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Research review paper

Production of foreign proteins using plastid transformation ☆

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ABSTRACT

In the past decades, the progress made in plant biotechnology has made possible the use of plants as a novel production platform for a wide range of molecules. In this context, the transformation of the plastid genome has given a huge boost to prove that plants are a promising system to produce recombinant proteins. In this review, we provide a background on plastid genetics and on the principles of this technology in higher plants. Further, we discuss the most recent biotechnological applications of plastid transformation for the production of enzymes, therapeutic proteins, antibiotics, and proteins with immunological properties. We also discuss the potential of plastid biotechnology and the novel tools developed to overcome some limitations of chloroplast transformation.

Contents

1. Introduction

A range of expression systems has been proposed for the production of recombinant proteins with pharmaceutical, industrial and agricultural interest (Demain and Vaishnav, 2009). The bacterium *Escherichia coli* among prokaryotes and the Chinese Hamster Ovary (CHO) cells and yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris* and others) among eukaryotes have the longest history of use and represent 39, 35 and 15%, respectively, of biopharmaceuticals produced (Rader, 2008). Additional systems include other bacteria (*e.g. Bacillus* spp.), filamentous fungi (*Aspergillus* spp., etc.), insect cells (mainly from the Lepidoptera *Spodoptera frugiperda*), other mammalian cells, protozoa (*Leishmania* spp. and others), transgenic animals, microalgae, and plants. Both stable and transient transgene expression systems have been developed in mammalian cells. Advantages and disadvantages of different systems, and relevant factors for protein yield and general efficiency, have been recently reviewed (Demain and Vaishnav, 2009; Fernandez-Robledo and Vasta, 2010; Hacker et al., 2009; Houdebine, 2009; Potvin and Zhang, 2010; Surzycki et al., 2009).

Higher plants have been proposed as an economic, safe and easily scalable production system. Plants are eukaryotic organisms and so they can produce complex proteins and process them post-translationally similarly, except for some differences in the glycosylation pattern, to animals. In addition, they are considered GRAS ("Generally Recognized

Abbreviations: FW, Fresh Weight; DW, Dry Weight; GlyBet, Glycine betaine; NEP, Nuclear Encoded Polymerase; PEP, Plastid Encoded Polymerase; TCP, Total Cellular Protein; TLP, Total Leaf Protein; TSP, Total Soluble Proteins; UTR, Untraslated Region; VLP, Virus-Like Particle.

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As Safe") organisms and can be exploited for oral and mucosal delivery of vaccines and other biopharmaceuticals (Demain and Vaishnav, 2009; Karg and Kallio, 2009; Mett et al., 2008; Rybicki, 2009; Sharma and Sharma, 2009; Tiwari et al., 2009; Yusibov and Rabindran, 2008). Using a range of biological and physical methods, plants can be transformed stably, inserting transgenes either in the nuclear or the plastid genome, or transiently. While in case of plastid transformation proteins are synthesized and accumulated in the same organelles, those derived from nuclear genes are synthesized in the cytoplasm, but they can be also secreted or directed to various sub-cellular compartments using appropriate signal peptides. On the other hand, using appropriate promoter sequences, it is possible to express the transgene in different plant organs, usually leaves or seeds. The choice of the plant organ and sub-cellular compartment, as well as the host plant species, depends on the recombinant protein to be expressed, but it can be of paramount importance for the accumulation level eventually obtained (Daniell et al., 2009b; Karg and Kallio, 2009; Lau and Sun, 2009; Rao et al., 2009; Sharma and Sharma, 2009; Tiwari et al., 2009). Besides whole plants grown in open field or greenhouses, cell suspensions, hairy root cultures and aquatic plants grown in bioreactors have been developed for better containment of the recombinant product (Franconi et al., 2010; Huang and McDonald, 2009; Ono and Tian, 2011; Shih and Doran, 2009; Xu et al., 2011). The biocontainment of transgenes and the level and stability of protein yield are the main concerns related to the use of plants as biofactories. Hence, both aspects have been deeply investigated and several strategies to improve them have been developed (Desai et al., 2010; Jamal et al., 2009; Murphy, 2007; Streatfield, 2007).

In this review, we focus on plastid transformation in higher plants as a recombinant protein production platform for different purposes. Compared to nuclear transformation, the main advantages of this technology are the high and stable production level of proteins as well as the natural containment of transgenes (Meyers et al., 2010). Since the pioneering work of Svab and colleagues in tobacco (Svab et al., 1990; Svab and Maliga, 1993), the technology has been extended to several other species and used for a wide range of objectives (Maliga and Bock, 2011). Herein, we give some background information on the technology and we discuss its potential and limitations. However, due to space constraints and to the availability of recent reviews (*e.g.* Bock, 2007a; Bock and Warzecha, 2010; Cardi et al., 2010; Daniell et al., 2009b; Hasunuma et al., 2009; Maliga and Bock, 2011), we limited our search of recombinant proteins produced in transgenic plastids to the last three years.

2. Plastids and plastid genomes

Morphology and function of higher plant plastids vary in different plant tissues, *e.g.* green chloroplasts are present in photosynthetically active cells, red/orange/yellow chromoplasts in fruits and flowers, colorless plastids in storage organs (Pyke, 2007). All plastid types derive from a small and unpigmented organelle named 'proplastid' and present in meristematic cells.

The plastid genome (ptDNA or plastome) is usually represented as a circular molecule of double-stranded DNA, with a size range of 120–160 kb in higher plants. Most plastomes show a tetrapartite genome organization with a large single copy region (LSC) and a small single copy region (SSC) separated by two inverted repeat regions (IRs) that differ only in their relative orientation (Fig. 1).

Plastids have an endosymbiotic origin. Compared to their cyanobacterial ancestors, they retain only a small portion of genes (110–120) which can be classified in three major groups: protein synthesis genes (encoding for rRNAs, RNA polymerase subunits, tRNAs, ribosomal proteins), photosynthesis-related genes (encoding for subunits of enzymatic protein complexes and other proteins involved in photosynthetic reactions), and other genes (genes with other functions, conserved and other open reading frames). Despite its small size, ptDNA represents a significant fraction of total cellular DNA (about 10–20%) due to its presence in high copy numbers; it has been estimated that, depending on species, tissue, developmental stage and environmental conditions, a plant cell can carry up to 10,000 identical copies of ptDNA (Bock, 2007b).

Besides several prokaryotic features, plastids acquired novel characters that make the regulation of gene expression rather complex. In fact, plastid gene expression is controlled at several transcriptional and post-transcriptional steps (Eberhard et al., 2002; Kahlau and Bock, 2008; Schmitz-Linneweber and Small, 2008; Valkov et al., 2009).

