionine excision and FtsH protease is essential for normal chloroplast development and function in Arabidopsis. Plant Cell 23:3745–3760

Agrawal P, Verma D, Daniell H (2011) Expression of *Trichoderma reesei* β-mannanase in tobacco chloroplasts and its utilization in lignocellulosic woody biomass hydrolysis. PLoS ONE 6:e29302

Ahmad N, Michoux F, McCarthy J, Nixon PJ (2012a) Expression of the affinity tags, glutathione-S-transferase and maltose-binding protein, in tobacco chloroplasts. Planta 235:863–871

Ahmad N, Michoux F, Nixon PJ (2012b) Investigating the production of foreign membrane proteins in tobacco chloroplasts: expression of an algal plastid terminal oxidase. PLoS ONE 7:e41722

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breeding 15:305–327

Apel W, Bock R (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin a conversion. Plant Physiol 151:59–66

Apel W, Schulze WX, Bock R (2010) Identification of protein stability determinants in chloroplasts. Plant J 63:636–650

Arai Y, Shikanai T, Doi Y, Yoshida S, Yamaguchi I, Nakashita H (2004) Production of polyhydroxybutyrate by polycistronic expression of bacterial genes in tobacco plastid. Plant Cell Physiol 45:1176–1184

Arlen PA, Falconer R, Cherukumilli S, Cole A, Cole AM, Oishi KK, Daniell H (2007) Field production and functional evaluation of chloroplast-derived interferon-α2b. Plant Biotechnol J 5:511–525

Bally J, Paget E, Droux M, Job C, Job D, Dubald M (2008) Both the stroma and thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide bonds in recombinant proteins. Plant Biotechnol J 6:46–61

Barkan A (2011) Expression of plastid genes: organelle-specific elaborations on a prokaryotic scaffold. Plant Physiol 155:1520–1532

Barone P, Zhang XH, Widholm JM (2009) Tobacco plastid transformation using the feedback-insensitive anthranilate synthase [alpha]-subunit of tobacco (ASA2) as a new selectable marker. J Exp Bot 60:3195–3202

Bendich AJ (2004) Circular chloroplast chromosomes: the grand illusion. Plant Cell 16:1661–1666

Bienvenut WV, Espagne C, Martinez A, Majeran W, Valot B, Zivy M, Vallon O, Adam Z, Meinnel T, Giglione C (2011) Dynamics of post-translational modifications and protein stability in the stroma of Chlamydomonas reinhardtii chloroplasts. Proteomics 11:1734–1750

Birch-Machin I, Newell CA, Hibberd JM, Gray JC (2004) Accumulation of rotavirus VP6 protein in chloroplasts of transplastomic tobacco is limited by protein stability. Plant Biotechnol J 2:261–270

Blanco NE, Ceccoli RD, Segretin ME, Poli HO, Voss I, Melzer M, Bravo-Almonacid FF, Scheibe R, Hajirezaei MR, Carrillo N (2011) Cyanobacterial flavodoxin complements ferredoxin deficiency in knocked-down transgenic tobacco plants. Plant J 65:922–935

Bock R (2007) Structure, function, and inheritance of plastid genomes. In: Ralph B (ed) Cell and molecular biology of plastids. Topics in current genetics. Springer, Berlin, vol 19, pp 29–63

Bohmert-Tatarev K, McAvoy S, Daughtry S, Peoples OP, Snell KD (2011) High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate. Plant Physiol 155:1690–1708

Boyhan D, Daniell H (2011) Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. Plant Biotechnol J 9:585–598

Cardi T, Giegé P, Kahlau S, Scotti N (2012) Expression profiling of organellar genes. In: Bock R, Knoop V (eds) Genomics of chloroplasts and mitochondria, advances in photosynthesis and respiration. Springer, vol 35, pp 323–355

Cardi T, Lenzi P, Maliga P (2010) Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev Vaccines 9:893–911

Caroca R, Howell KA, Hasse C, Ruf S, Bock R (2013) Design of chimeric expression elements that confer high-level gene activity in chromoplasts. Plant J 73:368–379

Ceasar SA, Ignacimuthu S (2012) Genetic engineering of crop plants for fungal resistance: role of antifungal genes. Biotechnol Lett 34:995–1002

Ceccoli RD, Blanco NE, Segretin ME, Melzer M, Hanke GT, Scheibe R, Hajirezaei MR, Bravo-Almonacid FF, Carrillo N (2012) Flavodoxin displays dose-dependent effects on photosynthesis and stress tolerance when expressed in transgenic tobacco plants. Planta 236:1447–1458

