The chloroplast transformation toolbox: selectable markers and marker removal

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Abstract

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, [...]
Review article
The chloroplast transformation toolbox: selectable markers and marker removal

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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 plasts 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 Costa 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 proplasts, 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 plasts. 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, plasts 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 endosymbiotics 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 focused 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 alfalfa 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 polyplody 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 tabacum* (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 (Swab 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; Swab 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), Swab 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'-adenytransferase 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 (Swab 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 homoplasy. These are not readily achieved for transplastomic shoots isolated using somatic embryogenesis.
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

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.
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 plastid. For chloroplast gene inactivation or site-directed mutagenesis, a portable selectable cassette inserted within or near the gene of interest may be difficult to obtain. This is particularly during selection, so that lines that are homoplasmic for the gene of interest favours co-segregation of the desired mutation with the portable selectable cassette inserted within or near the gene of interest. This is particu-
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<td>Trp analogues</td>
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It has been argued that non-lethal selection is important for successful transformation in tobacco (Swab 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 (Lamtham 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 Prm 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., 1991; Barnes et al., 2005; Michelet 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 the wild-type gene, selection for photoautotrophic growth is then a possible approach for the isolation of true transformants from spontaneous mutants that will not contain transgenic plastid genomes. 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 and Maliga, 1993). 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 combined excision of a chloroplast gene (rbcL) and marker (Kode et al., 2006). The resulting lines were heterothropic 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 and Maliga, 1993). 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 combined excision of a chloroplast gene (rbcL) and marker (Kode et al., 2006). The resulting lines were heterothropic 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.
Markers for chloroplast transformation

Markers for chloroplast transformation

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, with 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; Kanveski and Maliga, 1994; Maliga, 2004). They are dominant and in tobacco give transformation frequencies that are two orders of magnitude higher than with rns 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 heteroplastidic 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′)-VII), 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 Prm 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 plastids 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 diketinotirile (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 (lamtham 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 feed-back 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 AS2 (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 AS2 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 amidotransferase 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 cadA 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 cadA gene effectively prevents callus growth and shoot regeneration and inhibits seedling germination. Negative selection against the cadA gene 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 rm and rps12 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 adaA 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 (lamtham 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 (lamtham 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 (lamtham 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 T0 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.
The frequency of excision of marker genes is raised by increasing the number of direct repeats in the transformation process. Recombination between the two 174-bp direct repeats gave rise to herbicide-resistant plants in the T2 generation. Marker-free tobacco plants obtained illustrate the frequency and timing of excision, which takes place at a higher frequency and gives rise to marker-free T1 seedlings (24% of total seedlings).

Figure 4 The frequency of excision of marker genes is raised by increasing the number of direct repeats in the transformation process. Recombination between the two 174-bp direct repeats gave rise to herbicide-resistant plants in the T2 generation. Marker-free tobacco plants obtained illustrate the frequency and timing of excision, which takes place at a higher frequency and gives rise to marker-free T1 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 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 Maligna, 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).

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., 2000, 2001; Lutz et al., 2006a). These unforeseen events included recombination between the 117 bp *rrm* promoter region of a transgene with the native *rrm* gene. *loxP* was also found to recombine with a recombination hotspot in the promoter region of the *rrs7/*3*rrs12* operon (Hajdukiewicz et al., 2001; Corneille et al., 2003). This is the result of a pseudo *loxP* site (*loxP-rrs12*) that resembles the true *loxP* sequence. An additional pseudo *loxP* site was found in the *psaB* promoter (*loxP-psaB*). 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 homoplasmity might be challenging using this segregation approach.

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 plastome 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 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 aphA6 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 T₀ 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 AS2A, 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.

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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


Markers for chloroplast transformation


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