The chloroplast transformation toolbox: selectable markers and marker removal

Day, Anil; Goldschmidt-Clermont, Michel P.

How to cite

DAY, Anil, GOLDSCHMIDT-CLERMONT, Michel P. The chloroplast transformation toolbox: selectable markers and marker removal. In: Plant biotechnology journal, 2011, vol. 9, n° 5, p. 540–553. doi: 10.1111/j.1467-7652.2011.00604.x

This publication URL: https://archive-ouverte.unige.ch/unige:33527
Publication DOI: 10.1111/j.1467-7652.2011.00604.x

Plant Biotechnology Journal

Plant Biotechnology Journal (2011) **9**, pp. 540–553 doi: 10.1111/j.1467-7652.2011.00604.x

Review article

The chloroplast transformation toolbox: selectable markers and marker removal

Anil Day[1] and Michel Goldschmidt-Clermont[2,*]

[1]*Faculty of Life Sciences, The University of Manchester, Manchester, UK*
[2]*Departments of Plant Biology and of Molecular Biology, University of Geneva, Genève, Switzerland*

Received 8 November 2010;
revised 29 January 2011;
accepted 31 January 2011.
**Correspondence* (Tel +41 22 379 6188;
fax +41 22 379 6868; email
michel.goldschmidt-clermont@unige.ch)

Keywords: plastid transformation, marker-free, *aadA*, *aphA-6*, *Chlamydomonas reinhardtii*, *Nicotiana tabacum*.

Summary

Plastid transformation is widely used in basic research and for biotechnological applications. Initially developed in Chlamydomonas and tobacco, it is now feasible in a broad range of species. Selection of transgenic lines where all copies of the polyploid plastid genome are transformed requires efficient markers. A number of traits have been used for selection such as photoautotrophy, resistance to antibiotics and tolerance to herbicides or to other metabolic inhibitors. Restoration of photosynthesis is an effective primary selection method in Chlamydomonas but can only serve as a screening tool in flowering plants. The most successful and widely used markers are derived from bacterial genes that inactivate antibiotics, such as *aadA* that confers resistance to spectinomycin and streptomycin. For many applications, the presence of a selectable marker that confers antibiotic resistance is not desirable. Efficient marker removal methods are a major attraction of the plastid engineering tool kit. They exploit the homologous recombination and segregation pathways acting on chloroplast genomes and are based on direct repeats, transient co-integration or co-transformation and segregation of trait and marker genes. Foreign site-specific recombinases and their target sites provide an alternative and effective method for removing marker genes from plastids.

Introduction

Plastid transformation offers an important tool to investigate many aspects of plant physiology and the regulation of gene expression. It has also gained strong interest for applications in biotechnology because of several advantages compared with transformation of the nuclear genome (Meyers *et al.*, 2010). The most prominent is that plastid transgene expression can be remarkably high and the desired recombinant protein may represent up to 70% of leaf protein (Daniell *et al.*, 2009; Oey *et al.*, 2009a; Ruhlman *et al.*, 2010). It is also important that in the majority of flowering plants including major crops, inheritance of the plastid genome is through the maternal parent (Corriveau and Coleman, 1988), and transmission of plastids through pollen is very rare (Ruf *et al.*, 2007; Svab and Maliga, 2007). Thus, plastid transformation provides a strong level of biological containment. Exceptional pollen transmission following transfer of a chloroplast marker to the nucleus is also a very rare event (Huang *et al.*, 2003; Stegemann *et al.*, 2003; Ruf *et al.*, 2007; Bock and Timmis, 2008). Another advantage is that the integration of a transgene in the plastid genome proceeds by homologous recombination and is therefore precise and predictable. Hence, variable position effects on gene expression or the inadvertent inactivation of a host gene by integration of the transgene are avoided. Furthermore, plastid genes are not subject to gene silencing or RNA interference. It is also noteworthy that multiple transgenes organized as a polycistronic unit can be expressed from the plastid genome (Staub and Maliga, 1995; De Cosa *et al.*, 2001; Quesada-Vargas *et al.*, 2005). Recent reviews have focused on the numerous applications of plastid transformation for the production of pharmaceuticals or biofuels, and on the development of transformation protocols in a rapidly increasing number of plant and algal species (Griesbeck *et al.*, 2006; Bock, 2007; Verma and Daniell, 2007; Verma *et al.*, 2008; Daniell *et al.*, 2009; Wang *et al.*, 2009; Cardi *et al.*, 2010; Meyers *et al.*, 2010; Specht *et al.*, 2010). Here, we focus on selectable markers, which are essential tools for chloroplast transformation.

Chloroplast transformation

Chloroplasts are specialized plant organelles best known to host photosynthesis, but that also harbour many other important biosynthetic pathways. During plant development, they arise by differentiation of proplastids, precursors that are found in meristematic tissues and can also develop into many other forms such as the amyloplasts in roots or the chromoplasts in fruits. Plastid transformation can involve delivery of DNA into chloroplasts or non-green plastids. Once stable transformation has been achieved, all plastid types within the plant will contain the same transgenic plastome. Thus, in flowering plants containing a variety of plastid developmental forms, the term plastid transformation is more accurate than chloroplast transformation. During evolution, plastids were most probably derived from an endosymbiotic cyanobacterium (Gould *et al.*, 2008). From this ancestor, plastids have retained a small autonomous genome that contains approximately a hundred genes in vascular plants and Chlamydomonas. From the Gram-negative cyanobacterium, the plastids also inherited the two membranes that constitute the envelope, which in secondary or tertiary endosymbiotes is surrounded by one or two additional membranes derived from the host (Gould *et al.*, 2008). Thus, for chloroplast transformation, DNA has to be delivered through the cell wall and through at least three membranes (the plasma membrane and the envelope). Stable transformation of plastids in flowering plants was preceded by reports of transient expression of genes in isolated chloroplasts (Daniell and McFadden, 1987; Daniell *et al.*, 1990; Ye *et al.*, 1990). Isolated chloroplasts have limited viability, and most subsequent work has focussed on stable plastid transformation *in vivo*, a challenge that can be most efficiently achieved with a gene gun (Boynton *et al.*, 1988), but alternatively by treatment of protoplasts with polyethylene glycol (PEG) (O'Neill *et al.*, 1993; Koop *et al.*, 1996), agitating cell wall-deficient Chlamydomonas with glass beads (Kindle *et al.*, 1991) or by microinjection (Knoblauch *et al.*, 1999). Only the gene gun, PEG and glass-bead methods give rise to stable chloroplast transformants. The gene gun is used to bombard plant cells and organs, or a lawn of algal cells on the surface of an agar plate, with micron-sized metal particles carrying the DNA of interest. Dubbed 'biolistics', this technique allows the delivery of transforming DNA directly within the chloroplast, where it can integrate by homologous recombination in the plastid genome of the host. The whole process is not very efficient, for example in Chlamydomonas, transformed cells appear with a frequency of approximately 10^{-5}. Selection of the cells that are successfully transformed thus requires efficient markers and low rates of false positives.

While the small Chlamydomonas cell only accommodates a single chloroplast, plant leaf cells can have up to a hundred plastids. Each plastid may in turn harbour a hundred copies of the plastid genome, the plastome. This high degree of polyploidy implies that after the initial integration of DNA into one copy, both the transformed plastome within the plastid and the transformed plastid within the cell have to be amplified and segregated to yield homoplasmic cells where all copies of the plastome are transformed (Figure 1). This is typically achieved by several rounds of propagation or subcloning of the transformants under selective pressure. In multicellular plants, there is the further complication that when tissue and organ explants, or callus, microcolonies and suspension culture cells, are used as transformation targets, resistant shoots might be chimeric and contain a residual amount of non-transformed cells. Transformation systems involving somatic embryogenesis compared with organogenesis are particularly challenging (Daniell *et al.*, 2005).

Historical perspective

The green eukaryotic alga *Chlamydomonas reinhardtii* has been a long-standing model for chloroplast genetics and molecular biology, which helped pave the way for the development of stable chloroplast transformation. It is in Chlamydomonas that chloroplast transformation was first achieved (Boynton *et al.*, 1988), that the *aadA* marker was developed (Goldschmidt-Clermont, 1991) and that methods for marker removal were first demonstrated (Fischer *et al.*, 1996). For higher plants, *Nicotiana tabaccum* (tobacco) played a leading role, because of the relative ease of its tissue culture and regeneration, and also because early success relied on previously characterized mutations of the plastid ribosomal RNA genes that confer resistance to certain antibiotics that served as selectable markers (Svab *et al.*, 1990). The breakthrough for chloroplast transformation came with the development by John Sanford and his colleagues of the gene gun, and its successful application to Chlamydomonas. Building on the well-developed chloroplast genetics of Chlamydomonas, the initial strategy made use of a mutant deleted for part of the *atpB* gene. Such mutants have lost photoautotrophy, but can be grown in the dark in the presence of acetate as a source of reduced carbon. The wild-type *atpB* gene was thus used by Boynton *et al.* (1988) to rescue the chloroplast deletion mutant, allowing selection for photosynthetic growth on minimal medium in the light.

To circumvent the requirement for a specific mutant host, a selection scheme based on antibiotic resistance was soon developed in Chlamydomonas in the laboratory of J. Boynton and N. Gillham, and in tobacco by the laboratory of P. Maliga (Newman *et al.*, 1990; Svab *et al.*, 1990). For Chlamydomonas, this scheme uses mutations in the gene for the 16S RNA (*rrnS*) that confer resistance to spectinomycin or streptomycin, or in the gene for 23S RNA (*rrnL*) that bestow resistance to erythromycin (Newman *et al.*, 1990). Spontaneous mutations to antibiotic resistance appear with a frequency similar to the genuine transformants, but the latter can be readily distinguished if the rDNA used for transformation carries two mutations for resistance to different antibiotics. In the first demonstration of chloroplast transformation in a higher plant, *Nicotiana tabacum* (tobacco), Svab *et al.* (1990) used a similar selection scheme, based on antibiotic resistance conferred by mutations in *rrnS*.

Such markers are recessive or semidominant, and their versatility for targeted gene integration or site-directed mutation is further limited because these markers necessarily reside in a specific locus, namely the rDNA in the inverted repeat region of the plastome. If the locus of the gene of interest is distant from the locus of the rDNA marker, the desired trait can segregate independently of the marker. These limitations were avoided with the development of a selectable cassette that was both portable and dominant. The cassette was based on the transgenic expression of the bacterial *aadA* gene, which encodes aminoglycoside 3′ adenyl transferase and confers high levels of resistance to spectinomycin and streptomycin (Goldschmidt-Clermont, 1991). Driven by chloroplast expression sequences, the resistance cassette could be targeted to any region of the plastome by providing flanking sequences that direct homologous recombination at the locus of interest. When the *aadA* selectable cassette was developed for plastid transformation in tobacco, the efficiency of transformation improved by two orders of magnitude compared with the efficiency obtained with the mutant rDNA markers (Svab and Maliga, 1993).