Transcription of plastid genes is carried out by three different RNA polymerases: RPOTp and RPOTmp have a sub-cellular localization only in plastids (p) or in plastids and mitochondria (mp), are monomeric, nuclear-encoded (NEP) and are homologous to the phage-type RNA polymerase. On the other hand, the plastid-encoded polymerase (PEP) is a multi-subunit enzyme, highly homologous to the eubacterial RNA polymerase (Lerbs-Mache, 2011). Although several studies demonstrated that NEP and PEP enzymes preferentially transcribe plastid genes encoding proteins involved in gene expression or photosynthesis-related genes, respectively, it has been shown that some genes contain promoters for both polymerases (for reviews see Lerbs-Mache, 2011; Liere and Börner, 2007). Post-transcriptional mechanisms, such as RNA processing and editing, are also important steps to regulate plastid gene expression (Bollenbach et al., 2007; Schmitz-Linneweber and Barkan, 2007).

Plastid translation shares various features with that of eubacteria, like initiation factors, rRNAs, tRNAs and 70S-type ribosomes, and its regulation is mainly controlled by the initiation phase through an interaction of sequence elements in the 5′-untranslated region (5′-UTR) of the mRNA and nuclear encoded translation factors (Marín-Navarro et al., 2007; Peled-Zehavi and Danon, 2007). Further, the ribosome recruitment to the mRNA is often mediated by ribosome-binding sites (also known as Shine-Dalgarno sequences), which display sequence complementarity to the 3′-end of the 16S ribosomal RNA (Kozak, 2005). Nevertheless, in tobacco chloroplasts, 30 of the 79 protein-coding genes do not contain a SD-like sequence, suggesting an alternative mechanism involving other *cis*-elements and *trans*-acting factors for correct translation initiation (Sugiura et al., 1998).

Major levels of post-translational regulation in plastid gene expression are protein degradation and stability. Biochemical and genetic studies identified several proteases that have two types of function: an endopeptidic activity, essential for maturation of most plastidial proteins post-translationally targeted to the organelle; and a gradual degradation of unnecessary and likely non-functional proteins (for review see Sakamoto, 2006). A very recent study identified protein stability determinants *in vivo* by producing transplastomic plants expressing the GFP protein with N- and C-termini modified. The authors proposed that the stability of plastid proteins is influenced by three factors: the action of methionine aminopeptidase involved in the removal of the initiator methionine, whose efficiency varies with sequence context; N-end-rule-like protein degradation pathway in which protein stability is dependent on the identity of the penultimate amino acid; and additional sequences in the N-terminal region (Apel et al., 2010). Understanding protein stability determinants in chloroplasts is important to increment the accumulation levels of recombinant proteins obtainable in transgenic plastids and is therefore pivotal for plastid transformation technology.

3. Plastid transformation

In the last few years, plastid transformation has opened new perspectives to plant biotechnologists for using plants as a protein production platform, due to several advantages offered by transplastomic plants compared to conventional transgenic plants (Bock and Warzecha, 2010; Cardi et al., 2010; Daniell et al., 2005; Maliga, 2002).

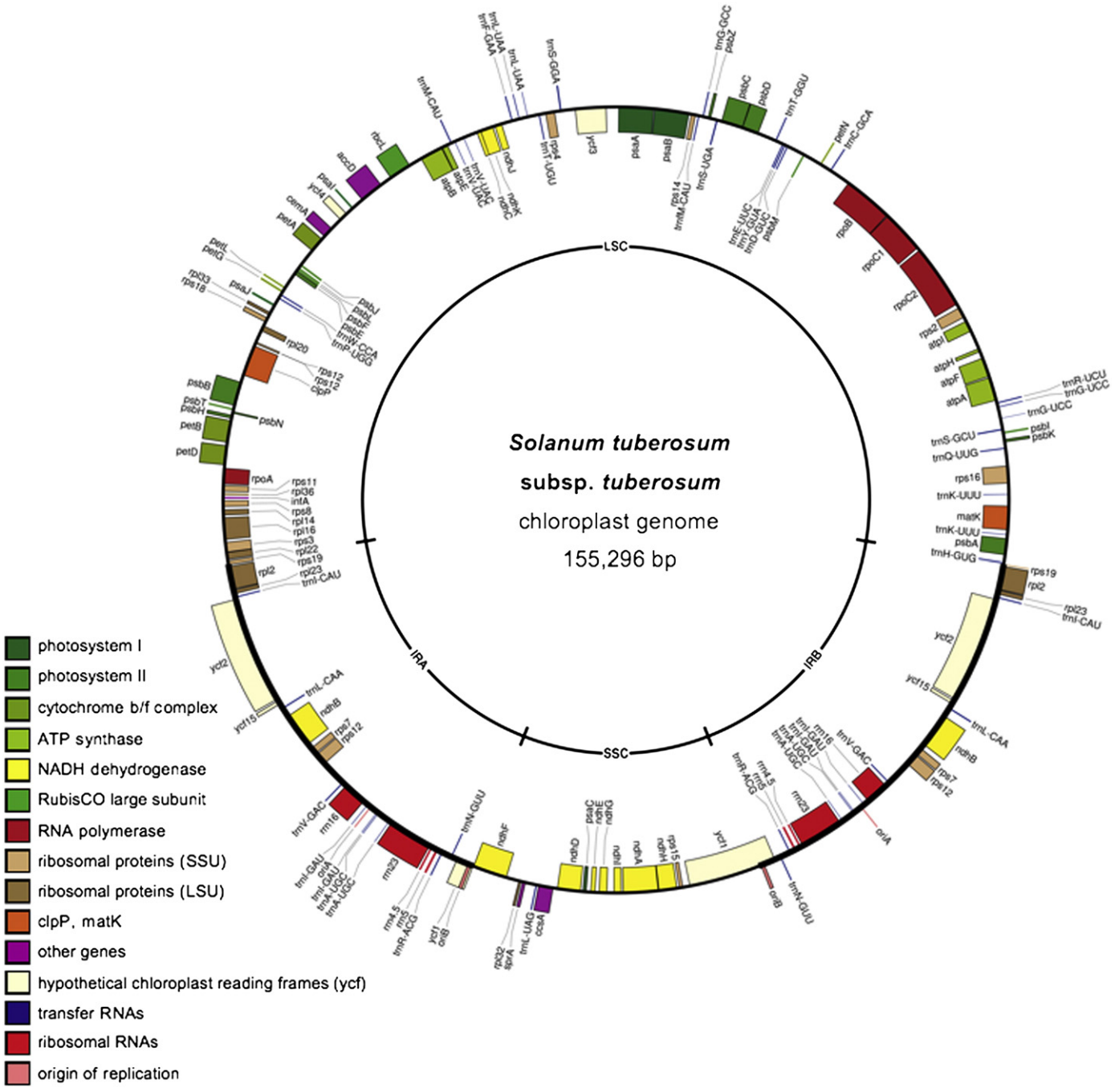

Fig. 1. Physical map, produced by OrganellarGenomeDRAW web-tool (Lohse et al., 2007), of the *Solanum tuberosum* (potato) plastid genome (Gargano et al., 2005) as an example of higher plant plastomes. Genes inside the circle are transcribed clockwise, whereas those outside the circle are transcribed counterclockwise. LSC = Large Single Copy region; SSC = Small Single Copy region; IRA and IRB = Inverted Repeat regions A and B, respectively.