Clarke J, Daniell H (2011) Plastid biotechnology for crop production: present status and future perspectives. Plant Mol Biol 76:211–220

Collinge DB, Jorgensen HJ, Lund OS, Lyngkjaer MF (2010) Engineering pathogen resistance in crop plants: current trends and future prospects. Annu Rev Phytopathol 48:269–291

Cominelli E, Tonelli C (2010) Transgenic crops coping with water scarcity. New Biotechnol 27:473–477

Craig W, Gargano D, Scotti N, Nguyen TT, Lao NT, Kavanagh TA, Dix PJ, Cardi T (2005) Direct gene transfer in potato: a comparison of particle bombardment of leaf explants and PEG-mediated transformation of protoplasts. Plant Cell Rep 24:603–611

Craig W, Lenzi P, Scotti N, De Palma M, Saggese P, Carbone V, Curran NM, Magee AM, Medgyesy P, Kavanagh TA, Dix PJ, Grillo S, Cardi T (2008) Transplastomic tobacco plants expressing a fatty acid desaturase gene exhibit altered fatty acid profiles and improved cold tolerance. Transgenic Res 17:769–782

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

Davarpanah S, Ahn J-W, Ko S, JUng S, Park Y-I, Liu J, Jeong W (2012) Stable expression of a fungal laccase protein using transplastomic tobacco. Plant Biotechnol Rep 6:305–312

Davoodi-Semiromi A, Schreiber M, Nalapalli S, Verma D, Singh ND, Banks RK, Chakrabarti D, Daniell H (2010) Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. Plant Biotechnol J 8:223–242

Day A, Goldschmidt-Clermont M (2011) The chloroplast transformation toolbox: selectable markers and marker removal. Plant Biotechnol J 9:540–553

Day A, Madesis P (2007) DNA replication, recombination, and repair in plastids. In: Bock R (ed) Cell and molecular biology of plastids, vol 19., Topics in current geneticsSpringer, Berlin, pp 65–119

De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19:71–74

De Marchis F, Pompa A, Bellucci M (2012) Plastid proteostasis and heterologous protein accumulation in transplastomic plants. Plant Physiol 160:571–581

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

Del L Yácono M, Farran I, Becher ML, Sander V, Sanchez VR, Martin V, Veramendi J, Clemente M (2012) A chloroplast-derived Toxoplasma gondii GRA4 antigen used as an oral vaccine protects against toxoplasmosis in mice. Plant Biotechnol J 10:1136–1144

Dix PJ, Kavanagh TA (1995) Transforming the plastome: genetic markers and DNA delivery systems. Euphytica 85:29–34

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

Egelkrout E, Rajan V, Howard JA (2012) Overproduction of recombinant proteins in plants. Plant Sci 184:83–101

Elghabi Z, Karcher D, Zhou F, Ruf S, Bock R (2011) Optimization of the expression of the HIV fusion inhibitor cyanovirin-N from the tobacco plastid genome. Plant Biotechnol J 9:599–608

Farran I, McCarthy-Suarez I, Rio-Manterola F, Mansilla C, Lasarte JJ, Mingo-Castel AM (2010) The vaccine adjuvant extra domain a from fibronectin retains its proinflammatory properties when expressed in tobacco chloroplasts. Planta 231:977–990

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

Gisby MF, Mellors P, Madesis P, Ellin M, Laverty H, O'Kane S, Ferguson MW, Day A (2011) A synthetic gene increases TGFβ3 accumulation by 75-fold in tobacco chloroplasts enabling rapid purification and folding into a biologically active molecule. Plant Biotechnol J 9:618–628

Gisby MF, Mudd EA, Day A (2012) Growth of transplastomic cells expressing d-amino acid oxidase in chloroplasts is tolerant to D-alanine and inhibited by D-valine. Plant Physiol 160:2219–2226

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

Gorantala J, Grover S, Goel D, Rahi A, Jayadev Magani SK, Chandra S, Bhatnagar R (2011) A plant based protective antigen [PA(dIV)] vaccine expressed in chloroplasts demonstrates protective immunity in mice against anthrax. Vaccine 29:4521–4533

Gray BN, Yang H, Ahner BA, Hanson MR (2011) An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. Plant Mol Biol 76:345–355

Hanson MR, Gray BN, Ahner BA (2013) Chloroplast transformation for engineering of photosynthesis. J Exp Bot 64:731–742

Hasunuma T, Kondo A, Miyake C (2009) Metabolic pathway engineering by plastid transformation is a powerful tool for production of compounds in higher plants. Plant Biotechnol 26:39–46

