Chapter 2
Plastid Transformation

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2.1 Introduction

The genome of eukaryotic cells is unevenly distributed and kept in different subcellular compartments. While the vast majority of genetic information is sheltered in the nucleus, small portions of DNA reside in organelles, namely the mitochondria and – in the case of plants – in the plastids. These unique organelles which in their most prominent manifestation are called chloroplasts, developed from cyanobacterial ancestors in a process described by the well accepted endosymbiosis theory (Gould et al. 2008). In brief, a pre-eukaryotic cell must have engulfed and taken up an ancestor of today's cyanobacteria and subsequently formed a close endosymbiotic relationship with the newly developing organelle. Residues of this evolutionary ancestry are still apparent today in certain prokaryotic characteristics retained by the plastids. There is for example the genome organization in operons, as well as the transcription and translation machinery with their 70S ribosomes, to name only a few features which resemble those of today's bacteria. However, during the adaption process which lasted several billion years the plastids lost their autonomy in that they transferred the majority of their genetic information and the capacity for its regulation. Genes were either lost or transferred to the nucleus, accompanied with the assembly of a regulatory network which operates most of the metabolic processes in plastids. What is present in contemporary plastids is a highly reduced genome retaining some integral features like DNA replication and protein biosynthesis. Furthermore, plastids and especially chloroplasts have a unique role in that they provide the primary energy source for the plants via photosynthesis and synthesize important compounds like aromatic amino acids. It has only recently become evident that plastids also have crucial roles in

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plant development and therefore additionally regulate processes in the cellular metabolism. Several reports provide evidence that plastid genes encode functions which reach beyond its borders, for example, chloroplast protein synthesis is mandatory for regular leaf development and its knock-down will result in aberrant phenotype (Ahlert et al. 2003).

Consequently, the genetic manipulation of chloroplasts became a major focus since it provides the option to study the function of this unique organelle in great detail. Also, the application of chloroplast transformation in biotechnology has advanced due to characteristics which make plastids a promising vehicle for the high-level production of recombinant proteins. The most prominent difference to nuclear genes is the mere number of transgenes which could be introduced into a single cell by transformation of the chloroplast genome. Usually, in green tissue every cell contains up to 100 chloroplasts. Every chloroplast itself contains up to 100 identical copies of the circular plastid DNA, organized in nucleoid structures of about ten aggregated copies (Thomas and Rose 1983). In total this makes up to a 10 000 copies of any gene, outnumbering every nuclear gene by far. This is one reason why chloroplast transformation often results in extraordinarily high levels of recombinant protein accumulation.

However, chloroplast transformation makes high demands on vector design, transformation method as well as plant regeneration. This is exemplified by the so far not solved problem to generate fertile transplastomic lines of the model plant *Arabidopsis thaliana* (Sikdar et al. 1998), an example depicting the obstacles of this technique which need to be overcome to gain broad applicability. On a routine basis, so far only tobacco chloroplasts are transformed, and therefore most examples given in this chapter refer to tobacco chloroplast transformation. Nevertheless, great progress has been made in expanding the range of this technique to other plant species. Today, there are reports of successful plastid transformation in about 16 species (Table 2.1) and studies with transplastomics give valuable insight into genetics and biochemistry of this unique organelle.

2.2 Delivery of Transforming DNA to the Chloroplast

Delivery of foreign DNA to the chloroplast requires its transport through several physical barriers: the cell wall, the cytoplasma membrane, and the chloroplast double-membrane system. Since no bacterial or viral pathogen is known which could be utilized for DNA delivery, transgene transmission needs to employ rather rigid physical methods. The most effective and widely used system utilizes microprojectile bombardment with plasmid-coated gold or tungsten particles, the so-called biolistic method, which was first used to transiently transform onion epidermal cells (Klein et al. 1987). Subsequent refinement of the technique eventually enabled the transformation of smaller cell types as well as subcellular targets, like plastids in the unicellular algae *Chlamydomonas* (Boynton et al. 1988) or in tobacco (Svab et al. 1990). Other sophisticated methods have also been developed,

Table 2.1 Plant species for which plastid transformation has been achieved. Common and scientific names are given, with the transgenes integrated so far, as well as the efficiency of the transformation process.