Unlike nuclear transformation, transgene integration into the plastome is based on two homologous recombination events between the targeting regions of the transformation vector and the wild-type ptDNA (Fig. 2). Recently, it has been demonstrated that the extent of similarity between the plastidial sequences involved in homologous recombination is important to ensure high transformation efficiency (Ruhlman et al., 2010; Valkov et al., 2011).

Transformation vectors derive from *E. coli* plasmids and, besides the two plastid sequences involved in recombination (flanking sequences A and B in Fig. 2), include the selectable marker gene (*smg*) and the gene of interest (*goi*) expression cassettes. To date, several plastid sequences have been used as transgene insertion sites (Maliga, 2004), even if three of them are the most employed in plastid transformation vectors: the *trnV-3′/rps12* and *trnI/trnA* regions located in the IRs (Daniell et al., 1998; Zoubenko et al., 1994), and the *trnfM/trnG* region in the LSC (Ruf et al., 2001).

The chimeric *aadA* gene, that confers resistance to spectinomycin and streptomycin, is the most common selectable marker included in transformation vectors. Kanamycin-resistance genes (*neo* and *aphA-6*, Carrer et al., 1993; Huang et al., 2002) and, more recently, genes conferring resistance to 4-methylindole and 7-methyl-DL-tryptophan (*asa2*, Barone et al., 2009) and chloramphenicol (*cat*, Li et al., 2011) have been also successfully employed as selective agents.

The expression cassettes usually consist of a 5′-regulatory region, which includes a strong promoter (P) and a translational control region (L, leader), and a 3′-regulatory region, composed of a terminator

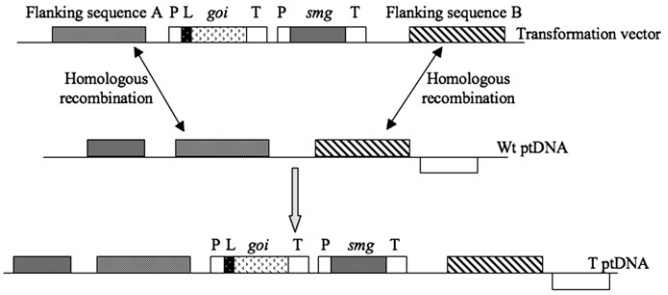

Fig. 2. Schematic representation of transgene sequence integration in a plastid genome through two homologous recombination events between the plastid flanking sequences in the vector (Flanking sequences A and B) and the corresponding sequences in the wild-type ptDNA (Wt ptDNA). P = promoter; L = leader; T = terminator; *smg* = selectable marker gene; *goi* = gene of interest; and T ptDNA = transformed ptDNA. Unmarked boxes in the Wt and T ptDNA represent additional genes not involved in transformation.

sequence (T) which corresponds to the mRNA 3′-untranslated region (3′-UTR) (Fig. 2). The leader sequences may contain either a 5′-UTR or a 5′-UTR and the N-terminus of the coding region (5′-TCR) (Maliga, 2002). In some cases, the use of a 5′-TCR was found to be essential to ensure protein production (Lenzi et al., 2008) or to obtain a notable increase in protein yield (Maliga, 2002; Scotti et al., 2009).

The transforming DNA can be delivered into the plastome either by the biolistic approach or the polyethylene glycol (PEG) treatment of protoplasts (Cardi et al., 2010). The first method is the system of choice in several laboratories due to its simplicity and the lack of an efficient shoot regeneration protocol from protoplasts in many crop species. Due to the polyploid nature of plastids, the primary regenerants are usually in a heteroplasmic state containing a mixed population of wild-type and transformed plastomes and plastids (Fig. 3A). Indeed, the homoplasmic state, with a homogeneous population of transformed plastids, is achieved after a sufficient number of cell divisions under high selective pressure (Fig. 3B). The identification of homoplasmic plants is usually carried out by PCR or Southern analyses (Fig. 3C); subsequently, molecular analyses are accomplished on these plants to evaluate gene expression (Fig. 3D). More recently, a visual marker gene (*aadA*^{au}) that allows both selection and detection of transplastomic sectors by leaf color has been developed in tobacco (Tungsuchat-Huang et al., 2011).

4. Applications of plastid transformation

Due to the high expression levels of recombinant proteins that can be attained, the plastid transformation technology is particularly suitable for the use of plants as biofactories. Hence, many proteins with pharmaceutical interest have been produced in transplastomic plants in the last three years (Tables 1 and 2). Several recombinant enzymes have been also expressed in transgenic plastids (Table 3). Finally, plastid transformation has been used for efficient metabolic engineering of several pathways located in this organelle as well as for the production of antimetabolites conferring insect resistance to plants (Table 3).

4.1. Therapeutic proteins and antibiotics

Recently, Lim et al. (2011) described the production of the human thioredoxin 1 in transplastomic lettuce and characterized the biologically active protein that reduced insulin disulfides. Boyhan and Daniell (2011) have expressed in tobacco and lettuce chloroplasts the cholera toxin B subunit (CTB) fused with the complete human proinsulin (A, B, C

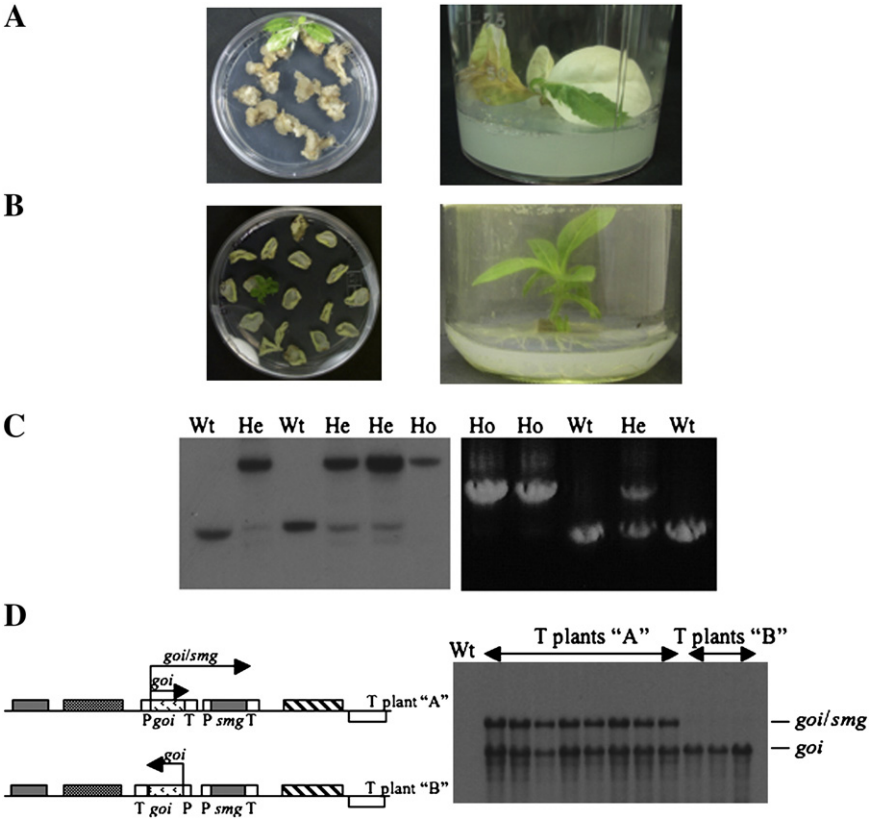

Fig. 3. Examples of regeneration of transplastomic plants and molecular analyses. A) Regeneration of a heteroplasmic shoot on selective medium. B) Regeneration of a homoplasmic shoot on selective medium. C) Southern and PCR analyses to identify homoplasmic plants. D) Northern analysis to show the accumulation of mono- or dicistronic transcripts. Wt = wild-type plants; He = heteroplasmic plants; Ho = homoplasmic plants; T plants "A" = transformed plants "A"; T plants "B" = transformed plants "B"; *goi* = monocistronic transcript; and *goi*/*smg* = dicistronic transcript.