Hasunuma T, Kondo A, Miyake C (2010) Metabolic engineering by plastid transformation as a strategy to modulate isoprenoid yield in plants. In: Fett-Neto AG (ed) Plant secondary metabolism engineering. Methods in molecular biology. Humana Press, vol 643, pp 213–227

Hasunuma T, Miyazawa S, Yoshimura S, Shinzaki Y, Tomizawa K, Shindo K, Choi SK, Misawa N, Miyake C (2008) Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. Plant J 55:857–868

Herz S, Füssl M, Steiger S, Koop HU (2005) Development of novel types of plastid transformation vectors and evaluation of factors controlling expression. Transgenic Res 14:969–982

Husaini AM, Rashid Z, Mir R, Aquil B (2011) Approaches for gene targeting and targeted gene expression in plants. GM Crops 2:150–162

Husken A, Prescher S, Schiemann J (2010) Evaluating biological containment strategies for pollen-mediated gene flow. Environ Biosafety Res 9:67–73

Hussein HS, Ruiz ON, Terry N, Daniell H (2007) Phytoremediation of mercury and organomercurials in chloroplast transgenic plants: enhanced root uptake, translocation to shoots, and volatilization. Environ Sci Technol 41:8439–8446

Inka Borchers AM, Gonzalez-Rabade N, Gray JC (2012) Increased accumulation and stability of rotavirus VP6 protein in tobacco chloroplasts following changes to the 5′ untranslated region and the 5′ end of the coding region. Plant Biotechnol J 10:422–434

Jansen R, Ruhlman T (2012) Plastid genomes of seed plants. In: Bock R, Knoop V (eds) Genomics of chloroplasts and mitochondria, Advances in photosynthesis and respiration. Springer, vol 35, pp 103–126

Jansen RK, Saski C, Lee SB, Hansen AK, Daniell H (2011) Complete plastid genome sequences of three Rosids (*Castanea, Prunus, Theobroma*): evidence for at least two independent transfers of rpl22 to the nucleus. Mol Biol Evol 28:835–847

Jin S, Kanagaraj A, Verma D, Lange T, Daniell H (2011) Release of hormones from conjugates: chloroplast expression of beta-glucosidase results in elevated phytohormone levels associated with significant increase in biomass and protection from aphids or whiteflies conferred by sucrose esters. Plant Physiol 155:222–235

Jin S, Zhang X, Daniell H (2012) *Pinellia ternata* agglutinin expression in chloroplasts confers broad spectrum resistance against aphid, whitefly, Lepidopteran insects, bacterial and viral pathogens. Plant Biotechnol J 10:313–327

Jones JD (2011) Why genetically modified crops? Philos Transact A Math Phys Eng Sci 369:1807–1816

Kahlau S, Bock R (2008) Plastid transcriptomics and translatomics of tomato fruit development and chloroplast-to-chromoplast differentiation: chromoplast gene expression largely serves the production of a single protein. Plant Cell 20:856–874

Kanagaraj AP, Verma D, Daniell H (2011) Expression of dengue-3 premembrane and envelope polyprotein in lettuce chloroplasts. Plant Mol Biol 76:323–333

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

Kim JY, Kavas M, Fouad WM, Nong G, Preston JF, Altpeter F (2011) Production of hyperthermostable GH10 xylanase Xyl10B from *Thermotoga maritima* in transplastomic plants enables complete hydrolysis of methylglucuronoxylan to fermentable sugars for biofuel production. Plant Mol Biol 76:357–369

Krech K, Ruf S, Masduki FF, Thiele W, Bednarczyk D, Albus CA, Tiller N, Hasse C, Schottler MA, Bock R (2012) The plastid genome-encoded Ycf4 protein functions as a nonessential assembly factor for photosystem I in higher plants. Plant Physiol 159:579–591

Krichevsky A, Meyers B, Vainstein A, Maliga P, Citovsky V (2010) Autoluminescent Plants. PLoS ONE 5:e15461

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

Kumar S, Hahn FM, Baidoo E, Kahlon TS, Wood DF, McMahan CM, Cornish K, Keasling JD, Daniell H, Whalen MC (2012) Remodeling the isoprenoid pathway in tobacco by expressing the cytoplasmic mevalonate pathway in chloroplasts. Metab Eng 14:19–28

Kuroda H, Maliga P (2001) Complementarity of the 16S rRNA penultimate stem with sequences downstream of the AUG destabilizes the plastid mRNAs. Nucleic Acids Res 29:970–975

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

Kwon KC, Nityanandam R, New JS, Daniell H (2013) Oral delivery of bioencapsulated exendin-4 expressed in chloroplasts lowers blood glucose level in mice and stimulates insulin secretion in beta-TC6 cells. Plant Biotechnol J 11:77–86