Other factors influencing successful plastid transformation include the tissue culture conditions used to promote division of resistant cells and their subsequent regeneration into shoots. These culture conditions required experimental optimization for each species in which stable plastid transformation has been established (Table 2). For example, fine-tuning of plant growth regulator concentrations was vital for isolating transplastomic commercial cultivars of lettuce (Ruhlman *et al.*, 2010). In addition, the plastid transformation regime may require the isolation of transplastomic shoots by organogenesis or somatic embryogenesis depending on the plant species (Daniell *et al.*, 2005). Organogenesis from leaves allows prolonged selection involving multiple cycles of shoot regeneration on selection media and the formation of shoots from small number of cells, conditions which promote homoplasmy. These are not readily achieved for transplastomic shoots isolated using somatic embryogenesis

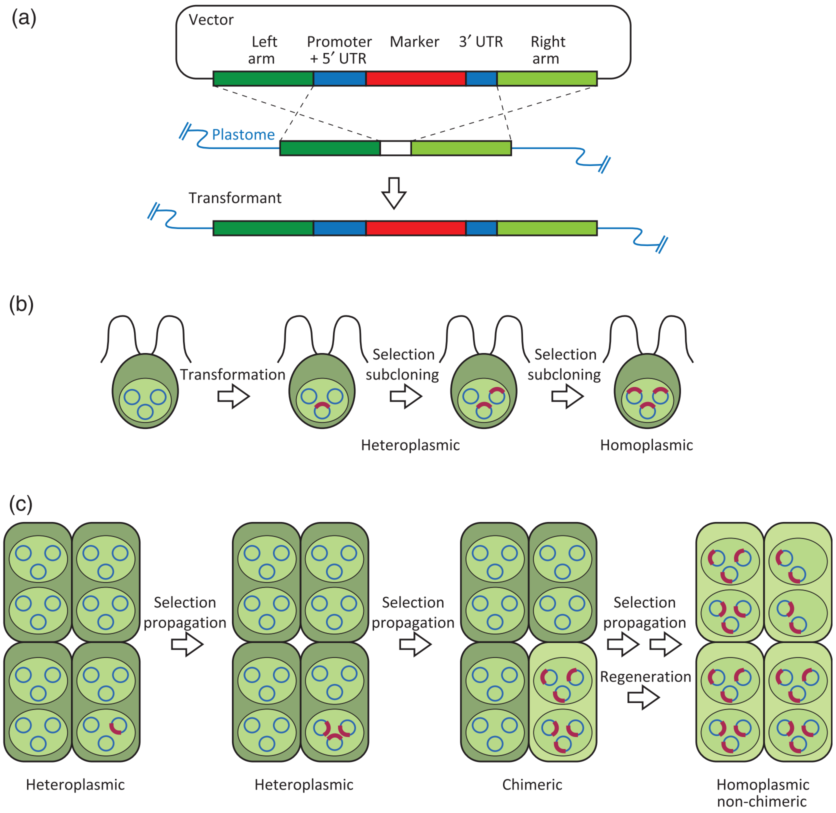

Figure 1 Chloroplast transformation. (a) In the transformation vector, a selectable marker (red) is placed under the control of plastid expression signals (promoter, 5′UTR, 3′UTR, shown in blue). Homologous recombination through the flanking targeting arms directs integration into the recipient plastid genome (plastome). The resulting transformant carries an insertion of the marker, or a substitution of the target sequence between the two arms (white bar). (b) The initial integration in only one copy of the polyploid plastome is heteroplasmic. In Chlamydomonas, the single chloroplast harbours approximately one hundred copies of its genome. Several rounds of subcloning and selection allow the recovery of homoplasmic clones. (This is only possible if no essential function of the plastome has been disrupted by the insertion, otherwise a heteroplasmic state retaining wild-type copies of the target sequence is maintained by a balance of selection for the marker and for the essential function.) (c) In multicellular plants, a similar situation prevails after transformation, but each cell contains multiple plastids. Repeated rounds of propagation and selection lead first to a homoplasmic plastid in a cell that may also contain non-transformed plastids, then to a cell with only homoplasmic plastids within a chimeric tissue and eventually to a non-chimeric homoplasmic plant. Regeneration from homoplasmic cells facilitates the recovery of homoplasmic plants.

(Dufourmantel *et al.*, 2004; Kumar *et al.*, 2004a). Yet another parameter is the optimal concentration of selection agent, which varies depending on the sensitivity of the species subjected to plastid transformation.

Selectable markers for plastid transformation

Over the years, a variety of selectable markers have been developed (Table 1). They differ in various properties that confer advantages and drawbacks, such as dominance, cell-autonomy or portability. Some markers are dominant, such as the *aadA* gene that confers resistance to spectinomycin and streptomycin by inactivating the antibiotics, while others are recessive, such as the point mutation in the ribosomal RNA genes (*rrnS* and *rrnL*) that confer resistance to various antibiotics by relieving the sensitivity of individual ribosomes. Dominance is of particular relevance for transformation of the highly polyploid plastome. Dominant markers increase the transformation frequency because they have an effect already at early stages during selection even though they may only be present in a minority of the plastomes. Conversely, recessive markers only confer resistance if random segregation has produced a plastid that has enough transformed copies of the plastome for the selectable phenotype to emerge. Because this is a rare event, recessive markers give lower transformation efficiencies than dominant ones.

Another important property of selectable markers is whether they are plastid- and cell-autonomous, such as the antibiotic-resistant *rrnS* or *rrnL* genes, which confer their phenotype only to the organelle or cell in which they reside. In contrast, genes that encode proteins that inactivate an antibiotic will also offer protection to neighbouring plastids and cells by locally decreasing the effective concentration of the drug. In this case, lines that emerge from a round of selection may still be heteroplasmic and plant tissues may be chimeric, with both transformed and wild-type sectors (Figure 1).

Some markers must integrate in a specific locus of the plastome, such as the *rrnS* or *rrnL* genes, while others are portable and autonomous and can be inserted in virtually any locus of the plastid genome, such as the *aadA* gene driven by chloroplast expression signals (promoter, 5′UTR and 3′UTR). Co-transformation with a marker and a gene of interest on separate vectors, followed by selection for the marker, can yield lines that carry both (Kindle *et al.*, 1991; Newman *et al.*, 1991; Carrer and Maliga, 1995; Ye *et al.*, 2003). However, if the two

Table 1 Selectable markers for plastid transformation

Marker	Selection	Organism	References	Notes
Photosynthesis				
atpB	Photoautotrophy	Chlamydomonas	Boynton *et al.* (1988)	
tscA	Photoautotrophy	Chlamydomonas	Goldschmidt-Clermont (1991); Kindle *et al.* (1991)	
psaA/B	Photoautotrophy	Chlamydomonas	Redding *et al.* (1998)	
petB	Photoautotrophy	Chlamydomonas	Cheng *et al.* (2005)	See Figure 2
petA, *ycf3*, *rpoA*	Photoautotrophy	Tobacco	Klaus *et al.* (2003)	Enhanced selection
rbcL	Photoautotrophy	Tobacco	Kode *et al.* (2006)	Enhanced selection
Antibiotic resistance				
rrnS	Spectinomycin streptomycin	Chlamydomonas	Kindle *et al.* (1991); Newman *et al.* (1990); Roffey *et al.* (1991)	
		Tobacco	Svab *et al.* (1990)	
		Tomato	Nugent *et al.* (2005)	
rrnL	Erythromycin	Chlamydomonas	Newman *et al.* (1990)	
aadA	Spectinomycin streptomycin	See Table 2	Goldschmidt-Clermont (1991)	See Table 2
nptII	Kanamycin	Tobacco	Carrer *et al.* (1993)	
		Cotton	Kumar *et al.* (2004b)	Double selection with *aphA-6*
aphA-6	Kanamycin, amikacin	Chlamydomonas	Bateman and Purton (2000)	
	Kanamycin	Tobacco	Huang *et al.* (2002)	
		Cotton	Kumar *et al.* (2004b)	Double selection with *nptII*
Herbicide resistance				
psbA	DCMU, metribuzin	Chlamydomonas	Newman *et al.* (1992); Przibilla *et al.* (1991)	
bar	Phosphinothricin	Tobacco	Iamtham and Day (2000)	Secondary selection; *aadA*-free
AHAS	Sulfometuron methyl	*Porphyridium* sp.	Lapidot *et al.* (2002)	
EPSP	Glyphosate	Tobacco	Ye *et al.* (2003)	Secondary selection; *aadA*-free
HPPD	Diketonitrile	Tobacco	Dufourmantel *et al.* (2007)	Secondary selection; *aadA*-free
Metabolism				
BADH	Betaine aldehyde	Tobacco	Daniell *et al.* (2001b)	
codA	5-fluorocytosine	Tobacco	Serino and Maliga (1997)	Negative selection
ARG9	Arg autotrophy	Chlamydomonas	Remacle *et al.* (2009)	
ASA2	Trp analogues	Tobacco	Barone *et al.* (2009)	

are not closely genetically linked, they can segregate independently during selection, so that lines that are homoplasmic for the gene of interest may be difficult to obtain. This is particularly true if the gene of interest is under negative selection pressure because it exerts an adverse effect on the chloroplast. For chloroplast gene inactivation or site-directed mutagenesis, a portable selectable cassette inserted within or near the gene of interest favours co-segregation of the desired mutation with the marker and facilitates the recovery of homoplasmic mutant lines.

Some markers confer a phenotype that is strong enough to allow direct selection of transformants. Relevant to this issue is whether selection is lethal, such that non-transformed cells or tissues are rapidly killed, or selection is non-lethal and growth is only retarded. The latter is thought to allow the sorting and amplification of transformed plastomes until a sufficient proportion is attained to confer the selectable phenotype, so that vigorous growth of the transformed cells will resume. In the case of non-lethal selection with spectinomycin, untransformed cells survive but are bleached, while transformed sectors are green and form shoots, so they are readily recognized. While spectinomycin inhibits plastid protein synthesis and causes bleaching in dicots, its action on cell proliferation is variable within the dicots. Spectinomycin inhibits cell proliferation in *Solanaceous* species such as tobacco but not in *Brassicas* (Zubko and Day, 1998). It has been argued that non-lethal selection is important for successful transformation in tobacco (Svab and Maliga, 1993), but this may not be essential (Maliga, 2004; Verma and Daniell, 2007). In some cases, a marker will confer a phenotype that is not strong enough for primary selection after transformation, but can still be used for secondary selection once established in a sufficient proportion of plastomes. The markers that confer tolerance to herbicides provide a typical example as discussed below (Iamtham and Day, 2000; Ye *et al.*, 2001; Dufourmantel *et al.*, 2007). Another possibility is to use such markers to enhance selection with a primary marker, as was exploited with photosynthesis markers (see next section).