Table 1
Overview of the therapeutic proteins and antibiotics recently produced in plant chloroplasts (2008–2011).

Protein	Source of transgene	Plant species	Protein yield	Protein functionality	References
CTB-proinsulin	Human	Tobaacco Lettuce	47% TLP 53% TLP	Verified Verified	Boyhan and Daniell, 2011
Insulin like growth factor-1[a, b]	Human	Tobacco	32.7% TSP[a] 32.4% TSP[b]	Verified	Daniell et al., 2009a
Cardiotrophin-1	Human	Tobacco	4.8% TSP	Verified	Farran et al., 2008
Aprotinin[a]	Bovine	Tobacco	0.5% TSP	Verified	Tissot et al., 2008
Alpha1-antitrypsin[a]	Human	Tobacco	2% TSP	Verified	Nadai et al., 2009
CTB-F.IX (Coagulation factor IX)	Human	Tobacco	3.8% TSP	Verified	Verma et al., 2010b
Thioredoxin 1	Human	Lettuce	1% TSP	Verified	Lim et al., 2011
Retrocyclin-101-GFP	Human	Tobacco	38% TSP	Verified	Lee et al., 2011
Protegrin-1-GFP	Porcine	Tobacco	26% TSP	Verified	Lee et al., 2011
Endolysin Cpl-1[a]	Phage infecting *Streptococcus pneumoniae*	Tobacco	10% TSP	Verified	Oey et al., 2009b
Endolysin Pal[a]	Phage infecting *S. pneumoniae*	Tobacco	30% TSP	Verified	Oey et al., 2009b
Endolysin PlyGBS[a]	Phage infecting *S. agalactiae*	Tobacco	70% TSP	Verified	Oey et al., 2009a

[a] Synthetic genes with plastid-optimized codon usage.
[b] Native insulin like growth factor 1 gene.

peptides). The fusion of proinsulin to CTB should protect the N-terminus of insulin from degradation, provide a means to facilitate purification and facilitate the uptake of orally delivered insulin into the gut-associated lymphoid tissue (GALT). Oral or injectable delivery of the transplastomic materials into mice showed reduction in blood glucose levels similar to commercial insulin (Table 1). In addition, Verma et al. (2010b) expressed in plastids CTB fused with the coagulation factor IX (CTB-F.IX) to aid in treatment of hemophilia B. After oral administration, F.IX was found in different tissues of the GALT, specifically the ileum, Peyer's patches, liver and plasma within 2–5 h.

An alternative strategy was followed for the production in plastids of the therapeutic protein aprotinin, a bovine serine protease inhibitor, that contains an arginine N-terminal residue and whose structure depends on the correct formation and pairing of three disulphide bonds (Tissot et al., 2008). The recombinant protein was targeted to the lumen of thylakoids using signal peptides derived from *Arabidopsis* nuclear genes that encode luminal proteins. Translocation of aprotinin into the thylakoid lumen was attempted using two different secretion pathways: the general secretion (Sec) and the twin-arginine translocation (Tat) pathways. In both cases, the plant-made aprotinin was secreted within the lumen of thylakoids and was produced with its N-terminal arginine residue. The recombinant plastid-made protein had an active conformation able to bind trypsin, suggesting the formation and pairing of the three disulphide bonds (Tissot et al., 2008). More recently, Daniell et al. (2009a) expressed the native and the optimized synthetic IGF-1 (Insulin like Growth Factor-1) genes in *E. coli* and in transplastomic plants. Expression of both genes was obtained only in transgenic tobacco plant lines where the expression of IGF-1 reached up to 32% Total Soluble Protein (TSP) using plastid light regulatory elements and under continuous

Table 2
Overview of the proteins with immunological properties recently produced in plant chloroplasts (2008–2011).

Protein	Source of transgene	Plant species	Protein level	Protein immunogenicity	References
p24-Nef[a]	HIV-1	Tobacco Tomato	40% TSP 2.5% TSP	NR[b] NR	Zhou et al., 2008
$Pr55^{gag}$	HIV-1	Tobacco	7–8% TSP	NR	Scotti et al., 2009
p24[a]	HIV-1	Tobacco (Maryland Mammoth)	4.5% TSP	NR	McCabe et al., 2008
L1_2xCysM (Mutated L1)	HPV-16	Tobacco	1.5% TSP	NR	Waheed et al., 2011
L1[a]	HPV-16	Tobacco	1.5% TSP	NR	Lenzi et al., 2008
L1	HPV-16	Tobacco	24% TSP	Immunogenic in mice	Fernández-San Millán et al., 2008
E7-CP	HPV-16	Tobacco	0.5% TSP	NR	Morgenfeld et al., 2009
A27L	Vaccinia virus	Tobacco	18% TSP	NR	Rigano et al., 2009
F1-V	*Yersinia pestis*	Tobacco	14.8% TSP	Immunogenic in mice	Arlen et al., 2008
VP-βGUS	Footh-and-mouth disease virus	Tobacco	51% TSP	Immunogenic in Mice	Lentz et al., 2010
E2	Classical swine fever virus	Tobacco	1–2% TSP	Immunogenic in mice	Shao et al., 2008
2 L21-TD	Canine parvovirus	Tobacco	6% TSP	Immunogenic in mice	Ortigosa et al., 2010
DPT	*Corynebacterium Diphtheria, Bordetella pertussis, Clostridium tetani*	Tobacco	0.8% TSP	Immunogenic in mice	Soria-Guerra et al., 2009
CTB-AMA1	*Vibrio cholerae-Plasmodium falciparum*	Tobacco Lettuce	13.2% TSP 7.3% TSP	Immunogenic in mice	Davoodi-Semiromi et al., 2010
CTB-MSP1	*V. cholerae-P. falciparum*	Tobacco Lettuce	10.1% TSP 6.1% TSP	Immunogenic in mice	Davoodi-Semiromi et al., 2010
LTB-ST[a]	*Escherichia coli*	Tobacco	2.3% TSP	Immunogenic in mice	Rosales-Mendoza et al., 2009
LTB-HNE[a]	Newcastle disease virus	Tobacco	0.5%TSP	NR	Sim et al., 2009
Adjuvant					
EDA (Extra domain A-fibronectin)	Mouse	Tobacco	2% TCP	Retain proinflammatory properties	Farran et al., 2010

[a] Synthetic genes with plastid-optimized codon usage.
[b] Not reported.