Species	Transgenes integrated	Explants per bombardment (efficiency)	Reference
Tobacco (*Nicotiana tabacum* Petit Havana SM1)	*aadA* + *uidA*[a]	1/1 (100%)	Zoubenko et al. (1994)[a]
Potato (*Solanum tuberosum* FL1607; *S. tuberosum* cv Desiree)	*aadA* + *gfp* *aadA* + *gfp*	3/104 (2.8%) 14/435 (3.2%)	Sidorov et al. (1999) Nguyen et al. (2005)
Tomato (*S. lycopersicum* var. IAC-Santa Clara)	*aadA*	1–3/20 (5–15%)	Ruf et al. (2001)
Petunia (*Petunia hybrida* var. Pink Wave)	*aadA* + *gusA*	3/31 (9.6%)	Zubko et al. (2004)
Soybean (*Glycine max* L. cv "Jack")	*aadA*	11/80 (13.7%)	Dufourmantel et al. (2004)
Lettuce (*Lactuca sativa* L. cv Cisco)	*aadA*/*gfp*	5/85 (5.8%)	Kanamoto et al. (2006)
Lesquerella fendleri	*aadA* + *gfp*	2/51 (3.9%)	Skarjinskaia et al. (2003)
Carrot (*Daucus carota* L. cv Half Long)	*aadA* + *badh*	1/7 (14%)	Kumar et al. (2004a)
Cotton (*Gossypium hirsutum*)	*aphA-6* + *nptII*	1/2.4 (41.6%)	Kumar et al. (2004b)
Poplar (*Populus alba*)	*aadA* + *gfp*	44/120 (36.6%)	Okumura et al. (2006)
Sugar beet (*Beta vulgaris* ssp. *vulgaris*)	*aadA* + *gfp*	3/40 (7.5%)	De Marchis et al. (2008)
Rice (*Oryza sativa japonica*)	*aadA* + *gfp*	2/100 (2%)	Lee et al. (2006)
Cabbage (*Brassica oleracea* L. var. *capita* L.)	*aadA* + *uidA*	3–5/150 (2–3%)	Liu et al. (2007)
Canola (*B. napus*)	*aadA* + *cry1Aa10*	4/1000 (0.4%)	Hou et al. (2003)
Cauliflower (*B. oleracea* var. *botrytis*)	*aadA*	1/5[b]	Nugent et al. (2006)
Arabidopsis thaliana ecotype RLD	*aadA*	2/201 (0.9%)	Sikdar et al. (1998)

[a]For tobacco, numerous more transformations have been reported
[b]Transformation was achieved by PEG-mediated transformation

like polyethylene glycol (PEG)-mediated transformation of protoplasts (Golds et al. 1993) or even the direct injection of DNA into the organelle via a femtoliter syringe (Knoblauch et al. 1999). Although plastid transformation with PEG requires some experience in the enzymatic digestion of the cell wall and the treatment of protoplasts as well as the regeneration of plants, it can be basically performed with standard laboratory equipment. Micromanipulation of cells on the other hand requires specialized equipment, which is limiting for its use; and so far no reports have shown the successful regeneration of transplastomic plants from this particular gene transfer method. However, the most widely and successfully used method is the biolistic transfer of DNA, depicted by the successful transformation of the plastids of numerous plant species (Table 2.1).