When the marker is a foreign gene that is inserted in the plastid genome, it is necessary to provide native expression signals such as a chloroplast promoter and a 5′UTR with translation initiation signals and a 3′UTR. The choice of such elements and of chloroplast vectors has been reviewed recently (Lutz *et al.*, 2007; Verma and Daniell, 2007; Verma *et al.*, 2008). In plants, the *Prrn* promoter with the ribosome binding site from gene 10 of bacteriophage T7 or the *psbA* promoter and 5′UTR are commonly used for high levels of expression. In Chlamydomonas, a number of different chloroplast expression signals have been employed such as those from *atpA*, *psaA*, *psbA*, or *psbD* (Ishikura *et al.*, 1999; Barnes *et al.*, 2005; Michelet *et al.*, 2010).

Table 2 List of species in which *aadA*-based plastid transformation has been demonstrated

Organism		Marker	Resistance	References	Notes
Chlamydomonas	*Chlamydomonas reinhardtii*	*aadA*	Spectinomycin streptomycin	Goldschmidt-Clermont (1991)	
Tobacco	*Nicotiana tabacum*	*aadA*	Spectinomycin streptomycin	Svab and Maliga (1993)	
Tobacco	*Nicotiana tabacum*	*aadA_GFP*	Spectinomycin	Khan and Maliga (1999)	
Arabidopsis	*Arabidopsis thaliana*	*aadA*	Spectinomycin	Sikdar *et al.* (1998)	Sterile
Rice	*Oryza sativa*	*aadA_GFP*	Streptomycin	Khan and Maliga (1999)	Heteroplasmic
		aadA	Streptomycin	Lee *et al.* (2006)	Heteroplasmic
Potato	*Solanum tuberosum*	*aadA*	Spectinomycin streptomycin	Sidorov *et al.* (1999)	
Euglena	*Euglena gracilis*	*aadA*	Spectinomycin streptomycin	Doetsch *et al.* (2001)	
Tomato	*Solanum lycopersicum*	*aadA*	Spectinomycin streptomycin	Ruf *et al.* (2001)	
Oilseed rape	*Brassica napus*	*aadA*	Spectinomycin	Hou *et al.* (2003)	Heteroplasmic
		aadA	Streptomycin	Cheng *et al.* (2005)	Heteroplasmic
Lesquerella	*Lesquerella fendleri*	*aadA_GFP*	Spectinomycin streptomycin	Skarjinskaia *et al.* (2003)	Grafted on *B. napus*
Carrot	*Daucus carota*	*aadA*	Spectinomycin	Kumar *et al.* (2004b)	
Soybean	*Glycine max*	*aadA*	Spectinomycin	Dufourmantel *et al.* (2004)	
Petunia	*Petunia hybrida*	*aadA*	Spectinomycin streptomycin	Zubko *et al.* (2004)	
Physcomitrella	*Physcomitrella patens*	*aadA*	Spectinomycin	Sugiura and Sugita (2004)	
Lettuce	*Lactuca sativa*	*aadA*	Spectinomycin	Lelivelt *et al.* (2005); Ruhlman *et al.* (2010)	Polyethylene glycol/protoplast
Cauliflower	*Brassica oleracea* var. *botrytis*	*aadA*	Spectinomycin	Nugent *et al.* (2006)	
Poplar	*Populus alba*	*aadA*	Spectinomycin	Okumura *et al.* (2006)	
Cabbage	*Brassica capitata*	*aadA*	Spectinomycin streptomycin	Liu *et al.* (2007)	
Sugarbeet	*Beta vulgaris*	*aadA*	Spectinomycin	De Marchis *et al.* (2009)	
Eggplant	*Solanum melongena* L.	*aadA*	Spectinomycin streptomycin	Singh *et al.* (2010)	

Photosynthesis

Chloroplast genes that encode subunits of the photosynthetic complexes are often strictly essential for photosynthesis. In Chlamydomonas, after transformation of a mutant host with the wild-type gene, selection for photoautotrophic growth is very stringent. Furthermore, if a mutant with a chloroplast deletion is used, its genetic stability ensures that the reversion rate is extremely low. This scheme was used with *atpB* in the initial demonstration of chloroplast transformation (Boynton *et al.*, 1988). A deletion spanning the *tscA* gene, which encodes a small RNA involved in *psaA trans*-splicing, was also used as a host for transformation with the wild-type gene and selection for restoration of photoautotrophic growth (Goldschmidt-Clermont, 1991; Kindle *et al.*, 1991).

Chlamydomonas mutants with defects in photosystem I (PSI) have a high sensitivity to light-induced oxidative damage. This property was used to facilitate site-directed mutagenesis of *psaA* and *psaB*, the chloroplast genes for the major subunits of PSI (Redding *et al.*, 1998). A host strain deleted for both genes was first constructed by sequentially substituting the two chloroplast genes with an antibiotic resistance marker (*aadA*) that was subsequently excised. This double deletion line was then co-transformed with mutant versions of both *psaA* and *psaB*, each flanked by an *aadA* marker. Selection for antibiotic resistance as well as tolerance to moderate levels of light then allowed recovery of transformants with modifications in both *psaA* and *psaB*, as long as the desired mutations allowed residual activity of PSI (Redding *et al.*, 1998).

A similar approach was also developed in tobacco by generating photosynthesis-deficient lines through the insertion of a selectable marker for antibiotic resistance (*aadA*) in one of several different chloroplast genes (Klaus *et al.*, 2003) or by the combined excision of a chloroplast gene (*rbcL*) and marker (Kode *et al.*, 2006). The resulting lines were heterotrophic and had visible phenotypes of pigment deficiency. The photosynthesis mutant plants containing *aadA* were transformed with an *aph*-A6 marker gene together with a wild-type copy of the chloroplast gene (Klaus *et al.*, 2003). Marker-free *rbcL* deletion mutants were re-transformed using *aadA* and the wild-type *rbcL* gene (Kode *et al.*, 2006). The photoautotrophic transformed cells formed recognizable green sectors that could be chosen for subcloning, and they also had a growth advantage so that the recovery of homoplasmic lines was facilitated. Early transfer of resistant plants to soil is possible, without prolonged antibiotic selection, because selection for photosynthesis maintains transgenic plastid genomes.

Antibiotic resistance

The translation machinery of the plastid has retained prokaryotic features, so that mutations in ribosomal proteins or in rRNA can afford resistance to several antibiotics, such as spectinomycin, streptomycin and erythromycin. These provide markers for chloroplast transformation, but can also cause problems because the rate of spontaneous mutation to antibiotic resistance may be of the same order of magnitude or even higher than the frequency of transformation. Mutations of the 16S rRNA gene (*rrnS*) to spectinomycin or streptomycin resistance can be used singly or in combination to facilitate the discrimination of true transformants from spontaneous mutants that will usually be resistant to only one of the antibiotics (Newman *et al.*, 1990; Svab *et al.*, 1990). A mutation in *rps12(3')*, encoding a protein component of the small subunit of the plastid ribosome, can confer resistance to streptomycin, but whether this can be used as a marker for primary selection was not reported (Staub and Maliga, 1992). Another approach is to use marker genes encoding enzymes that chemically modify and inactivate antibiotics. The most successful marker to date is based on bacterial aminoglycoside 3′′-adenyl transferase (*aadA*), which confers resistance to spectinomycin and streptomycin. When this bacterial gene is flanked by appropriate chloroplast expression signals (promoter and UTRs), it confers high levels of resistance to the antibiotics in Chlamydomonas (Goldschmidt-Clermont, 1991) and in plants (Svab and Maliga, 1993). Because the enzyme can modify spectinomycin and streptomycin, after selection with one antibiotic, true transformants can be distinguished from spontaneous mutants on the basis of their resistance to the other. Such selectable *aadA* cassettes are portable and can be inserted at any locus, so they are widely used for gene inactivation or site-directed mutagenesis (Takahashi *et al.*, 1991; Kanevski and Maliga, 1994; Maliga, 2004)). They are dominant and in tobacco give transformation frequencies that are two orders of magnitude higher than with *rrnS* markers (Svab and Maliga, 1993), but they are not cell-autonomous. The *aadA* marker has been used successfully for plastid transformation in a wide and still expanding range of plant species (Table 2). The concentrations of spectinomycin dihydrochloride used can vary greatly from 0.5 mg/L in tobacco (Svab and Maliga, 1993) to 0.1 mg/L in lettuce (Ruhlman *et al.*, 2010). Cereals are resistant to spectinomycin, but are sensitive to streptomycin, which can be used for selection of *aadA* as tested in rice (Khan and Maliga, 1999). In this work, a gene fusion of *aadA* with *gfp* was used (encoding a modified green fluorescent protein) so that transgenic plastids could be recognized by fluorescence microscopy. Whether the heteroplasmic leaf sectors could further be subcultured to yield homoplasmic rice lines was not reported. Streptomycin alone is rarely used to select plastid transformants in tobacco where spectinomycin selection is more efficient. Both antibiotics can be used to select transformants in tobacco but double selection delays the isolation of resistant shoots. The relative inefficiency of streptomycin may hinder the recovery of plastid transformants in cereals. The inclusion of a second marker gene to allow dual or stepwise selection on streptomycin and a second agent (Table 1) may expedite the emergence of transplastomic cereal cells.

The bacterial *nptII* (*neo*) gene from the transposon Tn7 encodes neomycin phosphotransferase and confers resistance to antibiotics such as neomycin and kanamycin. It can be used to construct a selectable cassette for plant plastid transformation and selection on kanamycin, as first demonstrated in tobacco (Carrer *et al.*, 1993). The bacterial gene *aphA-6*, encoding aminoglycoside (3′) transferase (APH(3′)-VI), also provides resistance to kanamycin. Assembled into a selectable cassette, it allows chloroplast transformation in Chlamydomonas with selection on kanamycin or amikacin (Bateman and Purton, 2000). The *aphA-6* gene can also be used for plant plastid transformation as first established in tobacco (Huang *et al.*, 2002). For plastid transformation of cotton, double selection was used based on *aphA-6* and *nptII*, markers that both confer resistance to kanamycin (Kumar *et al.*, 2004b). The *aphA-6 gene* was driven by the *Prrn* promoter and a 5′UTR containing the ribosome binding region of bacteriophage T7 gene 10 for ubiquitous expression. The *nptII* gene was placed under the *psbA* promoter and 5′UTR to enhance expression in green photosynthetic tissues in the light. This combination allowed selection of transgenic cotton lines using somatic embryogenesis.

A recent addition to the catalogue of antibiotic resistance markers is the bacterial *cat* gene, which encodes chloramphenicol acetyl transferase and can be used as a selectable cassette for plant plastid transformation as demonstrated in tobacco (Li *et al.* 2010).