Table 3
Overview of the enzymes recently produced in plant chloroplasts (2008–2011).

Enzyme	Source of transgene	Plant species	Protein yield	Enzyme activity	References
Lycopene β-cyclase	*Erwinia herbicola*	Tomato	NR[a]	Low	Apel and Bock, 2009
Lycopene β-cyclase	*Narcissus pseudonarcissus*	Tomato	NR	1 mg β-carotene/g DW	Apel and Bock, 2009
Endoglucanase	*Clostridium thermocellum*	Tobacco	NR	493 U/mg in crude TSP	Verma et al., 2010a
Exoglucanase	*C. thermocellum*	Tobacco	NR	442 U/mg in crude TSP	Verma et al., 2010a
Lipase	*Mycobacterium tuberculosis*	Tobacco	NR	Detected	Verma et al., 2010a
Pectate lyase A	*Fusarium solani*	Tobacco	NR	2.81 U/mg in crude TSP	Verma et al., 2010a
Pectate lyase B	*F. solani*	Tobacco	NR	2.42 U/mg in crude TSP	Verma et al., 2010a
Pectate lyase D	*F. solani*	Tobacco	NR	2.31 U/mg in crude TSP	Verma et al., 2010a
Cutinase	*F. solani*	Tobacco	NR	15 U/mg in crude TSP	Verma et al., 2010a
Endoglucanase	*Trichoderma reesei*	Tobacco	NR	339 U/mg in crude TSP	Verma et al., 2010a
Swollenin	*T. reesei*	Tobacco	NR	Detected	Verma et al., 2010a
Xylanase	*T. reesei*	Tobacco	NR	421 U/mg in crude TSP	Verma et al., 2010a
Acetyl xylan esterase	*T. reesei*	Tobacco	NR	Detected	Verma et al., 2010a
Beta glucosidase	*T. reesei*	Tobacco	NR	14 U/mg in crude TSP	Verma et al., 2010a
GH10 xylanase Xyl10B	*Thermotoga maritima*	Tobacco	13% TSP	22.9–61.9 U/g DW	Kim et al., 2011
Endo-β-1,4-glucanase E1 catalytic domain	*Acidothermus celluloyticus*	Tobacco	12% TSP (based on enzymatic activity)		Ziegelhoffer et al., 2009
Endoglucanase Cel6A	*Thermobifida fusca*	Tobacco	10.7% TSP	Detected	Gray et al., 2009
1-Deoxy-D-xylulose reductoisomerase	*Synechosystis* sp. Strain PCC6803	Tobacco	7.4% total stroma protein	3.5 U/mg protein	Hasunuma et al., 2008b
Δ^9 desaturase	*Solanum commersonii* and *Anacystis nidulans*	Tobacco Tobacco	NR	Detected	Craig et al., 2008
Choline monooxygenase	*Beta vulgaris*	Tobacco	NR	250 nmol GlyBet/g FW	Zhang et al., 2008
L-aspartate-α-decarboxylase	*Escherichia coli*	Tobacco	NR	2.3 nmol/mg protein/h (before stress)	Fouad and Altpeter, 2009
β-carotene ketolase and β-carotene hydroxylase[b]	*Brevundimonas* sp. SD212	Tobacco	NR	5.44 mg/g DW astaxanthin	Hasunuma et al., 2008a
β-glucosidase	*T. reesei*	Tobacco	NR	44.4 U/g fresh leaves	Jin et al., 2011
Acetolactate synthase (mutated)	*Arabidopsis thaliana*	Tobacco	NR	Detected	Shimizu et al., 2008
Chitinase BjCHl1	*Brassica juncea*	Tobacco	NR	0028 U/μg/min	Guan et al., 2008
Lux operon	*Photobacterium leiognathi*	Tobacco	NR	82×10^6 photons/minute	Krichevsky et al., 2010
Fructose-1,6/sedoheptulose-1, 7-bisphosphatase	*Synechococcus PCC7942*	Lettuce	3.5% TSP	<7 μmol/min/mg chl.	Ichikawa et al., 2010
Endoglucanase Cel9A[c]	*T. fusca*	Tobacco	40% TSP	<30 nmol glucose equivalents	Petersen and Bock, 2011
Exoglucanase Cel6B[c]	*T. fusca*	Tobacco	5% TSP	<30 nmol glucose equivalents	Petersen and Bock, 2011
β-glucosidase Bgl1C	*T. fusca*	Tobacco	Between 5 and 40% TSP[c]	<200 mU/ml protein sample	Petersen and Bock, 2011
Xyloglucanase Xeg74	*T. fusca*	Tobacco	Between 5 and 40% TSP[c]	<60 nmol glucose equivalents	Petersen and Bock, 2011
β-glucosidase BglC	*T. fusca*	Tobacco	12% TSP	detected	Gray et al., 2011

[a] Not reported.
[b] Synthetic genes with rape-optimized codon usage.
[c] Synthetic genes with plastid-optimized codon usage.

illumination (Daniell et al., 2009a). In the same year, Nadai et al. (2009) reported the expression of the human alpha1-antitrypsin, a serine-protease inhibitor, in transgenic chloroplasts. The plastid-made therapeutic protein was fully active and was able to bind to porcine pancreatic elastase (Nadai et al., 2009).

Plastid transformation has been recently used also as a novel strategy for large-scale, cost-effective production of next-generation antibiotics for topical and systemic treatments (Lee et al., 2011; Oey et al., 2009a, b). Two recent studies from the same laboratory group explored the feasibility of producing endolysin-type protein antibiotics in transgenic tobacco chloroplasts (Oey et al., 2009a,b). Expression of two genes encoding phage endolysin proteins was tested in tobacco plastids using a new strategy (called toxin shuttle) that allows the production in transgenic chloroplasts of proteins that are toxic to *E. coli* (Oey et al., 2009b). Expression cassettes that confer high-level protein expression in plastids usually drive high-level protein expression also in bacterial cloning hosts; therefore, cloning of genes encoding for proteins with antibacterial activities can be problematic. The toxin shuttle strategy developed in the latter study was based on preventing lethal transgene transcription in *E. coli* by inducing premature transcription termination upstream of the transgene coding region using bacterial transcription terminators. The bacterial terminators were flanked by *loxP* sites and can therefore be excised *in planta* by site-specific recombination after chloroplast transformation. Using the toxin shuttle strategy, Oey et al. (2009b) produced two lysine-type protein antibiotics (Cpl-1 and Pal) against *Streptococcus pneumoniae*. The 2 phage-derived protein antibiotics, that proved to be unclonable into standard plastid expression cassettes, accumulated at high-level (up to 30% TSP) in transgenic plastids and efficiently killed pathogenic strains of *S. pneumoniae*. In the same year, the same research group described the production of the lysine PlyGBS in tobacco chloroplasts. Expression levels of the synthetic *plyGBS* gene in chloroplasts were so high (more than 70% TSP, the highest foreign protein accumulation level ever obtained in a transgenic plant) that the production of endogenous chloroplast proteins was severely compromised. The authors speculated that this was due to the fact that massive production of the recombinant protein exhausted the plastid gene expression capacity. The plastid-made PlyGBS protein antibiotic was highly active and efficiently killed a pathogenic strain of *S. pyogenes* (Oey et al., 2009a).