Le Martret B, Poage M, Shiel K, Nugent GD, Dix PJ (2011) Tobacco chloroplast transformants expressing genes encoding dehydroascorbate reductase, glutathione reductase, and glutathione-S-transferase, exhibit altered anti-oxidant metabolism and improved abiotic stress tolerance. Plant Biotechnol J 9:661–673

Lee SB, Kim Y, Lee J, Oh K-J, Byun M-O, Jeong M-J, Bae S-C (2012) Stable expression of the sweet protein monellin variant MNEI in tobacco chloroplasts. Plant Biotechnol Rep 6:285–295

Lee SB, Li B, Jin S, Daniell H (2011) Expression and characterization of antimicrobial peptides Retrocyclin-101 and Protegrin-1 in chloroplasts to control viral and bacterial infections. Plant Biotechnol J 9:100–115

Lentz EM, Garaicoechea L, Alfano EF, Parreño V, Wigdorovitz A, Bravo-Almonacid FF (2012) Translational fusion and redirection to thylakoid lumen as strategies to improve the accumulation of a camelid antibody fragment in transplastomic tobacco. Planta 236:703–714

Lentz EM, Mozgovoj MV, Bellido D, dus santos mj, Wigdorovitz A, Bravo-Almonacid FF (2011) VP8* antigen produced in tobacco transplastomic plants confers protection against bovine rotavirus infection in a suckling mouse model. J Biotechnol 156:100–107

Lentz EM, Segretin ME, Morgenfeld MM, Wirth SA, Dus Santos MJ, Mozgovoj MV, Wigdorovitz A, Bravo-Almonacid FF (2010) High expression level of a foot and mouth disease virus epitope in tobacco transplastomic plants. Planta 231:387–395

Lenzi P, Scotti N, Alagna F, Tornesello M, Pompa A, Vitale A, De Stradis A, Monti L, Grillo S, Buonaguro F, Maliga P, Cardi T (2008) Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances antigen accumulation in transplastomic tobacco. Transgenic Res 17:1091–1102

Lerbs-Mache S (2011) Function of plastid sigma factors in higher plants: regulation of gene expression or just preservation of constitutive transcription? Plant Mol Biol 76:235–249

Li W, Ruf S, Bock R (2011) Chloramphenicol acetyltransferase as selectable marker for plastid transformation. Plant Mol Biol 76:443–451

Liere K, Börner T (2007) Transcription and transcriptional regulation in plastids. In: Ralph B (ed) Cell and molecular biology of plastids, vol 19., Topics in current geneticsSpringer, Berlin, pp 121–174

Liu CW, Lin CC, Yiu JC, Chen JJ, Tseng MJ (2008) Expression of a *Bacillus thuringiensis* toxin (cry1Ab) gene in cabbage (*Brassica oleracea* L. var. capitata L.) chloroplasts confers high insecticidal efficacy against *Plutella xylostella*. Theor Appl Genet 117:75–88

Lössl A, Bohmert K, Harloff H, Eibl C, Muhlbauer S, Koop HU (2005) Inducible trans-activation of plastid transgenes: expression of the *R. eutropha phb* operon in transplastomic tobacco. Plant Cell Physiol 46:1462–1471

Lössl A, Eibl C, Harloff HJ, Jung C, Koop HU (2003) Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of polyhydroxybutyrate are associated with growth reduction. Plant Cell Rep 21:891–899

Lössl AG, Waheed MT (2011) Chloroplast-derived vaccines against human diseases: achievements, challenges and scopes. Plant Biotechnol J 9:527–539

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

Madesis P, Osathanunkul M, Georgopoulou U, Gisby MF, Mudd EA, Nianiou I, Tsitoura P, Mavromara P, Tsaftaris A, Day A (2010) A hepatitis C virus core polypeptide expressed in chloroplasts detects anti-core antibodies in infected human sera. J Biotechnol 145:377–386

Madoka Y, Tomizawa K, Mizoi J, Nishida I, Nagano Y, Sasaki Y (2002) Chloroplast transformation with modified accD operon increases acetyl-CoA carboxylase and causes extension of leaf longevity and increase in seed yield in tobacco. Plant Cell Physiol 43:1518–1525

Magee AM, Aspinall S, Rice DW, Cusack BP, Sémon M, Perry AS, Stefanović S, Milbourne D, Barth S, Palmer JD, Gray JC, Kavanagh TA, Wolfe KH (2010) Localized hypermutation and associated gene losses in legume chloroplast genomes. Genome Res 20:1700–1710

Maier RM, Schmitz-Linneweber C (2004) Plastid genomes. In: Daniell H, Chase C (eds) Molecular biology and biotechnology of plant organelles. Springer, Dordrecht, pp 115–150