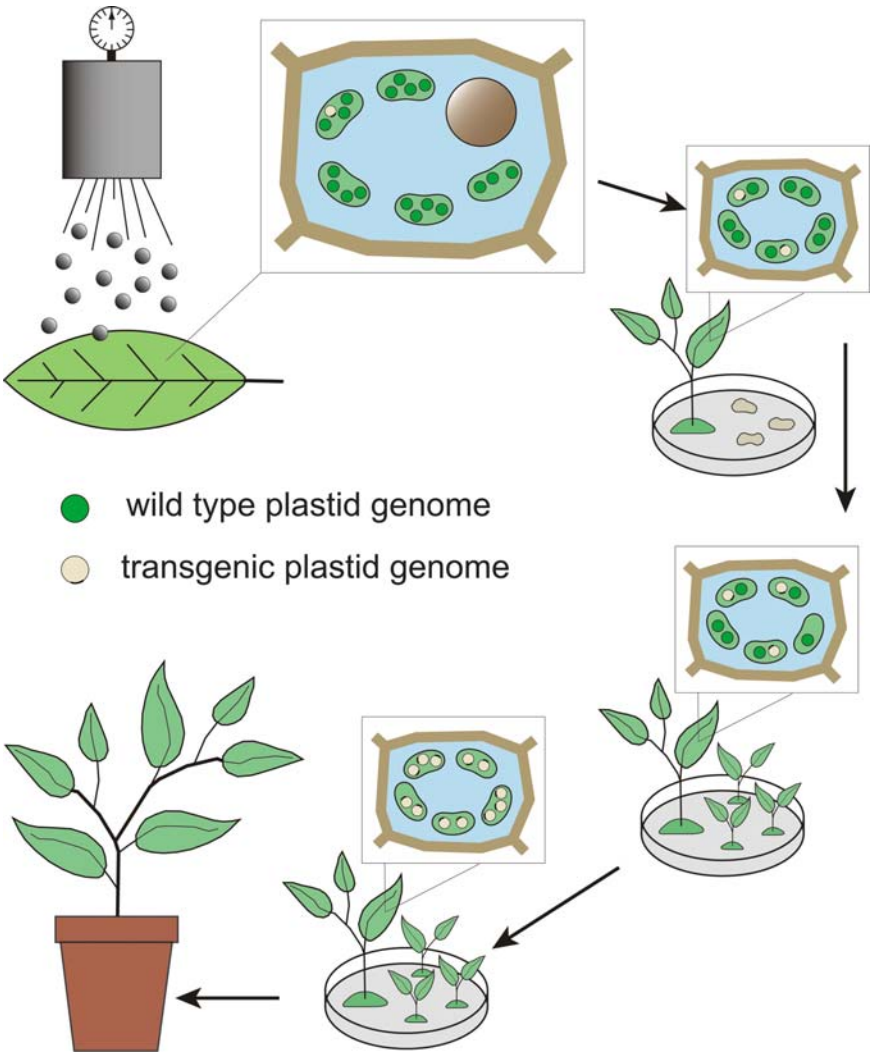

Fig. 2.1 Schematic drawing of the plastid transformation and regeneration process. An explant, usually a leaf, is bombarded with DNA-coated tungsten or gold particles. When the DNA is delivered to one chloroplast, integration of the transgene takes place, generating a heteroplastomic cell in which a small number of plastid genomes are transgenic (open circles). Subsequent differentiation and shoot regeneration from this cell results in a heteroplastomic plantlet. To obtain a homoplastomic transgenic plant requires several cycles of regeneration under selection

Once the transgene DNA has been delivered to the chloroplast (Fig. 2.1), stable integration via homologous recombination has to take place (see below) to generate a stable transgenic trait which will be passed on after plastid division to its descendants. Every chloroplast harbors up to a hundred copies of its genome, grouped in nucleoids representing aggregates of 7–10 copies. Since cells can contain up to