Lee et al. (2011) investigated the expression of two potent disulphide-bonded antimicrobial peptides in transgenic tobacco plastids: Retrocyclin-101 (RC101) and Protegrin-1 (PG1). To facilitate expression, detection and quantification of the peptides RC101 and PG1, each peptide was expressed in transgenic plastids fused with GFP. The antibacterial

activity of RC101 and PG1 expressed in transplastomic plants was confirmed by *in planta* bioassay with *Erwinia carotovora*. In addition, RC101 expressed in transgenic plastids conferred resistance to tobacco mosaic virus infection, confirming RC101 antiviral activity (Lee et al., 2011).

4.2. Proteins with immunological properties

Plastid-made vaccines proved to be fully functional and able to elicit the appropriate immune responses in experimental animals and to protect against toxin or pathogen challenges. In addition, transplastomic plants were recently used for the production of adjuvants such as the vaccine adjuvant extra domain A from fibronectin (Farran et al., 2010). Two recent reviews (Bock and Warzecha, 2010; Cardi et al., 2010) describe the production of vaccine antigens in transplastomic plants. Herein, we will concentrate on the recombinant antigens produced most recently (Table 2).

Zhou et al. (2008) reported the expression of HIV-1 antigens p24 and Nef in transplastomic tobacco and tomato plants. High antigen accumulation (up to 40% TSP in tobacco and tomato leaves and 2.5% TSP in green tomato fruits) was achieved when the p24-nef fusion gene was inserted in the plastome using an improved plastid transformation vector designed to prevent rearrangements of inserted sequences and facilitate selectable marker gene removal. On the other hand, the fusion protein p24-nef was not detectable in red ripe fruits, possibly because ripe fruits contain chromoplasts that are less active in plastid gene expression than chloroplasts (Zhou et al., 2008). In the same year, McCabe et al. (2008) expressed the protein p24 in transgenic plastids of Maryland Mammoth, a large tobacco variety that can produce 1–2 kg of leaf biomass per plant prior to flowering. Scotti et al. (2009) investigated the possibility of expressing the HIV structural polyprotein precursor protein $Pr55^{gag}$ using chloroplast and nuclear transformation. In transplastomic tobacco plants, the level of expression of $Pr55^{gag}$ was increased up to 7–8% TSP when the transgene was expressed using a vector with *rbcl* 5′-UTR followed by the first 42 nucleotides of the plastid photosynthetic RbcL protein. By comparison, when the protein was translated in the cytoplasm and imported into the chloroplasts, recombinant protein levels were between 0.01 and 0.1% TSP, suggesting that high accumulation levels achieved by plastid transformation were due to higher synthesis with the latter system rather than to higher protein stability. The $Pr55^{gag}$ protein produced in the plastids assembled into spherical particles resembling VLPs (Virus-Like Particles) produced from baculovirus and *E. coli* expression systems.

In the past few years, several studies investigated the feasibility of producing plant-made vaccines against HPV (human papillomavirus) based on the major capsid L1 protein that self assembles into immunogenic capsomeres and VLPs (Table 2). Waheed et al. (2011) recently described the production of capsomers in transplastomic plants, since these are considered a cost-saving alternative to VLP-based HPV vaccines. The authors expressed in tobacco plants a mutated L1 (L1_2xCysM) gene, which encoded an L1 protein with two cysteines replaced by serines, therefore retaining the ability to assemble into capsomers. Previously, Lenzi et al. (2008) produced transplastomic tobacco plants after transformation with several constructs carrying either the native viral or a plastid-optimized L1 sequence. The highest protein yield (1.5% TSP) was achieved with vectors that included the 5′-UTR and the first 42 nucleotides of the plastid *rbcL* gene. ELISA assays and electron microscopy analysis indicated that the plant-made L1 assembled in higher order structures. Fernández-San Millán et al. (2008) reported the highest expression level of L1 protein achieved in transplastomic plants (24% TSP). Immunization experiments in Balb/c mice demonstrated that the plastid-derived HPV-16 L1 VLPs were highly immunogenic after intraperitoneal injection, and neutralizing antibodies were detected.

Recently, Rigano et al. (2009) described the production in tobacco of the A27L immunogenic protein of vaccinia virus using stable transformation of the nuclear or plastid genome. In transplastomic plants, the vaccinia virus protein accumulated to about 18% TSP, a level of accumulation 500-fold higher than levels obtained in nuclear transformed plants. The A27L protein was expressed in transplastomic plants in a soluble form and was localized in the stroma. In addition, the plastid-made protein formed oligomers and was recognized by serum from a patient previously infected by a zoonotic orthopoxviruses.

The plastid transformation technology has been used for the production of peptides expressed as fusions with carrier protein in order to increase stability and accumulation levels of the target peptides. For example, Lentz et al. (2010) described *in planta* production of a small immunogenic epitope containing amino acid residues 135–160 of the structural protein VP1 of FMDV (foot and mouth disease virus) fused with the β-glucuronidase reporter gene. A very high expression level (51% TSP) of the FMDV epitope was obtained in transgenic plastids and the fusion peptide was immunogenic in mice. Following the same strategy, Ortigosa et al. (2010) produced transplastomic plants carrying a fusion between the immunogenic peptide 2 L21 derived from the canine parvovirus VP2 protein and the 42 amino acid tetramerisation domain (TD) from the human transcription factor p53.

Co-delivery with targeting proteins such as the heat-labile toxin B subunit from *E. coli* (LTB) and the cholera toxin B subunit from *Vibrio cholerae* (CTB) can also increase the ability of the mucosal immune system to recognize plant-derived, orally delivered antigens. For instance, Rosales-Mendoza et al. (2009) reported the production of a chloroplast-derived vaccine against enterotoxigenic *E. coli* (ETEC) by production of a fusion protein comprising LTB and the heat-stable toxin (ST). The plastid-made LTB-ST was able to bind to the GM1 gangliosides and elicited protective immune responses against the cholera toxin in orally immunized mice. In another study, Davoodi-Semiromi et al. (2010) expressed CTB fused to malarial vaccine antigens apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1) in tobacco and lettuce plastids, in order to produce a low-cost multivalent vaccine. Balb/C mice were immunized both orally with transplastomic leaves and subcutaneously with plastid-derived purified antigen. Sera of immunized mice blocked proliferation of the malarial parasite in red blood cells. In addition, CTB-IgG1 and CTB-IgA in orally immunized mice and only CTB-IgG1 in subcutaneous immunized mice conferred immunity against CT challenge. Another multicomponent vaccine was produced by Soria-Guerra et al. (2009) that expressed a multi-epitope diphtheria, pertussis and tetanus (DPT) fusion protein in transplastomic tobacco plants. The sequences of six immunoprotective exotoxin epitopes of *Corynebacterium diphtheriae, Bordetella pertussis* and *Clostridium tetani* were fused together to form a multiepitope protein and the DPT gene was inserted into a plastid expression cassette. The plastid-made DPT protein induced specific systemic and mucosal antibody responses in mice orally immunized with freeze-dried transplastomic leaves.