Majeran W, Friso G, Asakura Y, Qu X, Huang M, Ponnala L, Watkins KP, Barkan A, van Wijk KJ (2012) Nucleoid-enriched proteomes in developing plastids and chloroplasts from maize leaves: a new conceptual framework for nucleoid functions. Plant Physiol 158:156–189

Maliga P (2002) Engineering the plastid genome of higher plants. Curr Opin Plant Biol 5:164–172

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

Maliga P (2012) Plastid transformation in flowering plants. In: Bock R, Knoop V (eds) Genomics of Chloroplasts and Mitochondria. Advances in photosynthesis and respiration. Springer, vol 35, pp 393–414

Maliga P, Bock R (2011) Plastid biotechnology: food, fuel, and medicine for the 21st century. Plant Physiol 155:1501–1510

Malik Ghulam M, Zghidi-Abouzid O, Lambert E, Lerbs-Mache S, Merendino L (2012) Transcriptional organization of the large and the small ATP synthase operons, atpI/H/F/A and atpB/E, in Arabidopsis thaliana chloroplasts. Plant Mol Biol 79:259–272

Manimaran P, Ramkumar G, Sakthivel K, Sundaram RM, Madhav MS, Balachandran SM (2011) Suitability of non-lethal marker and marker-free systems for development of transgenic crop plants: present status and future prospects. Biotechnol Adv 29:703–714

Manuell AL, Quispe J, Mayfield SP (2007) Structure of the chloroplast ribosome: novel domains for translation regulation. PLoS Biol 5:e209

Marchfelder A, Binder S (2004) Plastid and plant mitochondrial RNA processing and RNA stability. In: Daniell H, Chase C (eds) Molecular biology and biotechnology of plant organelles. Springer, Dordrecht, pp 261–294

Marín-Navarro J, Manuell AL, Wu J, Mayfield PS (2007) Chloroplast translation regulation. Photosynth Res 94:359–374

McBride KE, Svab Z, Schaaf DJ, Hogan 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 (N Y) 13:362–365

Meyers B, Zaltsman A, Lacroix B, Kozlovsky SV, Krichevsky A (2010) Nuclear and plastid genetic engineering of plants: comparison of opportunities and challenges. Biotechnol Adv 28:747–756

Michoux F, Ahmad N, Hennig A, Nixon PJ, Warzecha H (2013) Production of leafy biomass using temporary immersion bioreactors: an alternative platform to express proteins in transplastomic plants with drastic phenotypes. Planta 237:903–908

Michoux F, Ahmad N, McCarthy J, Nixon PJ (2011) Contained and high-level production of recombinant protein in plant chloroplasts using a temporary immersion bioreactor. Plant Biotechnol J 9:575–584

Nakashita H, Arai Y, Shikanai T, Doi Y, Yamaguchi I (2001) Introduction of bacterial metabolism into higher plants by polycistronic transgene expression. Biosci Biotechnol Biochem 65:1688–1691

Naqvi S, Farré G, Sanahuja G, Capell T, Zhu C, Christou P (2010) When more is better: multigene engineering in plants. Trends Plant Sci 15:48–56

Oey M, Lohse M, Kreikemeyer B, Bock R (2009a) Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. Plant J 57:436–445

Oey M, Lohse M, Scharff LB, Kreikemeyer B, Bock R (2009b) Plastid production of protein antibiotics against pneumonia via a new strategy for high-level expression of antimicrobial proteins. Proc Natl Acad Sci USA 106:6579–6584

Ortigosa SM, Diaz-Vivancos P, Clemente-Moreno MJ, Pinto-Marijuan M, Fleck I, Veramendi J, Santos M, Hernandez JA, Torne JM (2010a) Oxidative stress induced in tobacco leaves by chloroplast over-expression of maize plastidial transglutaminase. Planta 232:593–605

Ortigosa SM, Fernández-San Millán A, Veramendi J (2010b) Stable production of peptide antigens in transgenic tobacco chloroplasts by fusion to the p53 tetramerisation domain. Transgenic Res 19:703–709

Orzaez D, Monforte AJ, Granell A (2010) Using genetic variability available in the breeder's pool to engineer fruit quality. GM Crops 1:120–127

Peled-Zehavi H, Danon A (2007) Translation and translational regulation in chloroplasts. In: Bock R (ed) Cell and molecular biology of plastids, vol 19., Topics in current genetic-sSpringer, Berlin, pp 249–281

Petersen K, Bock R (2011) High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. Plant Mol Biol 76:311–321