100 chloroplasts, a single integration event creates a transplastomic cell in which only a minority of genomes is altered, the so-called heteroplastomic state. For the generation of a stable transplastomic plant, wild-type plastid genomes have to be winnowed. As the sorting of plastid DNA during cell division in shoot-regeneration is a stochastic process a small percentage of altered homoplastomic plants can be generated in the absence of selection pressure (5.6%; Lutz and Maliga 2008). To increase the efficiency of transformation routinely, a homoplastomic state of the engineered plants is reached by successive regeneration under strong selective pressure with an appropriate antibiotic. It is estimated that it takes between 20 and 30 cell divisions to deplete the undesired wild-type chloroplast genomes (Maliga 2004; Verma and Daniell 2007). Since this number could not be reached in a single plant regeneration process, explants have to go through several cycles of regeneration under selection (Fig. 2.1). So far a given plants ability to regenerate from fully differentiated tissue is the biggest obstacle for applying the plastid transformation to a large number of plant species. Tobacco is by far the best analyzed system regarding plastid transformation, and therefore most experiments referred to in this section are made in tobacco.

2.3 Vector Design

2.3.1 Flanking Regions

Agrobacterium-mediated transformation utilizes universal vector systems (Lee and Gelvin 2008; see also Chapter 1) in which the transgene expression cassette is flanked by two rather short sequence stretches, termed left border (LB) and right border (RB). These sequences facilitate almost random insertion of the transgene cassette into the host genome, resulting in multiple individual lines differing in site and numbers of transgene integration. In absolutely contrast, insertion of foreign DNA into the chloroplast genome relies on targeted integration of transgenes by homologous recombination, facilitated by a bacterial recombination system inherited from the plastids cyanobacterial ancestors (Cerutti et al. 1992). Hence, a transgene could be targeted to virtually any site in the chloroplast genome by designing the flanking regions according to the desired location. This is not only a big advantage for the positioning of expression cassettes to defined locations but also enables the targeted inactivation of plastid genes for functional studies and gene knock-outs. For the former, preference is naturally given to intergenic regions to circumvent deleterious effects and interference with endogenous gene expression. For gene knock-out, the targeted sequence is mutated in vitro and reinserted into the plastome. In the case of tobacco, numerous studies describe the targeted knock-out of plastidal genes for functional studies (reviewed by Maliga 2004). Additionally, a total of 13 sites on the plastome has been utilized for the integration of an expression cassette (Fig. 2.2), demonstrating that modification and integration