Herbicide tolerance

Many herbicides owe their specificity to their inhibitory effect on processes that are plant-specific and many of which take place in the plastid. Tolerance to various herbicides has been used for the design of chloroplast selectable markers. In Chlamydomonas, the *psbA* gene with a mutation that alters binding of several herbicides can be used for the direct selection of transformants on metribuzin-containing media (Przibilla *et al.*, 1991). The *psbA* gene can carry additional mutations of interest, such as further changes that alter tolerance to herbicides of other classes.

Acetohydroxyacid synthase (AHAS) is the target of the herbicide sulfometuron methyl (SMM). In plants, the gene is usually nuclear, but in the red unicellular alga, *Porphyridium* sp., it is located in the plastome. A strain of *Porphyridium* resistant to SMM was isolated due to a mutation in the *AHAS* gene. The mutant *AHAS* could thus be used as a dominant marker for chloroplast transformation of *Porphyridium* and selection on SMM-containing media (Lapidot *et al.*, 2002).

In tobacco, after a round of primary selection with an *aadA* cassette, markers that confer resistance to certain herbicides have been used for secondary selection. The lethality of herbicide selection may prevent the successful use of these markers in the initial round, but once established in a sufficient proportion of the plastomes, they can be used in subsequent rounds, for instance for removal of the *aadA* marker. Over-expression of genes which encode metabolic enzymes that are the targets of herbicides has been employed in this way, for example EPSPS (5-enolpyruvylshikimate-3-phosphate synthase). This enzyme is required for aromatic amino acid biosynthesis and is inhibited by glyphosate. It was initially shown that the EPSPS gene from petunia, transformed into tobacco chloroplasts using *aadA*-based spectinomycin selection, conferred resistance to glyphosate (Daniell *et al.*, 1998). Secondary selection involving the gene for EPSPS from *Agrobacterium* CP4 was achieved by switching from *aadA*-based spectinomycin selection to glyphosate early in the transformation procedure (Ye *et al.*, 2003).

The enzyme HPPD (4-hydroxyphenylpyruvate dioxygenase) is involved in the biosynthesis of quinones and vitamin E. It is inhibited by diketonitrile (DKN), a metabolic derivative of the herbicide isoxaflutole (IFT) and by sulcatrione. Chloroplast over-expression of the *HPPD* gene from barley procures tolerance to sulcatrione in tobacco (Falk *et al.*, 2005), and the gene from *Pseudomonas fluorescens* provides tolerance to IFT both in tobacco and in soybean (Dufourmantel *et al.*, 2007). It can be used as a secondary selectable marker after excision of the *aadA* gene.

Another strategy is to use as a selectable marker a gene encoding an enzyme that inactivates the herbicide. Resistance to phosphinothricin (PPT, glufosinate or Basta) can be provided by the bacterial *bar* gene, which encodes PPT acetyl transferase (Iamtham and Day, 2000; Lutz *et al.*, 2001). This marker was used for secondary selections aimed at removing the *aadA* antibiotic resistance marker.

Metabolism

Some selectable markers for plastid transformation are based on plant genes rather than genes from bacteria. They have the advantage of addressing public concern about transgenic plants carrying bacterial genes that confer resistance to antibiotics or herbicides. Anthranilate synthase (AS) catalyses the first step in the biosynthetic pathway for tryptophane. The enzyme is feedback inhibited by the end product, tryptophane, and also by tryptophane analogues that are toxic to the cell because they down-regulate the pathway and cause Trp deficiency. A dominant feedback-insensitive mutant of *ASA2* (the gene for the alpha subunit of ASA) is resistant to the Trp analogue 7-methyl-DL-tryptophan (7MT) or to the toxic precursor 4-methylindole (4MI). This mutant *ASA2* was recently expressed in the chloroplast of tobacco and used as a marker for primary selection on 4MI or 7MT (Barone *et al.*, 2009).

In Chlamydomonas, mutations in the *ARG9* gene cause arginine auxotrophy because of a defect in *N*-acetyl ornithine aminotransferase activity. Using an *arg9* mutant host, the wild-type *ARG9* gene can be used as a marker for chloroplast transformation (Remacle *et al.*, 2009). While the nuclear genome of Chlamydomonas has a high GC content (~65%), the chloroplast genome has a low GC content (~35%) and transgenes that have the corresponding codon bias are expressed at significantly higher levels (Franklin *et al.*, 2002). Thus, the Arabidopsis *ARG9* gene, which has a lower GC content than the endogenous Chlamydomonas nuclear gene, was chosen as the marker.

The betaine aldehyde dehydrogenase gene (BADH) converts toxic betaine aldehyde to betaine, which is an osmoprotectant that is accumulated by some plants in dry or saline environments. The BADH gene from spinach was used for plastid transformation of tobacco and carrot, and selection on betaine aldehyde (Daniell *et al.*, 2001a; Kumar *et al.*, 2004a). Betaine aldehyde is relatively expensive and the BADH marker is not in widespread use. The BADH gene might be more useful as a trait gene to confer salt tolerance on salt-sensitive species (Kumar *et al.*, 2004a).

Markers that allow negative selection are useful in genetic screens or to facilitate the removal of genetic elements. The first example of negative selection in the plastid was developed in tobacco using the bacterial *codA* gene (Serino and Maliga, 1997). This gene encodes cytosine deaminase and is absent in plants. It can convert 5-fluoro cytosine (5FC) to 5-fluoro uracil, which is further metabolized to 5-fluoro dUMP, a toxic inhibitor of thymidylate synthase. When expressed in the plastid in the presence of 5FC, the *codA* gene effectively prevents callus growth and shoot regeneration and inhibits seedling germination. Negative selection against the *codA* transgene facilitates the identification of seedlings where the marker had been eliminated using the *cre/loxP* system in tobacco plastids (Corneille *et al.*, 2001).

Marker removal

Marker genes are clearly necessary to select transgenic plastids and to isolate stable plastid transformants following delivery of genes into plastids as described above. Once stable plastid transformants have been isolated, marker genes are useful for tracking transgenic plastids using selective agents to screen transplastomic plants or algae. This is particularly useful as a research tool to follow the transmission of transgenic plastids and to confirm the identity and homoplasmy (100% maternal transmission of resistance) of stocks. In microalgae, excision of markers is usually not required when chloroplast transformation is based on rescue of photosynthetic mutations using the cognate wild-type chloroplast genes as marker genes. If the marker gene serves a useful purpose in a transplastomic crop or alga, such as conferring herbicide resistance (Iamtham and Day, 2000; Ye *et al.*, 2001; Dufourmantel *et al.*, 2007), its retention is warranted. However, there are situations when it may be desirable to remove marker genes from a transplastomic plant, for example, to facilitate regulatory approval or to allow multiple rounds of plastid transformation using the same efficient marker gene.

Regulatory approval of transgenic crops is particularly problematic for marker genes conferring antibiotic resistance. Directive 2001/18/EC of the European Parliament and Council on the deliberate release of transgenic organisms into the environment requires the removal of resistance genes against antibiotics in medical or veterinary use that may have adverse effects on human health and the environment. The directive addresses concerns over the potential spread of antibiotic resistance genes from plants to bacteria and their subsequent transmission to pathogenic bacteria to create 'super bugs' resistant to antibiotics in clinical use. The high copy number of plastid DNA and the expression of plastid marker genes in bacteria owing to the prokaryotic nature of plastid regulatory elements (promoters, ribosome binding sites) enhance the possibility of marker gene transfer to bacteria (Kay *et al.*, 2002; Ceccherini *et al.*, 2003; Pontiroli *et al.*, 2009). Other concerns focus on the protein products of the antibiotic resistance genes, such as their unintended consequences on the metabolism of the plant, their toxicity and allergenicity, or their possible effect, once ingested, on the efficacy of an oral dose of the target antibiotic. While it can be argued, on a case-by-case basis, that the risk of a particular antibiotic resistance gene to human health and the environment are negligible, removing these genes from transgenic plastids provides the simplest solution to facilitate regulatory approval of a transplastomic crop.

While the availability of non-antibiotic resistance markers, and native mutant *rrn* and *rps*12 genes, for plastid transformation addresses some of the regulatory concerns related to human health and the environment, their safety would still require evaluation. Excision of marker genes has additional advantages beyond facilitating regulatory approval. Marker excision allows multiple rounds of transformation with the same marker gene (marker recycling). This is particularly useful when plastid transformation is reliant on a limited set of efficient marker genes such as the *aadA* gene, which is the most frequently reported plastid transformation marker in the scientific literature. Marker gene removal also addresses any growth penalty associated with the energetic costs of expressing an unnecessary marker protein in plastids that is not required in the crop.

Strategies for isolating transgenic chloroplasts without foreign marker genes

Marker rotation

Rotation of markers in Chlamydomonas enables multiple cycles of chloroplast transformation and isolation of marker-free transgenic strains. The availability of non-photosynthetic chloroplast DNA deletion mutants in Chlamydomonas allows their rescue with the corresponding wild-type chloroplast genes by chloroplast transformation. Selection based on restoration of photosynthesis using wild-type Chlamydomonas chloroplast genes as markers does not raise regulatory issues, thereby obviating the need for marker removal. A simple two-step scheme that combines *aadA*-based chloroplast transformation with rescue of photosynthetic mutants allows multiple cycles of chloroplast transformation (Figure 2). In the first step, *aadA* is used to make a knockout in a photosynthesis gene giving rise to a strain that can only grow on acetate media. In the second step, rescue of the mutation with the wild-type plastid gene enables photosynthetic growth on minimal media lacking acetate. This two-step procedure (Figure 2) removes *aadA* from the final transgenic strain. This allows the marker rotation cycle to be repeated by knocking out other photosynthesis genes in the Chlamydomonas chloroplast genome with *aadA* and using the resulting non-photosynthetic strains to act as recipients for chloroplast transformation. The procedure has been used to replace the chloroplast *chlL* gene with the *nifH* and *gusA* genes (Cheng *et al.*, 2005). The method relies on co-integration of the foreign *nifH* or *gusA* genes together with the adjacent wild-type *petB* gene. A crossover event that takes place in the intervening region between *petB* and foreign genes would repair *petB* without insertion of foreign genes. Therefore, it is important to minimize the distance between the location of the photosynthetic mutation and the site of integration of the foreign genes.

Marker excision

Recombination between directly repeated sequences excises the intervening DNA sequence and one copy of the direct repeat. The breakage and joining of DNA strands involved in recombination can be mediated by the native homologous recombination machinery present in plastids or by foreign site-specific recombinases (Figure 3). Once marker-free genomes have been produced, they can act as templates for gene conversion. Both recombination-mediated gene excision and gene conversion will lead to a uniform population of marker-free plastid genomes.