Pfalz J, Bayraktar OA, Prikryl J, Barkan A (2009) Site-specific binding of a PPR protein defines and stabilizes 5′ and 3′ mRNA termini in chloroplasts. EMBO J 28:2042–2052

Poage M, Le Martret B, Jansen MA, Nugent GD, Dix PJ (2011) Modification of reactive oxygen species scavenging capacity of chloroplasts through plastid transformation. Plant Mol Biol 76:371–384

Prikryl J, Rojas M, Schuster G, Barkan A (2011) Mechanism of RNA stabilization and translational activation by a pentatricopeptide repeat protein. Proc Natl Acad Sci USA 108:415–420

Que Q, Chilton MD, de Fontes CM, He C, Nuccio M, Zhu T, Wu Y, Chen JS, Shi L (2010) Trait stacking in transgenic crops: challenges and opportunities. GM Crops 1:220–229

Quesada-Vargas T, Ruiz ON, Daniell H (2005) Characterization of heterologous multigene operons in transgenic chloroplasts: transcription, processing, and translation. Plant Physiol 138:1746–1762

Reguera M, Peleg Z, Blumwald E (2012) Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops. Biochim Biophys Acta 1819:186–194

Rigano MM, Manna C, Giulini A, Pedrazzini E, Capobianchi M, Castilletti C, Di Caro A, Ippolito G, Beggio P, De Giuli Morghen C, Monti L, Vitale A, Cardi T (2009) Transgenic chloroplasts are efficient sites for high-yield production of the vaccinia virus envelope protein A27L in plant cells dagger. Plant Biotechnol J 7:577–591

Rigano MM, Scotti N, Cardi T (2012) Unsolved problems in plastid transformation. Bioengineered 3:329–333

Rogalski M, Carrer H (2011) Engineering plastid fatty acid biosynthesis to improve food quality and biofuel production in higher plants. Plant Biotechnol J 9:554–564

Rojas CA, Hemerly AS, Ferreira PC (2010) Genetically modified crops for biomass increase. Genes and strategies. GM Crops 1:137–142

Rosellini D (2012) Selectable markers and reporter genes: a well furnished toolbox for plant science and genetic engineering. Crit Rev Plant Sci 31:401–453

Rott M, Martins NF, Thiele W, Lein W, Bock R, Kramer DM, Schottler MA (2011) ATP synthase repression in tobacco restricts photosynthetic electron transport, CO2 assimilation, and plant growth by overacidification of the thylakoid lumen. Plant Cell 23:304–321

Rubio-Infante N, Govea-Alonso DO, Alpuche-Solis AG, Garcia-Hernandez AL, Soria-Guerra RE, Paz-Maldonado LM, Ilhuicatzi-Alvarado D, Varona-Santos JT, Verdin-Teran L, Korban SS, Moreno-Fierros L, Rosales-Mendoza S (2012) A chloroplast-derived C4V3 polypeptide from the human immunodeficiency virus (HIV) is orally immunogenic in mice. Plant Mol Biol 78:337–349

Ruhlman T, Verma D, Samson N, Daniell H (2010) The role of heterologous chloroplast sequence elements in transgene integration and expression. Plant Physiol 152:2088–2104

Ruiz ON, Alvarez D, Torres C, Roman L, Daniell H (2011) Metallothionein expression in chloroplasts enhances mercury accumulation and phytoremediation capability. Plant Biotechnol J 9:609–617

Ruiz ON, Daniell H (2005) Engineering cytoplasmic male sterility via the chloroplast genome by expression of {beta}-ketothiolase. Plant Physiol 138:1232–1246

Ruiz ON, Hussein HS, Terry N, Daniell H (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiol 132:1344–1352

Sanz-Barrio R, Millan AF, Corral-Martinez P, Segui-Simarro JM, Farran I (2011) Tobacco plastidial thioredoxins as modulators of recombinant protein production in transgenic chloroplasts. Plant Biotechnol J 9:639–650

Saski C, Lee SB, Fjellheim S, Guda C, Jansen RK, Luo H, Tomkins J, Rognli OA, Daniell H, Clarke JL (2007) Complete chloroplast genome sequences of Hordeum vulgare, *Sorghum bicolor* and *Agrostis stolonifera*, and comparative analyses with other grass genomes. Theor Appl Genet 115:571–590

Scharff LB, Childs L, Walther D, Bock R (2011) Local absence of secondary structure permits translation of mRNAs that lack ribosome-binding sites. PLoS Genet 7:e1002155

Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci 13:663–670

Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci 3:175–180

Schuster G, Stern D (2009) RNA polyadenylation and decay in mitochondria and chloroplasts. In: Condon C (ed) Progress in molecular biology and translational science. Molecular biology of RNA processing and decay in prokaryotes. Elsevier Inc., vol 85, pp 393–422