4.3. Enzymes and other proteins

Hasunuma et al. (2008a) used plastid transformation to alter carotenoid compositions and contents in plants (Table 3). The authors expressed in tobacco plastids two genes encoding for the enzymes β-carotene ketolase and β-carotene hydroxylase from the marine bacterium *Brevundimonas* sp. SD212. In the tobacco transformants, there was an accumulation in leaves of large quantities of astaxanthin (up to 5.44 mg g^{-1} DW, corresponding to more than 70% of the total carotenoids), while total carotenoid level was 2.1-fold higher than in wild-type plants. Transplastomic plants synthesized also the novel carotenoids 4-ketoantheraxanthin and a rare carotenoid fritschiellaxanthin. In the same year, the same research group used plastid metabolic engineering to manipulate the yield of isoprenoids in plants

by over-expression of the enzyme 1-Deoxy-D-xylulose reductoisomerase (DXR) from the cyanobacterium *Synechosystis* sp. strain PCC6803 (Hasunuma et al., 2008b). More recently, Apel and Bock (2009) introduced the lycopene β-cyclase genes from the eubacterium *Erwinia herbicola* and the plant daffodil (*Narcissus pseudonarcissus*) into the tomato plastid genome under the control of the promoter *rrn* and the T7g10 5′-UTR in order to increase the contents of the antioxidant provitamin A. While the expression of the bacterial enzyme did not strongly alter carotenoid composition, the expression of the plant enzyme in fruits of transplastomic tomato plants triggered efficient conversion of lycopene to provitamin A (β-carotene) and led to a >50% increase in total fruit carotenoid accumulation. In a previous work, Wurbs and colleagues (2007) expressed the lycopene β-cyclase genes from *E. herbicola* and from the zyomycete fungus *Phycomyces blakesleeanus* under the control of the promoter *atpI* in transplastomic tomatoes. In that case, the fungal gene constructs did not result in measurable changes of carotenoid biosynthesis while expression of the bacterial genes resulted in an increase in β-carotene accumulation, but in a lower total carotenoid content than with the plant gene (Apel and Bock, 2009). Results obtained in the latter studies demonstrate that the successful application of chloroplast engineering depends not only on the gene used for metabolic engineering but also on the origin of the gene and of the expression cassette. A differential effect was also observed when the *dxs* gene (encoding the 1-Deoxy-D-xylulose synthase enzyme) was cloned from different sources and inserted in tobacco chloroplasts. While transplastomic plants with the sequence from *E. coli* showed severe alterations in chloroplast ultrastructure and reduced growth, plants with the same gene from *Synechocystis* spp. did not show any detrimental effect (Scotti et al., unpublished data).

Today, biofuel production from lignocellulosic materials is limited by the lack of technology, infrastructures and high cost of enzymes. One interesting option for low-cost production of cellulolytic enzymes is the use of transplastomic plants. Examples are the expression of an endoglucanase and a β-glucosidase from *Thermobifida fusca* and of the endo-β-1,4-glucanase E1 catalytic domain of *Acidothermus celluloyticus* in transplastomic tobacco plants (Gray et al., 2009; Gray et al., 2011; Ziegelhoffer et al., 2009). Recently, Petersen and Bock (2011) produced in transplastomic plants a set of four thermostable cell wall-degrading enzymes from the bacterium *Thermobifida fusca*. Verma et al. (2010a) used plastid transformation for the expression of the enzymes endoglucanases, exoglucanase, pectate lysases, cutinase, swollenin, xylanase, acetyl xylan esterase, beta glusosidase and lipase from bacteria and fungi and tested the plant-derived enzyme cocktails for the production of fermentable sugars from lignocellulosic biomass. Alternatively, Kim and colleagues (2011) expressed the GH10 xylanase Xyl10B from *Thermotoga maritima* in tobacco chloroplasts in order to maximize conversion of methylglucuronoxylan, the major components of hemicellulose in woody biofuel crops, to fermentable sugars.

Chloroplast transformation has been used also for the improvement of pest resistance in crop plants (Table 3). For example, the *Bacillus thuringiensis* toxin (*cry1AB*) was expressed in the chloroplasts of transformed cabbage and conferred high insecticidal efficacy against *Plutella xylostella* (Liu et al., 2008). In addition, several studies investigated new strategies to improve crop yield potential and the efficiency of photosynthesis using plastome transformation for genetic engineering of the Rubisco, the CO_2-fixing enzyme whose large catalytic subunit is encoded in the plastome (Whitney et al., 2011). A recent paper described the production of transplastomic lettuce with enhanced growth and high yield of the chloroplast expressing the cyanobacterial fructose-1,6/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) in the chloroplast (Ichikawa et al., 2010). Plastid engineering has been also conducted to enhance crop tolerance to different abiotic stresses. For example, the expression of the enzyme choline monooxygenase from *Beta vulgaris* in transplastomic tobacco plants improved salt and drought tolerance through accumulation of glycine betaine, a compound that functions as osmoprotectant in plants (Zhang et al., 2008). In the same year, Craig et al. (2008) tested the feasibility of using plastid transformation to engineer lipid metabolic pathways and improve cold tolerance by production of a Δ^9 desaturase gene from either *Solanum commersonii* (a wild potato species) or the cyanobacterium *Anacystis nidulans* in tobacco plastids. Transplastomic plants showed altered fatty acid profiles and an increase in their unsaturation level both in vegetative and reproductive tissues. The work from Craig and colleagues (2008) represents the first example of transplastomic plants expressing agronomically relevant genes generated using "binding-type" vectors based on antibiotic-insensitive point mutations in chloroplast genes. Transplastomic plants were obtained by PEG-mediated transformation of leaf protoplasts.

Novel biotechnology applications using plastid transformation have been recently described by Jin et al. (2011). The authors expressed a fungal β-glucosidase (Bgl-1) in transplastomic tobacco plants. Plastid-transformed plants flowered one month earlier and showed an increase in biomass, height, internode length and leaf area compared to untransformed plants. In addition, the transplastomic lines showed an increase in density of leaf globular trichomes that contain more sugar esters that confer protection from whitefly and aphid attack. In BGL-1 lines there was an increase in the level of IAA and trans-Zeatin, and protoplasts from those lines were able to divide and form calli without exogenous hormones.

Krichevsky and colleagues (2010) demonstrated that even a complete functional bacterial luciferase pathway of prokaryotes could be reconstituted in plant plastids. In this study the expression of the six genes of the *lux* operon (*luxCDABEG*) was reported in transgenic tobacco chloroplasts producing the first autoluminescent plant able to emit light visible by naked eye.