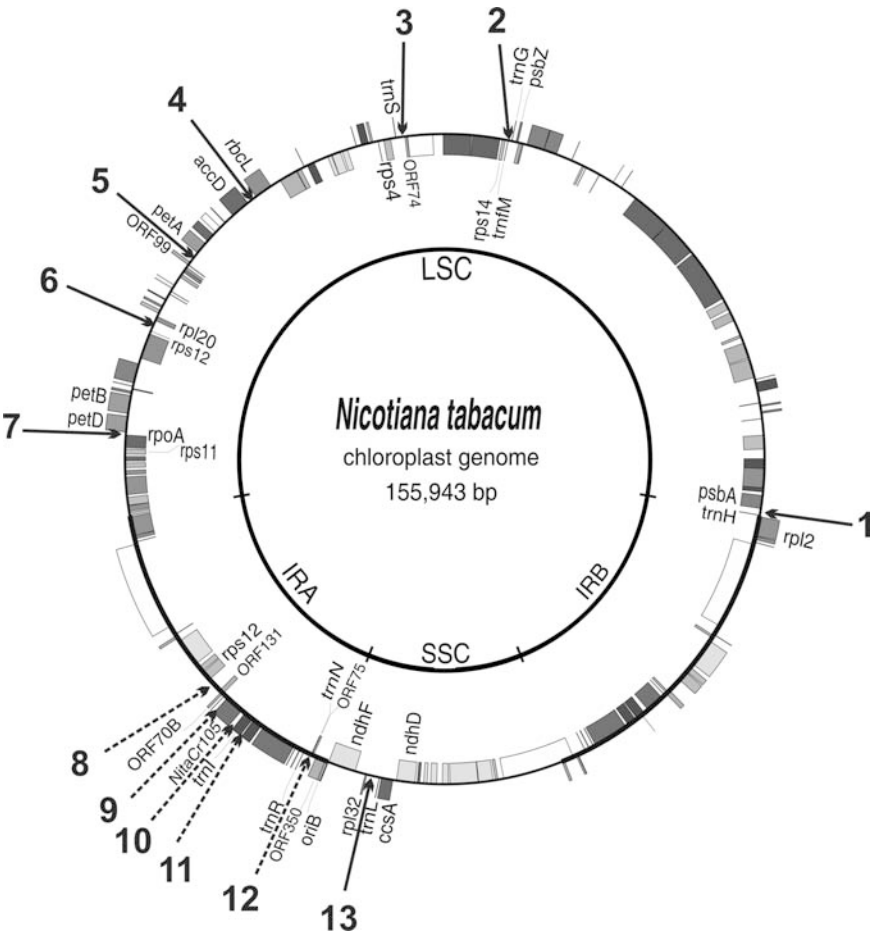

Fig. 2.2 Graphic map of the *Nicotiana tabacum* plastid genome (GeneBank accession number NC_001879), made with the web-based program OGDRAW (Lohse et al. 2007). Genes on the outside of the circle are transcribed counter-clockwise, those on the inside clockwise. *IRA* Inverted repeat A, *IRB* inverted repeat B, *LSC* large single copy region, *SSC* single copy region. *Numbered arrows* Transgene integration sites. *Dashed arrows* (numbers *8–13*) Site of integration into the inverted repeat region; therefore integration sites are in duplicate. First published reports are given: *1* Carrer and Maliga (1995), *2* Bock and Maliga (1995), *3* Huang et al. (2002), *4* Svab and Maliga (1993), *5* Huang et al. (2002), *6* Kuroda and Maliga (2003), *7* Suzuki and Maliga (2000) and Klaus et al. (2003), *8* Zoubenko et al. (1994), *9* Staub and Maliga (1993), *10* Svab et al. (1990), *11* Muhlbauer et al. (2002), *12* Huang et al. (2002) and Zou et al. (2003), *13* Koop et al. (1996) and Eibl et al. (1999)

of heterologous genes can be performed at many given sites in the circular plastid genome.

One of the unique features of the chloroplast genome is the presence of two large inverted repeat regions (IRA and IRB in Fig. 2.2). Integration of the transgene into this particular region leads under selection to a doubling of the gene by a process

called copy correction. Especially, sites in the *rrn* operon have been frequently chosen for transgene integration and gene expression from these sites has proved to be high in many cases (Verma et al. 2008).

To facilitate efficient recombination, flanking regions of about 1–2 kb endogenous DNA should frame the sequence to be inserted. The question whether a transformation vector for a certain plant species requires strain-specific flanking regions was clearly negated by Lutz et al. (2007). This is due to the sufficiently high sequence homology of plastid genomes between species to facilitate homologous recombination. In fact, vectors designed for transgene integration into the tobacco plastome could be also used for transformation of tomato (Ruf et al. 2001; Chapter 25), potato (Sidorov et al. 1999; Chapter 20), and petunia (Zubko et al. 2004; Chapter 19). Two recent papers describe convenient vector systems designed for chloroplast transformation (Lutz et al. 2007; Verma and Daniell 2007).

2.3.2 Promoters and UTRs

For the expression of heterologous genes, the choice of promoters and regulatory sequences is highly important. In general, chloroplasts mainly utilize a prokaryotic transcription and translation machinery, a heritage from their cyanobacterial ancestors. Gene organization in operons, an eubacterium-type RNA polymerase as well as sigma-like factors, and a specific codon-usage in the open reading frames are highly similar to those found in bacteria. However, during evolution novel mechanisms of gene organization and regulation of expression also evolved in plastids. Processes like intron splicing and RNA editing can be found in chloroplasts which are absent in bacteria. Aiming for the high-level expression of a given gene, regulatory sequences are required which provide efficient transcription, translation, and RNA stability. The strongest promoter described so far is the σ^{70}-type promoter of the ribosomal RNA operon (*Prrn*; Svab and Maliga 1993; Kuroda and Maliga 2001a, b). In the majority of vectors used to date, this particular promoter is used to drive the expression of the marker gene *aadA* (see below) to provide sufficient expression for selection of transformed cells. Another promoter in use is the endogenous *psbA* promoter (driving expression of the abundant D1-protein; Zoubenko et al. 1994).