Direct-repeat-mediated excision through homologous recombination. Direct-repeat-mediated excision has been used to remove *aadA* genes from Chlamydomonas (Fischer *et al.*, 1996) and tobacco chloroplasts (Iamtham and Day, 2000). Following transformation and once homoplasmy of transgenic plastid genomes is achieved, selection is removed allowing the accumulation of *aadA*-free plastid genomes with time. Excision is a spontaneous process, and the frequency is dependent on the length of direct repeats. In Chlamydomonas, direct repeats of 483 bp gave rise to excision frequencies that allowed efficient isolation of *aadA*-free lines, whereas direct repeats of 100 and 230 bp were ineffective in excising *aadA* (Fischer *et al.*, 1996). Because different sequences were used in the direct repeats, it remained possible that excision rates were influenced by the particular DNA sequence used.

Excision rates are also influenced by the number of direct repeats. In tobacco, two direct repeats of 418 bp were ineffective in excising *aadA* from the plastid genome (Iamtham and Day, 2000). When three direct repeats of 418 bp were combined with two direct repeats of 174 bp (Figure 4), high rates of recombination were observed between the outermost 418-bp repeats (Iamtham and Day, 2000). This allowed the isolation of a high percentage (24%) of marker-free transplastomic seedlings containing the *gusA* gene. In the experimental scheme used, spectinomycin plus streptomycin selection for *aadA* was switched to the herbicide PPT to select for the *bar* gene early in the transformation procedure. This promoted recombination between the two 174-bp repeats excising *gusA* and *aadA* to leave the *bar* gene in the plastid genome in two of 42 transplastomic lines isolated in the T_0 generation. While this example illustrates the potential of homology-based marker excision to provide several desirable outcomes from a single transformation event, in most cases a single set of direct repeats flanking the marker should suffice. Homology-based marker excision can be

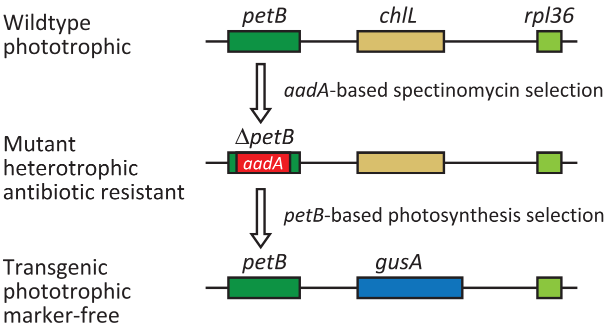

Figure 2 Marker rotation in Chlamydomonas (Cheng *et al.*, 2005). *aadA*-based spectinomycin selection is used to disrupt a photosynthesis gene (e.g. *petB*). Transformation of the resulting heterotrophic strain with the wild-type gene restores photosynthesis by removing *aadA* and allows integration of an adjacent foreign gene (e.g. *gusA*). The cycle can be repeated multiple times at different locations of the chloroplast genome.

Direct DNA repeats	Enzymes
>400 bp (650 bp)	Native plastid enzymes
34 bp *loxP* sites	Cre site-specific recombinase
34 bp *attB* and 39 bp *attP*	phage phi C31 site specific integrase

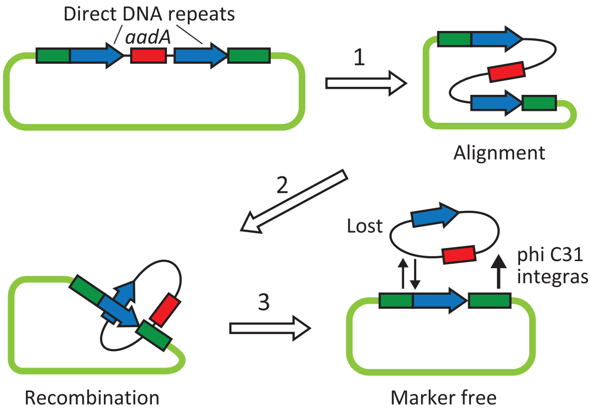

Figure 3 Recombination between direct DNA repeats bordering the *aadA* marker results in excision. The plastid homologous recombination machinery will act on a variety of DNA sequences (blue block arrows). Excision has been observed between 174-bp sequences but is more effective with larger DNA repeats (>400 bp), and increasing direct repeats to 650 bp gives rise to high excision frequencies. The Cre and phi C31 recombinases recognize specific short sequences, which if placed in direct orientation (blue block arrows) lead to *aadA* deletion. The *attB* and *attP* sites are not identical, and while orientation is important, they are not DNA direct repeats. The excised DNA circles are not stable and are lost. Loss of DNA circles or their physical separation following excision explains the limited impact of re-integration by reversible homologous and Cre-catalysed recombination events on marker removal. The phi C31 integrase reaction is unidirectional.

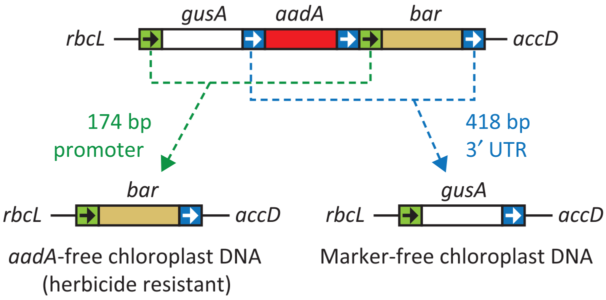

Figure 4 The frequency of excision of marker genes is raised by increasing the number of direct repeats (Iamtham and Day, 2000). Expression of *gusA*, *aadA* and *bar* genes is driven by duplicated *rrn* promoter (green) and triplicated 3′ *psbA* UTR regulatory elements (blue). Selection was switched from spectinomycin to herbicide (phosphinothricin) early in the transformation process. Recombination between the two 174-bp direct repeats (green dotted arrow) gave rise to herbicide (phosphinothricin)-resistant plants in the T_0 generation. Marker-free tobacco plants containing the *gusA* transgene are derived from recombination between the outermost 418-bp repeats (blue dotted arrow), which takes place at a higher frequency and gives rise to marker-free T_1 seedlings (24% of total seedlings).

visualized by using direct repeats of 649 bp to excise *aadA*, *gusA* and the native plastid *rbcL* gene resulting in pigment-deficient sectors (Figure 5). The number and sizes of sectors obtained illustrates the frequency and timing of excision, which takes place throughout plant development (Kode *et al.*, 2006). Sector formation also requires segregation of marker-free plastid genomes and marker-free plastids by cytoplasmic sorting during plant growth and development. In the schemes described above, excision of the *aadA* gene results in loss of antibiotic resistance, which is promoted by removing antibiotic selection. An alternative strategy is to promote direct-repeat-mediated excision of *aadA* by gain of function, such as herbicide resistance (Dufourmantel *et al.*, 2007). This is illustrated in Figure 6 where the *aadA* gene interrupts the coding region of the 4-hydroxyphenylpyruvate dioxygenase (*hppd*) gene. A partial duplication of the *hppd* gene creates 403-bp direct repeats that flank *aadA*. Recombination between these 403-bp repeats excises *aadA* and concomitantly restores the *hppd* coding sequence that is expressed and confers resistance to the herbicide DKN. Direct-repeat-mediated excision of marker genes exploits native homologous recombination pathways in plastids and provides a simple and effective method to excise marker genes from plastids. A similar scheme using a split *bar* gene flanking *aadA* and GFP genes has been used to isolate *aadA*-free transplastomic soybean plants resistant to PPT. The length of the duplicated *bar* repeat used was 367 bp (Lestrade *et al.*, 2009). Raising the length of direct repeats to over 600 bp increases the efficiency of the process, without comprising transformation frequency. The efficiency of marker removal using short direct repeats of less than 400 bp could be improved by including a negative selection marker (Serino and Maliga, 1997) in the excision cassette. Negative selection would be expected to favour segregation of plastids that have lost the excision cassette. Multiple cycles of transformation and marker excision are achievable by using unrelated direct-repeat sequences to flank the marker gene at each transformation step. Rigorous proof for *aadA* removal by direct-repeat excision has been obtained by re-transforming a transplastomic plant with the same *aadA* marker gene (Kode *et al.*, 2006). Predominance of homologous DNA recombination in plastids ranging from those found in algae to flowering plants suggests that direct-repeat-mediated excision will be widely applicable. Of the marker removal methods that have been demonstrated in tobacco, direct-repeat-mediated excision is the first to be also implemented in a major crop, namely soybean (Lestrade *et al.*, 2009).

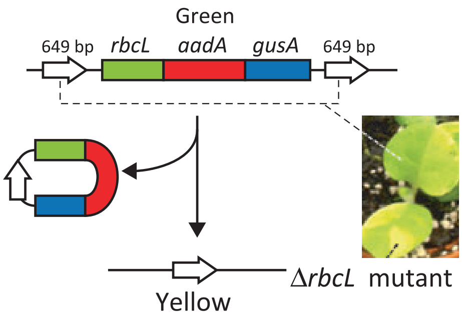

Figure 5 Direct-repeat-mediated excision takes place at all stages of leaf development in tobacco. The excision cassette contains *rbcL*, *aadA* and *gusA* genes. Loss of the photosynthetic *rbcL* gene arrests chloroplast development and gives rise to yellow cells, which are easily visualized (Kode *et al.*, 2006).

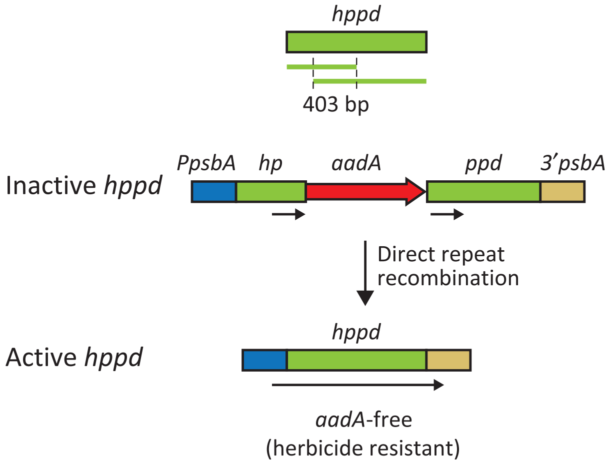

Figure 6 Excision of *aadA* mediated by gain of herbicide resistance in tobacco (Dufourmantel *et al.*, 2007). Initial selection for *aadA* on spectinomycin is replaced by selection on the herbicide DKN promoting the recombination event that deletes *aadA* and restores the *hppd* gene.