5. Potential and limitations

Since chloroplasts represent a highly polyploidy genetic system, plastid transformation generally ensures a higher transgene expression level compared to nuclear transformation. However, the examples described in the previous sections and in Tables 1–3 clearly demonstrate that the expression level can be affected by several factors: protein type, regulatory sequences included in the expression cassettes, plant tissue, development stage of the plant and protein stability. The levels of protein accumulation obtainable in transgenic plastids usually cannot be predicted, even in *E. coli* cells, which share similar expression mechanisms with plastids (Magee et al., 2004), and some foreign proteins proved to be difficult to express in transplastomic plants (Tables 1–3). For instance, the short peptide 2L21 from the canine parvovirus VP2 protein did not accumulate in transgenic plastids and was expressed in transplastomic plants only by fusion with the p53 tetramerisation domain (Ortigosa et al., 2010). However, recent studies suggest that some empiric rules can be followed in order to develop optimized vectors for plastid transformation, such as the choice of species-specific plastid sequences for transgene expression (Ruhlman et al., 2010), or of sequence determinants in the N-terminal region for protein stability (Apel et al., 2010). Gene expression in non-green plastids is generally low. Nevertheless, regulatory sequences potentially useful to increase transgene expression in tomato chromoplasts and potato amyloplasts have been identified (Kahlau and Bock, 2008; Valkov et al., 2009; Valkov et al., 2011).

Post-translational modifications, such as disulphide bond formations, can also influence the stability of the plastid-made recombinant proteins and hence influence protein accumulation (De Marchis et al., 2011; Tissot et al., 2008). In this regard, it is noteworthy the work of De Marchis and colleagues (2011). The authors expressed in transplastomic tobacco plants the fusion protein zeolin (the bean protein phaseolin fused to the first half of maize γ-zein) with its native signal peptide (SP)

and two mutated forms, one without SP and one without Cys residues (unable to form disulphide bonds). The signal peptide of phaseolin targeted zeolin to the thylakoid membranes, where it accumulated as trimers able to form disulphide bonds, a modification that contributed to protein accumulation.

Several post-translational modifications can occur in transgenic plastids, including protein lipidation, multimerization, N-terminal methionine excision and the already mentioned disulphide bond formation (De Marchis et al., 2011; Hennig et al., 2007; Rigano et al., 2009; Tissot et al., 2008). On the other hand, glycosylation is absent in plastids, preventing the production of glycosylated antigens. The absence of protein glycosylation could represent a possible limitation of the plastid transformation technology; however in some cases, for instance for the production of the therapeutic protein human alpha1-anti-trypsin (A1AT), the absence of glycosylation could be considered an advantage (Nadai et al., 2009).

Sometimes, the constitutive high expression level of transgenes can interfere with plant development, inducing phenotypic alterations and reduced growth (Rigano et al., 2009; Scotti et al., 2009; Zhou et al., 2008). For this reason, several approaches to induce transgene expression in transplastomic plants have been developed (Buhot et al., 2006; Lössl et al., 2005; Muhlbauer and Koop, 2005; Tungsuchat et al., 2006; Verhounig et al., 2010). Early systems have been recently reviewed (Cardi et al., 2010; Khan, 2006; Koop et al., 2007; Wang et al., 2009). The approach recently proposed by Verhounig and colleagues (2010) is based on an engineered riboswitch that functions as translational regulator of transgene expression in transformed plastids in response to the application of its ligand theophilline. Currently, all inducible systems described are proof-of-concept experiments developed in tobacco, the model plant for plastid transformation. Some optimizations are still needed before the application of these systems to molecular farming, especially considering the low protein yield induced.

An important issue regarding the use of plants as biofactories is transgene containment, which is one of the main advantages offered by plastid transformation. In fact, in most crops, plastids are maternally transmitted to the sexual progeny, and thus pollen does not contain ptDNA (Cardi et al., 2010). Although this feature ensures an efficient biocontainment that prevents undesired transgene transmission to other species, the growth of transplastomic plants outdoors is still a contentious issue. To address this concern, Michoux et al. (2011) evaluated the potential of tobacco transplastomic cell suspensions as a fully contained production system. The authors produced transplastomic cell suspensions from homoplasmic tobacco leaves expressing a modified GFP and the fragment C of tetanus toxin (TetC), loaded them into a 2-L bioreactor temporally submerged in MS media supplemented with Thidiazuron to induce shoot formation. This fully contained biomass production system yielded 660 mg/L (33% TSP) and 95 mg/L (8% TSP) for GFP and TetC, respectively, values slightly lower than those obtained with CHO cells.

Another potential advantage of plastid transformation is the possibility to achieve multigene engineering through expression of multiple transgenes in prokaryotic-like operons. This feature of chloroplast transformation allows co-expression of multiple antigens in plastids for the production of multivalent vaccines against multiple infectious diseases (Davoodi-Semiromi et al., 2010). In addition, the possibility of transgene stacking in operons makes chloroplast transformation an ideal tool for metabolic engineering (Hasunuma et al., 2009), as demonstrated by the expression in plastids of the complete bacterial luciferase pathway (*luxCDABEG*) leading to the production of a truly autonomously luminescent plant (Krichevsky et al., 2010).

Presently, besides tobacco, only two plant species have been used to successfully produce nutraceutical and biopharmaceutical proteins by plastid transformation: tomato (Apel and Bock, 2009; Wurbs et al., 2007; Zhou et al., 2008) and lettuce (Davoodi-Semiromi et al., 2010; Ichikawa et al., 2010; Lim et al., 2011; Ruhlman et al., 2007; Ruhlman et al., 2010). The development of improved selection/regeneration protocols and/or transformation vectors containing homologous flanking sequences increased the transformation efficiency in various plant species other than tobacco (Dufourmantel et al., 2004; Liu et al., 2007; Ruf et al., 2001; Ruhlman et al., 2010; Valkov et al., 2011; Zubko et al., 2004). Despite these recent advances, however, the extension of plastid transformation technology to other crops, especially those belonging to monocots, is still limited.

6. Conclusions and perspectives

More than 50 different recombinant proteins have been produced in transgenic plastids in the last three years (Tables 1–3). Although almost all reports in the literature used tobacco, in the same period there was a significant improvement of the technology in other crop species, now including important ones such as tomato, petunia, potato, soybean, lettuce and cabbage (Maliga and Bock, 2011). Considering the recent scientific and technological developments in plastid transformation technology, such as the use of additional marker genes and/or their removal after selection, the possibility to induce gene expression, the selection of novel regulatory sequences for expression in chloroplasts and in non-green plastids (*e.g.* chromoplasts and amyloplasts) (Maliga and Bock, 2011), it can be predicted that in the next future the plastid transformation approach will be applied to a larger set of species and for a wider range of purposes. This will allow exploiting the potential advantages offered by this technology in comparison to nuclear transformation. The chance of success will depend on the target protein and thus it will be wise to test several expression cassettes and transformation vectors in preliminary experiments. Some proteins, however, will remain not-expressible in transgenic plastids, *e.g.* those requiring glycosylation for their functionality.

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