In plastids there is also a high degree of translational control of gene expression which is in clear contrast to prokaryotes. Therefore, the 5′-UTRs and 5′-regulatory sequences have proven indispensable for RNA stability and efficient translation initiation. While in Eubacteria virtually all mRNAs contain a Shine–Delgarno sequence for accurate translation initiation, only 40% of chloroplast mRNAs contain such sequences, indicating that alternative pathways of regulation exist (Hager and Bock 2000). Numerous studies have investigated in detail the effect of swapping different regulatory sequences and determined the consequence on protein accumulation. For example, Eibl et al. (1999) could show that such variations in 5'-UTRs result in up to 100-fold differences in protein accumulation in the case

of the reporter gene *uidA* (encoding beta-glucoronidase). Not only the 5′-UTR but also the first codons of the open reading frame (the so-called "downstream box") seems to contribute to translation efficiency (Kuroda and Maliga 2001a, b), demonstrating that a high variability of expression levels could be expected for any given sequence and expression cassette. The highest expression level reported so far was over 70% of the total soluble protein (TSP) of a phage derived lytic protein driven by the *rrn* promoter fused to the *Escherichia coli* phage T7 gene 10 (T7g10) 5′-untranslated region (Melanie Oey 2008). This extraordinarily high content of recombinant protein almost exhausted the protein biosynthesis capacity of the chloroplasts and resulted in plants with impaired growth. Although extraordinarily high amounts of protein are often aspired, several proteins have been reported to be toxic to the plastid at elevated levels (Hennig et al. 2007), and therefore certain threshold levels might exist which should be taken into account to preserve the plants' viability.

2.4 Transgene Stacking and Control of Gene Expression

A demanding task in the generation of transgenic plants is the option of simultaneously introducing two or more genes into an organism. Conventional approaches require the combination of separate expression cassettes each containing a promoter and terminator region framing the gene of interest. In contrast, plastids are thought to offer an unique option of combining multiple ORFs under the control of one promoter, yielding a polycistronic transcript from which translation can be initiated independently (Staub and Maliga 1995). However, except for a few examples like the *cry2Aa2* operon from *Bacillus thuringiensis* (De Cosa et al. 2001; Quesada-Vargas et al. 2005), this technique has not been utilized for the simultaneous production of two or more recombinant proteins so far. This is probably due to the hitherto unpredictable secondary structure interactions in polycistronic transcripts, which determine the translatability and processing into monocistronic mRNAs and subsequently result in poor protein accumulation. Although the similarities between transcription and translation in bacteria and in plastids are striking, one cannot generally extrapolate results obtained in bacteria to plastids. For instance, an operon encoding for hemoglobin α- and β-subunits, which worked well in *E. coli*, did not lead to detectable expression when integrated into plastids (Magee et al. 2004). A recent study identified a so-called intercistronic expression element (IEE), a short sequence that mediates the cleavage of a polycistronic precursor into stable monocistronic transcripts (Zhou et al. 2007). It will be interesting to see whether this novel element leads to concerted high-level expression of recombinant proteins.

Another challenge is the regulation of gene expression in plastids, as it is state of the art in *E. coli*. It would be highly desirable to avoid deleterious or toxic effects of recombinant proteins on plant metabolism by initiating expression at will. Most promoters used so far are more or less constitutive (like the P*rrn*) or regulated by