Excision of marker genes using site-specific recombinases. The Cre site-specific recombinase promotes strand-exchange between 34-bp *loxP* sites and is derived from the P1 bacteriophage of *Escherichia coli* (Sternberg and Hamilton, 1981). When the *aadA* gene is flanked by directly repeated 34-bp *loxP* sites in plastid DNA, it can be removed by introducing Cre recombinase into plastids (Corneille *et al.*, 2001; Hajdukiewicz *et al.*, 2001; Lutz *et al.*, 2006b; Oey *et al.*, 2009b). The process appears to be efficient giving rise to a high frequency of *aadA*-free seedlings when a highly expressed nucleus-localized *cre* gene, whose product is targeted to plastids, is introduced into transplastomic plants. This can be achieved by stable or transient introduction of the *cre* gene into transplastomic plants using *Agrobacterium tumefaciens*, or by fertilization of transplastomic plants with pollen from plants expressing the nuclear *cre* gene. Cre-catalysed breakage and joining of *loxP* sites stimulates unintended recombination events resulting in deletions in the plastid genome (Corneille *et al.*, 2001, 2003; Hajdukiewicz *et al.*, 2001; Lutz *et al.*, 2006a). These unforeseen events included recombination between the 117 bp *rrn* promoter region of a transgene with the native *rrn* gene. *LoxP* was also found to recombine with a recombination hotspot in the promoter region of the *rps7/3′rps12* operon (Hajdukiewicz *et al.*, 2001; Corneille *et al.*, 2003). This is the result of a pseudo *loxP* site (*loxP-rps12*) that resembles the true *Lox*P sequence. An additional pseudo *lox*P site was found in the *psbA* promoter (*Lox*P-*psbA*). Introduction of plastid-targeted Cre by pollination was reported to reduce the frequency of unintended chloroplast genome deletions (Corneille *et al.*, 2001). Unexpected recombination events promoted by Cre are undesirable and would need to be monitored. However, the resulting deleted genomes do not appear to persist into the next seed generation once Cre is removed (Corneille *et al.*, 2003). Compared with direct-repeat excision mediated by native plastid enzymes, the Cre/*loxP* system requires the additional steps of first introducing and then removing plastid-targeted Cre recombinase from transplastomic plants. However, the timing of introducing a site-specific recombinase into plastids can be controlled and marker loss is rapid once the recombinase enters plastids. Cre removal can be accelerated by its transient expression in plastids, which deletes *aadA*, and gives rise to about 10% of marker-free shoots lacking the *cre* gene (Lutz *et al.*, 2006a). The retention of a *loxP* site following marker excision would hinder a second cycle of *aadA* integration and excision via flanking *loxP* sites at a distant site of the plastid genome. Excision of *aadA* from the plastid genome has also been achieved using the phiC31 bacteriophage site-specific integrase (Kittiwongwattana *et al.*, 2007) that recombines non-identical 34-bp *attB* and 39-bp *attP* target DNA sites (Groth *et al.*, 2000) that flank *aadA*. This system has the advantage of being unidirectional unlike the reversible Cre/*loxP* system where Cre would be expected to promote both excision and re-integration of *aadA* via *loxP* sites. Pseudo *attB* and *attP* sites are not present in the plastid genome reducing the possibility of unintended recombination events promoted by phiC31 integrase (Kittiwongwattana *et al.*, 2007). Plastid transformation vectors that include marker gene elimination cassettes based on Cre/*loxP* and phiC31/*attB*/*attP* sites have been developed (Lutz *et al.*, 2007). Site-specific recombinases are particularly useful for excising genes that might have an inherent selective advantage in plastids, such as essential plastid genes (Kuroda and Maliga, 2003).

Isolation of aadA-free plastid genomes using co-transformation and segregation. The approach relies on the fact that the plastome is highly polyploid. Co-transformation of *aadA* and herbicide resistance genes to distinct sites of the plastid genome will give rise to a proportion of plastid genomes only containing a single marker gene early in transformation (Figure 7). Switching antibiotic selection for *aadA* to herbicide selection for the *bar* or *EPSPS* genes early in transformation, when heteroplasmy is still present, gave rise to 50%–64% of plastid transformants co-transformed with both markers (Ye *et al.*, 2003). Of these co-transformants, 20% gave rise to herbicide-resistant segregants that were *aadA*-free (Ye *et al.*, 2003). The approach relies on segregation of plastid genomes from a heteroplasmic population and requires efficient co-transformation and effective early timing of the switch from antibiotic to herbicide selection. The efficiency of co-transformation can vary between experiments. When co-transformants were isolated using only *aadA*-based spectinomycin selection, the second gene was present in around 20% of *aadA* transformants (Carrer and Maliga, 1995). This approach has not been used to isolate an *aadA*-free transplastomic plant with a non-selectable trait gene. Driving a non-selectable trait gene to homoplasmy might be challenging using this segregation approach.

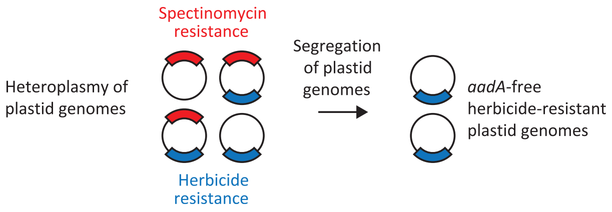

Figure 7 Co-transformation and segregation of marker-free plastid genomes in tobacco (Ye *et al.*, 2003). The *aadA* marker and herbicide resistance genes (*bar* or EPSPS genes) are targeted to different locations of the plastid genome. Switching from spectinomycin to herbicide selection while cells were heteroplasmic gave rise to herbicide-resistant plants of which about 20% lacked the *aadA* marker gene.

An elegant variant of this co-transformation approach targets *aadA* to disrupt the essential gene *rpoC2* (formerly known as ORF472) in Chlamydomonas (Fischer *et al.*, 1996; Rochaix, 1997). When spectinomycin selection is applied, both versions of the essential gene are required: the *aadA*-disrupted allele to confer resistance to spectinomycin and the intact allele to provide the essential function for cell viability. This results in heteroplasmy at the *aadA* integration site. When *aadA* was co-transformed with a chloroplast plasmid containing a mutant *psaB* gene, this replaced the WT *psaB* gene at a distant site of the chloroplast genome. Spectinomycin selection combined with screening single colonies allowed the isolation of clones that were homoplasmic for the mutant *psaB* gene. Release of spectinomycin selection resulted in loss of the *aadA* gene due to selection for the functional allele of *rpoC2* (Fischer *et al.*, 1996). This resulted in *aadA*-free strains containing the mutant *psaB* gene. The approach would also allow the insertion of a gene of interest and should be applicable to tobacco where a number of essential chloroplast genes have been identified (Drescher *et al.*, 2000; Shikanai *et al.*, 2001; Kuroda and Maliga, 2003; Kode *et al.*, 2005; Rogalski *et al.*, 2006).

Isolation of marker-free plants using transient co-integration of the marker gene (Klaus et al., 2004). Stable integration of a marker gene into plastid DNA requires targeting arms to enable a double crossover event in the homologous regions flanking the marker gene (Figure 1). If the plastid marker gene (*aphA6*) is inserted into the bacterial vector backbone, this mode of insertion is not possible**.** Integration of the *aphA6* gene into the plastid genome takes place through a single crossover event in either the left (shown in Figure 8) or right arms. This creates an unstable co-integrate containing large direct repeats of the left and right targeting arms. Recombination between the repeated right arms in the co-integrate results in excision of the marker genes leaving the *gusA* transgene together with a copy of the wild-type *rpoA* gene. The procedure requires the prior isolation of an *aadA* knockout in the plastid *rpoA* gene, which has an albino phenotype. Rescue of the *rpoA* mutation results in restoration of green pigmentation, allowing the identification of transformation events resulting from the integration of the *gusA* and *rpoA* genes (Figure 8). The shoots contain a complex heteroplasmic mixture of molecules. Green shoots removed from kanamycin selection rapidly lose the *aph*A6 gene. Mutations in other plastid genes, such as *petA*, resulting in a loss of pigmentation can be used in place of *rpoA* (Klaus *et al.*, 2004). The method requires extra effort to isolate and propagate (on sucrose medium) the chloroplast mutants needed to facilitate the identification of desirable recombination events. Use of the *aphA6* gene and kanamycin selection is not as efficient as *aadA*-based spectinomycin selection and requires biolistic transformation of microcolonies rather than leaves. Once the mutants are isolated, the method provides a rapid approach to isolate marker-free plants in the T_0 generation.

Conclusion

A varied repertoire of selectable markers has been developed over the two decades since the first report of plastid transformation. The most flexible of these markers rely on resistance to antibiotics. While these are efficient and convenient for fundamental research, antibiotic resistance is not a desirable trait in transgenic crops and has been legally banned from open fields in a number of countries and requires compliance with directive 2001/18/EC within the European Union. The problem can be circumvented with a number of efficient methods to excise undesired markers. An alternative is to use markers based on endogenous photosynthetic or metabolic markers, which is readily feasible in Chlamydomonas because many mutant hosts are available and selection is stringent. Such mutant hosts have been developed in tobacco, but because selection is not stringent enough, the photosynthetic markers have been used in combination with a conventional antibiotic resistance marker to facilitate screening. Another alternative is to use non-antibiotic marker genes, particularly native plant genes such as *ASA2*, which offers a dominant, portable selectable marker that can be used in a wild-type host (Barone *et al.*, 2009).

While many types of markers have been reported, to date most of them have been used only sporadically, with the exception of the *aadA* cassette. This marker is widely applied, in particular for the development of plastid transformation protocols in a rapidly expanding number of crop plants. It is not cell- or plastid-autonomous, which might be an important factor in its efficiency. However, this property does increase the time required to obtain homoplasmic and non-chimeric plants. Combining marker genes for stepwise antibiotic selection followed by herbicide selection provides one approach to improve the stringency of selection and allows the removal of antibiotic resistance genes. Conservation of plastid genomes and maintenance mechanisms would suggest that marker removal methods developed in Chlamydomonas and tobacco would be applicable to a wide range of flowering plants. Developmental differences between plants, especially between monocots and dicots, might impact on the segregation of marker-free plastid lineages during plant growth. Developing routine plastid transformation procedures in major crops remains a challenge. More efficient markers and selection is only one aspect that can be developed and will need to be combined with improving both the competence of cells and the subsequent proliferation and regeneration of cells with transformed plastids.

Thus, if the toolbox for chloroplast transformation contains adequate technology, there is still much space for improvement and in particular for the development of new markers.