Scotti N, Alagna F, Ferraiolo E, Formisano G, Sannino L, Buonaguro L, De Stradis A, Vitale A, Monti L, Grillo S, Buonaguro FM, Cardi T (2009) High-level expression of the HIV-1 Pr55(gag) polyprotein in transgenic tobacco chloroplasts. Planta 229:1109–1122

Scotti N, Gargano D, Lenzi P, Cardi T (2011) Transformation of the plastid genome in higher plants. In: Dan Y, Ow DW (eds) Historical technology developments in plant transformation. Bentham Science Publishers Ltd., pp 123–145

Scotti N, Rigano MM, Cardi T (2012) Production of foreign proteins using plastid transformation. Biotechnol Adv 30:387–397

Segretin ME, Lentz EM, Wirth SA, Morgenfeld MM, Bravo-Almonacid FF (2012) Transformation of *Solanum tuberosum* plastids allows high expression levels of beta-glucuronidase both in leaves and microtubers developed in vitro. Planta 235:807–818

Shanmugabalaji V, Besagni C, Piller LE, Douet V, Ruf S, Bock R, Kessler F (2013) Dual targeting of a mature plastoglobulin/fibrillin fusion protein to chloroplast plastoglobules and thylakoids in transplastomic tobacco plants. Plant Mol Biol 81:13–25

Shaver JM, Oldenburg DJ, Bendich AJ (2008) The structure of chloroplast DNA molecules and the effects of light on the amount of chloroplast DNA during development in medicago truncatula. Plant Physiol 146:1064–1074

Shen H, Qian B, Chen W, Liu Z, Yang L, Zhang D, Liang W (2010) Immunogenicity of recombinant F4 (K88) fimbrial adhesin FaeG expressed in tobacco chloroplast. Acta Biochim Biophys Sin (Shanghai) 42:558–567

Shimizu M, Goto M, Hanai M, Shimizu T, Izawa N, Kanamoto H, Tomizawa K, Yokota A, Kobayashi H (2008) Selectable tolerance to herbicides by mutated acetolactate synthase genes integrated into the chloroplast genome of tobacco. Plant Physiol 147:1976–1983

Singer SD, Liu Z, Cox KD (2012) Minimizing the unpredictability of transgene expression in plants: the role of genetic insulators. Plant Cell Rep 31:13–25

Soria-Guerra RE, Alpuche-Solis AG, Rosales-Mendoza S, Moreno-Fierros L, Bendik EM, Martinez-Gonzalez L, Korban SS (2009) Expression of a multi-epitope DPT fusion protein in transplastomic tobacco plants retains both antigenicity and immunogenicity of all three components of the functional oligomer. Planta 229:1293–1302

Stern DB, Goldschmidt-Clermont M, Hanson MR (2010) Chloroplast RNA metabolism. Annu Rev Plant Biol 61:125–155

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

Tangphatsornruang S, Birch-Machin I, Newell CA, Gray JC (2011) The effect of different 3′ untranslated regions on the accumulation and stability of transcripts of a gfp transgene in chloroplasts of transplastomic tobacco. Plant Mol Biol 76:385–396

Thyssen G, Svab Z, Maliga P (2012) Exceptional inheritance of plastids via pollen in *Nicotiana sylvestris* with no detectable paternal mitochondrial DNA in the progeny. Plant J 72:84–88

Tiller N, Weingartner M, Thiele W, Maximova E, Schöttler MA, Bock R (2012) The plastid-specific ribosomal proteins of *Arabidopsis thaliana* can be divided into non-essential proteins and genuine ribosomal proteins. Plant J 69:302–316

Tillich M, Hardel SL, Kupsch C, Armbruster U, Delannoy E, Gualberto JM, Lehwark P, Leister D, Small ID, Schmitz-Linneweber C (2009) Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs. Proc Natl Acad Sci USA 106:6002–6007

Tillich M, Krause K (2010) The ins and outs of editing and splicing of plastid RNAs: lessons from parasitic plants. New Biotechnol 27:256–266

Tungsuchat-Huang T, Slivinski KM, Sinagawa-Garcia SR, Maliga P (2011) Visual spectinomycin resistance (aadA(au)) gene for facile identification of transplastomic sectors in tobacco leaves. Plant Mol Biol 76:453–461

Valkov VT, Gargano D, Manna C, Formisano G, Dix PJ, Gray JC, Scotti N, Cardi T (2011) High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5' and 3' regulatory sequences. Transgenic Res 20:137–151