factors which could be hardly used for targeted expression initiation. In photosynthetically active chloroplasts especially the 5′-UTRs of several transcripts contribute to the regulation of translation. It has been shown that light regulates the translation of the *psbA* mRNA (Kim and Mullet 1994) while RNA levels are kept relatively constant (Shiina et al. 1998). However, since light cannot be withheld until the desired transgene expression needs to be initiated, it would be highly advantageous to have instead an inducible system at hand which relays on chemical or other physiological triggers. A sophisticated approach is to put the transgene under the control of the phage T7 promoter, which is per se not active in plastids. Expression can be only initiated by the appropriate T7 RNA polymerase, which needs to be introduced by genetic crossing with a plant line carrying the gene in the nucleus and fused to a plastid signal peptide (McBride et al. 1994). To add a regulatory element to this system, other studies used inducible nuclear promoters to control expression of the nuclear gene, e.g. the salicylic acid-inducible *PR-1a* promoter from tobacco (Magee et al. 2004) or an ethanol inducible promoter (Lossl et al. 2005). The disadvantage of this particular approach is that two subsequent transformations of different cellular compartments or genetic crossing of different transformants are necessary to obtain the final plant. Also the *E.coli lac* control system has been adopted for plastid expression. Therefore, the *lacI* repressor needed to be co-expressed together with the heterologous gene (*gfp*) under control of a modified *rrn*/T7g10 promoter inside the plastids (Muhlbauer and Koop 2005). Spraying of plants with isopropyl thiogalactoside (IPTG) indeed induced GFP-formation, but it needs to be established whether this method is applicable on a large scale.

2.5 Selection

2.5.1 Antibiotic Resistance Markers

For the selection of plastid transformants, aminoglycoside antibiotics have proven highly useful, and especially spectinomycin has become indispensable as a selective marker. Its mode of action is plastid-specific as it binds to the prokaryotic-type plastid ribosomes and inhibits protein synthesis. Mutations in the 16S rRNA, one of the target sites of spectinomycin, confer resistance to the antibiotic and early transformation vectors contained such mutant genes (Svab et al. 1990). Since the mutant form of an endogenous gene is recessive until the homoplasmic stage is reached, transformation efficiency with such marker genes is low. The use of a gene encoding an antibiotic detoxifying protein, namely the aminoglycoside-3″-adenyltransferase (AAD, encoded by the *aadA* gene) as a marker greatly expedites the development of transplastomic plants (Svab and Maliga 1993).

Several more resistance markers have been tested for their applicability in plastid transformation, especially as it was observed that spontaneous spectinomycin resistant lines were formed under strong selection. For kanamycin, no spontaneous

resistance in higher plants was reported and thus this particular marker was also adapted for selection of plastid transformants. First successful attempts using the *neo* gene (encoding for neomycin phosphotransferase II, NPTII) generated transplastomic tobacco, but at a lower efficiency than with *aadA* (Carrer et al. 1993). Koop and co-workers used the aminoglycoside phosphotransferase gene (*aphA*-6) gene from *Acinetobacter baumannii*, which yielded a much higher transformation efficiency (Huang et al. 2002). Both selection markers are in use, even in combination (Kumar et al. 2004a, b) but have not displaced the *aadA* gene as the most frequently used antibiotic resistance marker.

2.5.2 Other Selection Markers

Another approach is to insert a gene which confers resistance to a toxic compound, for example herbicides. This approach is widely used for nuclear transformation (see Chapters 3, 9), and it is thought to be also functional in plastid transformation. Resistance against glyphosate could be obtained via 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; Ye et al. 2001), and integration of the *bar* gene made tobacco plants resistant to phosphinothricin (Lutz et al. 2001). However, all expression cassettes also contained the *aadA* gene for initial selection on spectinomycin and herbicide resistance was only observed when nearly homoplastomic plants were obtained.

Another novel selective agent was used for plastid transformation, namely betaine aldehyde, for which a detoxifying gene was linked to the heterologous gene. The introduction of the spinach gene encoding for betaine aldehyde dehydrogenease (BADH) into tobacco chloroplasts proved to be an useful marker (Daniell et al. 2001). Since this marker gene is of plant origin, its use is thought to obviate the concerns about the use of antibiotic resistance in plant genetic engineering. Although this approach is highly promising there is still a lack of studies confirming the broad applicability of the BADH gene as a marker in plastid transformation (Maliga 2004). For the sake of efficient and reliable generation of transplastomic plants it seems to be wise to use antibiotic resistance markers and – after establishment of the transgenic plant line – remove the gene by marker gene excision.