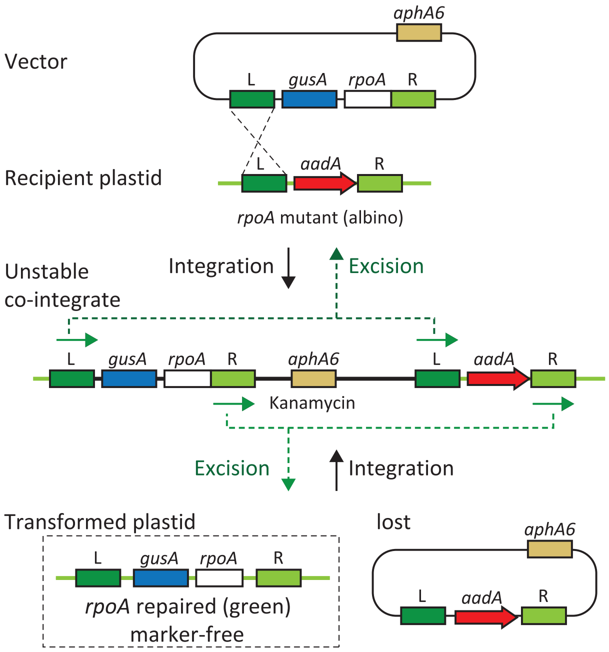

Figure 8 Scheme whereby transient co-integration of the *aphA6* marker gene leads to marker-free tobacco plants (Klaus *et al.*, 2004). The *aphA6* gene is located in the vector backbone and recipient albino plants contain an *aadA* knockout of the plastid *rpoA* gene. Kanamycin selection of transformants promotes integration via a single crossover event within the left (L) or right (R) targeting arms. Only the consequence of integration in the L-arm is shown. The resulting co-integrate is unstable. Recombination between the duplicate L-arms reverses integration, whereas recombination between the duplicated R-arms excises the marker genes and inserts the *gusA* transgene and wild-type *rpoA* gene. Rescue of the mutant *rpoA* gene restores pigmentation allowing marker-free transplastomic cells to be identified.

Acknowledgements

Work on chloroplast transformation in MGC's laboratory was supported by FP7 KBBE 2009-3 Sunbiopath (GA 245070), the Swiss NRP59 program (Benefits and Risks of the Deliberate Release of Genetically Modified Plants) and the Swiss National Science Foundation (3100AO-117712). AD was supported by the Biotechnology and Biological Sciences Research Council (BB/E020445/1). We thank Jean-David Rochaix for his critical comments on the manuscript and Nicolas Roggli for the preparation of the figures.

Note added in proof

While this manuscript was in press, Cui and coworkers reported stable plastid transformation in wheat (*Triticum aestivum*) using the *nptII* selectable marker and G418 selection (Cui *et al.*, 2011).

References

Barnes, D., Franklin, S., Schultz, J., Henry, R., Brown, E., Coragliotti, A. and Mayfield, S.P. (2005) Contribution of 5′- and 3′-untranslated regions of plastid mRNAs to the expression of *Chlamydomonas reinhardtii* chloroplast genes. *Mol. Genet. Genomics*, **274**, 625–636.

Barone, P., Zhang, X.H. and Widholm, J.M. (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.

Bateman, J.M. and Purton, S. (2000) Tools for chloroplast transformation in Chlamydomonas: expression vectors and a new dominant selectable marker. *Mol. Gen. Genet.* **263**, 404–410.

Bock, R. (2007) Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. *Curr. Opin. Biotechnol.* **18**, 100–106.

Bock, R. and Timmis, J.N. (2008) Reconstructing evolution: gene transfer from plastids to the nucleus. *Bioessays*, **30**, 556–566.

Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B., Sanford, J.C. (1988) Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. *Science*, **240**, 1534–1538.

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

Carrer, H. and Maliga, P. (1995) Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. *Bio-technology*, **13**, 791–794.

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

Ceccherini, M.T., Pote, J., Kay, E., Van, V.T., Marechal, J., Pietramellara, G., Nannipieri, P., Vogel, T.M. and Simonet, P. (2003) Degradation and transformability of DNA from transgenic leaves. *Appl. Environ. Microbiol.* **69**, 673–678.

Cheng, Q., Day, A., Dowson-Day, M., Shen, G.F. and Dixon, R. (2005) The *Klebsiella pneumoniae* nitrogenase Fe protein gene (nifH) functionally substitutes for the *chlL* gene in *Chlamydomonas reinhardtii*. *Biochem. Biophys. Res. Commun.* **329**, 966–975.

Corneille, S., Lutz, K., Svab, Z. and Maliga, P. (2001) Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system. *Plant J.* **27**, 171–178.

Corneille, S., Lutz, K.A., Azhagiri, A.K. and Maliga, P. (2003) Identification of functional lox sites in the plastid genome. *Plant J.* **35**, 753–762.

Corriveau, J.L. and Coleman, A.W. (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *Am. J. Bot.* **75**, 1443–1458.

Cui, C., Song, F., Tan, Y., Zhou, X., Zhao, W., Ma, F., Liu, Y., Hussain, J., Wang, Y., Yang, G. and He, G. (2011) Stable chloroplast transformation of immature scutella and inflorescences in wheat (Triticum aestivum L.). *Acta. Biochim. Biophys. Sin.* doi:10.1093/abbs/gmr008.

Daniell, H. and McFadden, B.A. (1987) Uptake and expression of bacterial and cyanobacterial genes by isolated cucumber etioplasts. *Proc. Natl Acad. Sci. USA*, **84**, 6349–6353.

Daniell, H., Vivekananda, J., Nielsen, B.L., Ye, G.N., Tewari, K.K. and Sanford, J.C. (1990) Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc. Natl Acad. Sci. USA*, **87**, 88–92.

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

Daniell, H., Muthukumar, B. and Lee, S.B. (2001a) Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr. Genet.* **39**, 109–116.

Daniell, H., Wiebe, P.O. and Millan, A.F. (2001b) Antibiotic-free chloroplast genetic engineering—an environmentally friendly approach. *Trends Plant Sci.* **6**, 237–239.

Daniell, H., Kumar, S. and Dufourmantel, N. (2005) Breakthrough in chloroplast genetic engineering of agronomically important crops. *Trends Biotechnol.* **23**, 238–245.

Daniell, H., Singh, N.D., Mason, H. and Streatfield, S.J. (2009) Plant-made vaccine antigens and biopharmaceuticals. *Trends Plant Sci.* **14**, 669–679.

De Cosa, B., Moar, W., Lee, S.B., Miller, M. and 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., Wang, Y., Stevanato, P., Arcioni, S. and Bellucci, M. (2009) Genetic transformation of the sugar beet plastome. *Transgenic Res.* **18**, 17–30.

Doetsch, N.A., Favreau, M.R., Kuscuoglu, N., Thompson, M.D. and Hallick, R.B. (2001) Chloroplast transformation in *Euglena gracilis*: splicing of a group III twintron transcribed from a transgenic psbK operon. *Curr. Genet.* **39**, 49–60.

Drescher, A., Ruf, S., Calsa, T., Carrer, H. and Bock, R. (2000) The two largest chloroplast genome-encoded open reading frames of higher plants are essential genes. *Plant J.* **22**, 97–104.

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

Dufourmantel, N., Dubald, M., Matringe, M., Canard, H., Garcon, F., Job, C., Kay, E., Wisniewski, J.P., Ferullo, J.M., Pelissier, B., Sailland, A. and Tissot, G. (2007) Generation and characterization of soybean and marker-free tobacco plastid transformants over-expressing a bacterial 4-hydroxyphenylpyruvate dioxygenase which provides strong herbicide tolerance. *Plant Biotechnol. J.* **5**, 118–133.

Falk, J., Brosch, M., Schafer, A., Braun, S. and Krupinska, K. (2005) Characterization of transplastomic tobacco plants with a plastid localized barley 4-hydroxyphenylpyruvate dioxygenase. *J. Plant. Physiol.* **162**, 738–742.

Fischer, N., Stampacchia, O., Redding, K. and Rochaix, J.D. (1996) Selectable marker recycling in the chloroplast. *Mol. Gen. Genet.* **251**, 373–380.

Franklin, S., Ngo, B., Efuet, E. and Mayfield, S.P. (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *Plant J.* **30**, 733–744.

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

Gould, S.B., Waller, R.F. and McFadden, G.I. (2008) Plastid evolution. *Annu. Rev. Plant Biol.* **59**, 491–517.

Griesbeck, C., Kobl, I. and Heitzer, M. (2006) *Chlamydomonas reinhardtii*: a protein expression system for pharmaceutical and biotechnological proteins. *Mol. Biotechnol.* **34**, 213–223.

Groth, A.C., Olivares, E.C., Thyagarajan, B. and Calos, M.P. (2000) A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl Acad. Sci. USA*, **97**, 5995–6000.

Hajdukiewicz, P.T.J., Gilbertson, L. and Staub, J.M. (2001) Multiple pathways for Cre⁄lox-mediated recombination in plastids. *Plant J.* **27**, 161–170.

Hou, B.K., Zhou, Y.H., Wan, L.H., Zhang, Z.L., Shen, G.F., Chen, Z.H. and Hu, Z.M. (2003) Chloroplast transformation in oilseed rape. *Transgenic Res.* **12**, 111–114.

Huang, F.C., Klaus, S.M., Herz, S., Zou, Z., Koop, H.U. and Golds, T.J. (2002) Efficient plastid transformation in tobacco using the *aphA-6* gene and kanamycin selection. *Mol. Genet. Genomics*, **268**, 19–27.

Huang, C.Y., Ayliffe, M.A. and Timmis, J.N. (2003) Direct measurement of the transfer rate of chloroplast DNA into the nucleus. *Nature*, **422**, 72–76.

Iamtham, S. and Day, A. (2000) Removal of antibiotic resistance genes from transgenic tobacco plastids. *Nat. Biotechnol.* **18**, 1172–1176.

Ishikura, K., Takaoka, Y., Kato, K., Sekine, M., Yoshida, K. and Shinmyo, A. (1999) Expression of a foreign gene in *Chlamydomonas reinhardtii* chloroplast. *J. Biosci. Bioeng.* **87**, 307–314.

Kanevski, I. and Maliga, P. (1994) Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proc. Natl Acad. Sci. USA*, **91**, 1969–1973.

Kay, E., Vogel, T.M., Bertolla, F., Nalin, R. and Simonet, P. (2002) In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Appl. Environ. Microbiol.* **68**, 3345–3351.

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

Kindle, K.L., Richards, K.L. and Stern, D.B. (1991) Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **88**, 1721–1725.

Kittiwongwattana, C., Lutz, K., Clark, M. and Maliga, P. (2007) Plastid marker gene excision by the phiC31 phage site-specific recombinase. *Plant Mol. Biol.* **64**, 137–143.

Klaus, S.M., Huang, F.C., Eibl, C., Koop, H.U. and Golds, T.J. (2003) Rapid and proven production of transplastomic tobacco plants by restoration of pigmentation and photosynthesis. *Plant J.* **35**, 811–821.

Klaus, S.M., Huang, F.C., Golds, T.J. and Koop, H.U. (2004) Generation of marker-free plastid transformants using a transiently cointegrated selection gene. *Nat. Biotechnol.* **22**, 225–229.

Knoblauch, M., Hibberd, J.M., Gray, J.C. and van Bel, A.J. (1999) A galinstan expansion femtosyringe for microinjection of eukaryotic organelles and prokaryotes. *Nat. Biotechnol.* **17**, 906–909.

Kode, V., Mudd, E.A., Iamtham, S. and Day, A. (2005) The tobacco *accD* gene is essential and is required for leaf development. *Plant J.* **44**, 237–244.