Valkov VT, Scotti N, Kahlau S, MacLean D, Grillo S, Gray JC, Bock R, Cardi T (2009) Genome-wide analysis of plastid gene expression in potato leaf chloroplasts and tuber amyloplasts: transcriptional and posttranscriptional control. Plant Physiol 150:2030–2044

van Wijk KJ, Baginsky S (2011) Plastid proteomics in higher plants: current state and future goals. Plant Physiol 155:1578–1588

Verhounig A, Karcher D, Bock R (2010) Inducible gene expression from the plastid genome by a synthetic riboswitch. Proc Natl Acad Sci USA 107:6204–6209

Verma D, Kanagaraj A, Jin S, Singh ND, Kolattukudy PE, Daniell H (2010a) Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars. Plant Biotechnol J 8:332–350

Verma D, Moghimi B, LoDuca PA, Singh HD, Hoffman BE, Herzog RW, Daniell H (2010b) Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proc Natl Acad Sci USA 107:7101–7106

Waheed MT, Thönes N, Müller M, Hassan SW, Gottschamel J, Lössl E, Kaul HP, Lössl AG (2011a) Plastid expression of a double-pentameric vaccine candidate containing human papillomavirus-16 L1 antigen fused with LTB as adjuvant: transplastomic plants show pleiotropic phenotypes. Plant Biotechnol J 9:651–660

Waheed MT, Thönes N, Müller M, Hassan SW, Razavi NM, Lössl E, Kaul HP, Lössl AG (2011b) Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines. Transgenic Res 20:271–282

Walter M, Piepenburg K, Schöttler MA, Petersen K, Kahlau S, Tiller N, Drechsel O, Weingartner M, Kudla J, Bock R (2010) Knockout of the plastid RNase E leads to defective RNA processing and chloroplast ribosome deficiency. Plant J 64:851–863

Wang Y, Yau YY, Perkins-Balding D, Thomson JG (2011) Recombinase technology: applications and possibilities. Plant Cell Rep 30:267–285

Webster DE, Thomas MC (2012) Post-translational modification of plant-made foreign proteins; glycosylation and beyond. Biotechnol Adv 30:410–418

Whitney SM, Kane HJ, Houtz RL, Sharwood RE (2009) Rubisco oligomers composed of linked small and large subunits assemble in tobacco plastids and have higher affinities for CO_2 and O_2. Plant Physiol 149:1887–1895

Wicke S, Schneeweiss G, de Pamphilis C, Müller K, Quandt D (2011) The evolution of the plastid chromosome in land plants: gene content, gene order, gene function. Plant Mol Biol 76:273–297

Wurbs D, Ruf S, Bock R (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. Plant J 49:276–288

Yabuta Y, Tanaka H, Yoshimura S, Suzuki A, Tamoi M, Maruta T, Shigeoka S (2013) Improvement of vitamin E quality and quantity in tobacco and lettuce by chloroplast genetic engineering. Transgenic Res 22:391–402

Yang H, Gray BN, Ahner BA, Hanson MR (2013) Bacteriophage 5′ untranslated regions for control of plastid transgene expression. Planta 237:517–527

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

Youm JW, Jeon JH, Kim H, Min SR, Kim MS, Joung H, Jeong WJ, Kim HS (2010) High-level expression of a human β-site APP cleaving enzyme in transgenic tobacco chloroplasts and its immunogenicity in mice. Transgenic Res 19:1099–1108

Zerges W (2004) Regulation of translation in chloroplasts. In: Daniell H, Chase C (eds) Molecular biology of plant organelles. Springer, Dordrecht, pp 443–490

Zhang J, Ruf S, Hasse C, Childs L, Scharff LB, Bock R (2012) Identification of cis-elements conferring high levels of gene expression in non-green plastids. Plant J 72:115–128

Zhang J, Tan W, Yang XH, Zhang HX (2008) Plastid-expressed choline monooxygenase gene improves salt and drought tolerance through accumulation of glycine betaine in tobacco. Plant Cell Rep 27:1113–1124

Zhang XH, Webb J, Huang YH, Lin L, Tang RS, Liu A (2011) Hybrid Rubisco of tomato large subunits and tobacco small subunits is functional in tobacco plants. Plant Sci 180:480–488

Zhou F, Karcher D, Bock R (2007) Identification of a plastid intercistronic expression element (IEE) facilitating the expression of stable translatable monocistronic mRNAs from operons. Plant J 52:961–972

Zoschke R, Kroeger T, Belcher S, Schöttler MA, Barkan A, Schmitz-Linneweber C (2012) The pentatricopeptide repeat-SMR protein ATP4 promotes translation of the chloroplast atpB/E mRNA. Plant J 72:547–558