2.6 Marker Gene Excision

In principle, any marker gene becomes dispensable as soon as the homoplastomic stage is achieved. Sophisticated protocols to subsequently remove the marker flank the sequence to be removed by two directly oriented recombinase target sites. Examples include the 34-bp *loxP* sites, which are recognized by the Cre recombinase derived from the P1 bacteriophage (Corneille et al. 2001; Hajdukiewicz et al.

2001), and the *attP* (215 bp) and *attB* (54 bp) sites, recognized by the phiC31 phage integrase Int (Lutz et al. 2004). As long as no integrase is present, the genomes harboring those sites are stable. To initiate excision, a second, nuclear transformation step has to be performed. Using a construct in which the recombinase is genetically fused to a chloroplast transit peptide will target the protein to all chloroplasts and initiate site specific excision of the unwanted genes. Further backcrosses are required where also the nuclear transgene is removed to obtain a marker-free plant line. Several more methods have been described for marker gene removal, and an excellent overview is given by Lutz and Maliga (2007).

It needs to be emphasized that, in plastids, homologous recombination takes place between virtually all directly oriented sequences of sufficient length. This effect can also be used for marker gene excision (Iamtham and Day 2000), but more often it is the cause of unwanted rearrangement and transgene loss in transformation experiments (Svab and Maliga 1993). Initially this effect was observed when the integrated expression cassette contained two homologous sequences oriented in the same direction, e.g. duplicate promoter or terminator sequences, or when homologous sequences integrated were in the same direction as endogenous plastid genome sequences. When the choice of regulatory elements to be used is limited, it might be advisable to utilize interspecies regulatory elements to avoid unwanted recombination products (Nadai et al. 2008).

2.7 Analysis

Initial plastid transformation and subsequent regeneration of transplastomic plants need to be carefully monitored by different techniques. While presence of the transgene can be easily checked by PCR, no precise statement about the integration site can be made with gene-specific primers. To rule out an accidental insertion of the expression cassette into the nuclear genome, a PCR with a primer combination bridging the transgene/genome border is advisable. Differentiation between the homoplastomic and heteroplastomic states can be made by restriction fragment length polymorphism (RFLP) analysis. Restriction digestion of plastid genomes eventually generates fragments of variable length, regarding the integration of the transgene, and therefore resulting in a specific pattern on a subsequent DNA blot hybridized with a specific probe. The gradually disappearance of the wild-type signal and attainment of the homoplastomic condition can be monitored with this technique. However, the occurrence of promiscuous plastid DNA in the nucleus sometimes feigns a heteroplastomic state and generates a need for isolating chloroplast DNA prior to analysis (Ruf et al. 2000). An unambiguous test for plastid transformation is testing for maternal inheritance of the resistance trait. Transgenic plants pollinated with wild-type pollen generate uniformly green seedlings on a selective medium only if they are homoplastomic.

Testing the gene expression usually gives highly heterogeneous results, depending on the expression cassette and the gene itself. Accumulation rates inside the

chloroplast also greatly depend on the stability of the given protein to proteolytic degradation (Birch-Machin et al. 2004).

Once a transplastomic line has been established there is in principle no need for screening numerous other lines, as would be necessary for *Agrobacterium*-mediated nuclear transformation. This is due to the targeted integration of the transgene, resulting in uniformly modified plant lines.

2.8 Conclusions

During the past years tremendous progress has been made in developing and improving plastid transformation techniques and in expanding the methodology to various plant species. Although the method is time-consuming and tedious, it can be established without great effort. Especially for the production of recombinant proteins, plastid transformation has become a valuable tool, notably due to the enormous rate of protein accumulation reported. The next years will show whether this particular technique can be applied to more crop plant species as a standard method.

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