Kode, V., Mudd, E.A., Iamtham, S. and Day, A. (2006) Isolation of precise plastid deletion mutants by homology-based excision: a resource for site-directed mutagenesis, multi-gene changes and high-throughput plastid transformation. *Plant J.* **46**, 901–909.

Koop, H.U., Steinmuller, K., Wagner, H., Rossler, C., Eibl, C. and Sacher, L. (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. *Planta*, **199**, 193–201.

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

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

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

Lapidot, M., Raveh, D., Sivan, A., Arad, S.M. and Shapira, M. (2002) Stable chloroplast transformation of the unicellular red alga Porphyridium species. *Plant Physiol.* **129**, 7–12.

Lee, S.M., Kang, K., Chung, H., Yoo, S.H., Xu, X.M., Lee, S.B., Cheong, J.J., Daniell, H. and Kim, M. (2006) Plastid transformation in the monocotyledonous cereal crop, rice (*Oryza sativa*) and transmission of transgenes to their progeny. *Mol. Cells*, **21**, 401–410.

Lelivelt, C.L., McCabe, M.S., Newell, C.A., Desnoo, C.B., van Dun, K.M., Birch-Machin, I., Gray, J.C., Mills, K.H. and Nugent, J.M. (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.). *Plant Mol. Biol.* **58**, 763–774.

Lestrade, C., Pelissier, B., Roland, A. and Dubald, M. (2009) Construct for obtaining transplastomic plant or plant cell comprises at least chimeric gene encoding selectable marker and chimeric color gene, or chimeric gene encoding luminescent protein, or chimeric gene encoding a negative marker: Bayer Cropscience Ag (Farb). Patent application number WO 2010079117 A2.

Li, W., Ruf, S., Bock, R. (2010) Chloramphenicol acetyltransferase as selectable marker for plastid transformation. *Plant Mol Biol*. doi:10.1007/s11103-010-9678-4.

Liu, C.W., Lin, C.C., Chen, J.J. and Tseng, M.J. (2007) Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. *capitata* L.) by particle bombardment. *Plant Cell Rep.* **26**, 1733–1744.

Lutz, K.A., Knapp, J.E. and Maliga, P. (2001) Expression of bar in the plastid genome confers herbicide resistance. *Plant Physiol.* **125**, 1585–1590.

Lutz, K.A., Bosacchi, M.H. and Maliga, P. (2006a) Plastid marker-gene excision by transiently expressed CRE recombinase. *Plant J.* **45**, 447–456.

Lutz, K.A., Svab, Z. and Maliga, P. (2006b) Construction of marker-free transplastomic tobacco using the Cre-loxP site-specific recombination system. *Nat. Protoc.* **1**, 900–910.

Lutz, K.A., Azhagiri, A.K., Tungsuchat-Huang, T. and Maliga, P. (2007) A guide to choosing vectors for transformation of the plastid genome of higher plants. *Plant Physiol.* **145**, 1201–1210.

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

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

Michelet, L., Lefebvre-Legendre, L., Burr, S.E., Rochaix, J.D. and Goldschmidt-Clermont, M. (2010) Enhanced chloroplast transgene expression in a nuclear mutant of Chlamydomonas. *Plant Biotechnol. J.* doi:10.1111/j.1467-7652.2010.00564.x.

Newman, S.M., Boynton, J.E., Gillham, N.W., Randolph-Anderson, B.L., Johnson, A.M. and Harris, E.H. (1990) Transformation of chloroplast ribosomal RNA genes in Chlamydomonas: molecular and genetic characterization of integration events. *Genetics*, **126**, 875–888.

Newman, S.M., Gillham, N.W., Harris, E.H., Johnson, A.M. and Boynton, J.E. (1991) Targeted disruption of chloroplast genes in *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **230**, 65–74.

Newman, S.M., Harris, E.H., Johnson, A.M., Boynton, J.E. and Gillham, N.W. (1992) Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: evidence for a hotspot and an adjacent cold region. *Genetics*, **132**, 413–429.

Nugent, G.D., Ten Have, M., van der Gulik, A., Dix, P.J., Uijtewaal, B.A. and Mordhorst, A.P. (2005) Plastid transformants of tomato selected using mutations affecting ribosome structure. *Plant Cell Rep.* **24**, 341–349.

Nugent, G.D., Coyne, S., Nguyen, T.T., Kavanagh, T.A. and Dix, P.J. (2006) Nuclear and plastid transformation of *Brassica oleracea* var. *botrytis* (cauliflower) using PEG-mediated uptake of DNA into protoplasts. *Plant Sci.* **170**, 135–142.

Oey, M., Lohse, M., Kreikemeyer, B. and 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, L.B., Kreikemeyer, B. and 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.

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

O'Neill, C., Horvath, G.V., Horvath, E., Dix, P.J. and Medgyesy, P. (1993) Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic delivery systems. *Plant J.*, **3**, 729–738.

Pontiroli, A., Rizzi, A., Simonet, P., Daffonchio, D., Vogel, T.M. and Monier, J.M. (2009) Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco. *Appl. Environ. Microbiol.* **75**, 3314–3322.

Przibilla, E., Heiss, S., Johanningmeier, U. and Trebst, A. (1991) Site-specific mutagenesis of the D1 subunit of photosystem II in wild-type Chlamydomonas. *Plant Cell*, **3**, 169–174.

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

Redding, K., MacMillan, F., Leibl, W., Brettel, K., Hanley, J., Rutherford, A.W., Breton, J. and Rochaix, J.D. (1998) A systematic survey of conserved histidines in the core subunits of Photosystem I by site-directed mutagenesis reveals the likely axial ligands of P700. *EMBO J.* **17**, 50–60.

Remacle, C., Cline, S., Boutaffala, L., Gabilly, S., Larosa, V., Barbieri, M.R., Coosemans, N. and Hamel, P.P. (2009) The *ARG9* gene encodes the plastid-resident *N*-acetyl ornithine aminotransferase in the green alga *Chlamydomonas reinhardtii*. *Eukaryot. Cell*, **8**, 1460–1463.

Rochaix, J.-D. (1997) Chloroplast reverse genetics: new insights into the function of plastid genes. *Trends Plant Sci.* **2**, 419–425.

Roffey, R.A., Golbeck, J.H., Hille, C.R. and Sayre, R.T. (1991) Photosynthetic electron transport in genetically altered photosystem II reaction centers of chloroplasts. *Proc. Natl Acad. Sci. USA*, **88**, 9122–9126.

Rogalski, M., Ruf, S. and Bock, R. (2006) Tobacco plastid ribosomal protein S18 is essential for cell survival. *Nucleic Acids Res.* **34**, 4537–4545.

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

Ruf, S., Karcher, D. and Bock, R. (2007) Determining the transgene containment level provided by chloroplast transformation. *Proc. Natl Acad. Sci. USA*, **104**, 6998–7002.

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

Serino, G. and Maliga, P. (1997) A negative selection scheme based on the expression of cytosine deaminase in plastids. *Plant J.* **12**, 697–701.

Shikanai, T., Shimizu, K., Ueda, K., Nishimura, Y., Kuroiwa, T. and Hashimoto, T. (2001) The chloroplast *clp*P gene, encoding a proteolytic subunit of ATP-dependent protease, is indispensable for chloroplast development in tobacco. *Plant Cell Physiol.* **42**, 264–273.

Sidorov, V.A., Kasten, D., Pang, S.Z., Hajdukiewicz, P.T., Staub, J.M. and Nehra, N.S. (1999) Technical advance: stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant J.* **19**, 209–216.

Sikdar, S.R., Serino, G., Chaudhuri, S. and Maliga, P. (1998) Plastid transformation in *Arabidopsis thaliana*. *Plant Cell Rep.* **18**, 20–34.

Singh, A.K., Verma, S.S. and Bansal, K.C. (2010) Plastid transformation in eggplant (*Solanum melongena* L.). *Transgenic Res.* **19**, 113–119.

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

Specht, E., Miyake-Stoner, S. and Mayfield, S. (2010) Micro-algae come of age as a platform for recombinant protein production. *Biotechnol. Lett.* **32**, 1373–1383.

Staub, J.M. and Maliga, P. (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. *Plant Cell*, **4**, 39–45.

Staub, J.M. and Maliga, P. (1995) Expression of a chimeric *uidA* gene indicates that polycistronic mRNAs are efficiently translated in tobacco plastids. *Plant J.* **7**, 845–848.

Stegemann, S., Hartmann, S., Ruf, S. and Bock, R. (2003) High-frequency gene transfer from the chloroplast genome to the nucleus. *Proc. Natl Acad. Sci. USA*, **100**, 8828–8833.

Sternberg, N. and Hamilton, D. (1981) Bacteriophage-P1 site specific recombination. 1. Recombination between LoxP sites. *J. Mol. Biol.* **150**, 467–486.

Sugiura, C. and Sugita, M. (2004) Plastid transformation reveals that moss tRNA(Arg)-CCG is not essential for plastid function. *Plant J.* **40**, 314–321.

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

Svab, Z. and Maliga, P. (2007) Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proc. Natl Acad. Sci. USA*, **104**, 7003–7008.

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

Takahashi, Y., Goldschmidt-Clermont, M., Soen, S.Y., Franzen, L.G. and Rochaix, J.D. (1991) Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilizes photosystem I. *EMBO J.* **10**, 2033–2040.

Verma, D. and Daniell, H. (2007) Chloroplast vector systems for biotechnology applications. *Plant Physiol.* **145**, 1129–1143.

Verma, D., Samson, N.P., Koya, V. and Daniell, H. (2008) A protocol for expression of foreign genes in chloroplasts. *Nat. Protoc.* **3**, 739–758.

Wang, H.H., Yin, W.B. and Hu, Z.M. (2009) Advances in chloroplast engineering. *J. Genet. Genomics*, **36**, 387–398.

Ye, G.N., Daniell, H. and Sanford, J.C. (1990) Optimization of delivery of foreign DNA into higher plant chloroplasts. *Plant Mol. Biol.* **15**, 809–819.

Ye, G.N., Hajdukiewicz, P.T., Broyles, D., Rodriguez, D., Xu, C.W., Nehra, N. and Staub, J.M. (2001) Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. *Plant J.* **25**, 261–270.

Ye, G.N., Colburn, S.M., Xu, C.W., Hajdukiewicz, P.T. and Staub, J.M. (2003) Persistence of unselected transgenic DNA during a plastid transformation and segregation approach to herbicide resistance. *Plant Physiol.* **133**, 402–410.

Zubko, M.K. and Day, A. (1998) Stable albinism induced without mutagenesis: a model for ribosome-free plastid inheritance. *Plant J.* **15**, 265–271.

Zubko, M.K., Zubko, E.I., van Zuilen, K., Meyer, P. and Day, A. (2004) Stable transformation of petunia plastids. *Transgenic Res.* **13**, 523–530.