Regeneration and plastid transformation approaches in *Arabidopsis thaliana* and Rapid-Cycling *Brassica rapa*

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1 Zusammenfassung

Plastidentransformation ist eine wertvolle Methode in der Biotechnologie, weil sie hohe Expressionsraten rekombinanter Proteine ermöglicht und weil die Transgene nicht durch Pollen verbreitet werden können. In der Grundlagenforschung können die Funktion und Regulation von plastidären Genen mit Hilfe der Plastidentransformation näher untersucht werden.
Diese Studie konzentriert sich auf die drei wichtigsten Schritte bei der Plastidentransformation in *Arabidopsis thaliana* und in Rapid-Cycling *Brassica rapa* (RCBr); die Etablierung eines Regenerationsprotokolls, die Konstruktion von artspezifischen Vektoren und die Verwendung unterschiedlicher Transformationsprotokolle.

Um die Erzeugung von fertilen Pflanzen aus transformierten Zellen zu ermöglichen, wurde zuerst ein zuverlässiges Regenerationsprotokoll etabliert. Fertile Pflanzen von *Arabidopsis thaliana* wurden mit hoher Regenerationsrate aus Kotyledonen, Kalli von Samen und Protoplasten regeneriert. In RCBr konnten fertile Pflanzen aus Gewebekulturen von Hypokotylen regeneriert werden.

Für die Übertragung von Genen in das Genom der Plastiden wurden geeignete Vektoren mit verschiedenen selektiven und visuellen Markern konstruiert. Diese sorgen für eine ortsspezifische Integration der gewünschten Sequenzen und ermöglichen die Selektion der transformierten Zelllinien. In dieser Studie wurden artspezifische Vektoren für *Nicotiana tabacum*, *Arabidopsis thaliana* und RCBr konstruiert und kloniert, die Aminoglykosid-Resistenz-Marker wie das *aadA*-Gen (Resistenz gegen Spectinomycin und Streptomycin), das *nptII*- oder das *aphA6*-Gen (Resistenz gegen Kanamycin) enthielten. Um mit den Herbiziden Phosphinothricin oder Bialaphos selektieren zu können, wurde das *bar*-Gen eingeführt. Darüber hinaus wurden Fluoreszenz-Marker wie GFP, DsRed und AmCyan als visuelle Marker hergenommen.

Nach der Etablierung eines Regenerationsprotokolls und der Konstruktion spezifischer Vektoren wurden zwei unterschiedliche Protokolle für die Plastidentransformation, die Particle Gun- oder die PEG (Polyethylenglykol)-

vermittelte Transformationsmethode, angewandt. Allerdings konnten bisher keine transformierten Pflanzen von *Arabidopsis thaliana* und RCBr erhalten werden. Dass das Transformationsprotokoll grundsätzlich funktioniert, konnte durch die Verwendung von *Nicotiana tabacum* gezeigt werden. Plastiden von *Nicotiana tabacum* wurden erfolgreich mit beiden Transformationsmethoden transformiert, und AmCyan konnte als neuer visueller Marker etabliert werden. Bei diesen Experimenten konnten fertile, homoplasmatische Tabakpflanzen erhalten werden, was durch Southern-Blot-Analyse und reziproke Kreuzungen bestätigt wurde. Somit konnte die Funktionalität der verwendeten transgenen Expressions-kassetten eindeutig nachgewiesen werden.

2 Summary

Plastid transformation is a well-established tool for biotechnology as it allows high expression levels of proteins and as it provides biological transgene containment because of maternal inheritance of cytoplasmic genes in most crops. In basic research the function and regulation of plastid encoded genes can be further studied with the help of plastid transformation.

This study is focused on the three most critical steps to achieve plastid transformation in *Arabidopsis thaliana* and Rapid-Cycling *Brassica rapa* (RCBr), the establishment of a regeneration protocol, the construction of species specific vectors and the use of different transformation protocols.

First, to allow the generation of fertile plants out of transformed cells, a reliable regeneration protocol was established. Fertile plants of *Arabidopsis thaliana* were obtained with high regeneration efficiencies from cotyledons, seed derived calli and protoplasts. In RCBr, fertile plants could be regenerated from tissue culture of hypocotyls.

For introduction of genes into plastids, appropriate vectors with different selection and visual markers were constructed. These ensure site specific integration of desired sequences and enable selection of transformed cell lines. In this study, species specific vectors for *Nicotiana tabacum*, *Arabidopsis thaliana* and RCBr were designed and cloned, using aminoglycoside resistance markers such as the *aadA* gene (confers resistance to spectinomycin and streptinomycin), the *nptII* or the *aphA6* gene (conferring resistance to kanamycin). To allow the selection through the herbicides phosphinothricin or bialaphos, the *bar* gene was employed. In addition, fluorescence markers such as GFP, DsRed and AmCyan were taken as visual markers.

After the establishment of a regeneration protocol and the construction of specific vectors, two different protocols for plastid transformation, the particle gun- or PEG (Polyethylene glycol)-mediated transformation method, were applied. However, no transformed plant in *Arabidopsis thaliana* and RCBr could be

recovered so far. That the transformation protocol does work in principal could be shown by the use of *Nicotiana tabacum*. Plastids of *Nicotiana tabacum* were successfully transformed using both transformation methods and AmCyan as new visual marker. Out of these experiments, fertile homoplasmic tobacco plants could be obtained as was shown by Southern blot analysis and reciprocal crosses. Thus, the functionality of the used transgene expression cassettes was clearly proven.

3 Introduction

3.1 Plastids

Plastids are the defining feature in plants and algae. According to the endosymbiosis theory, plastids were free-living cyanobacteria over one billion years ago, before becoming endosymbionts.

Plastids are double-membrane organelles developed from undifferentiated proplastids in dividing meristematic cells (Whatley, 1978). Proplastids differentiate into several plastid types like chloroplasts (chlorophyll), chromoplasts (carotenoids), amyloplasts (starch), elaioplasts (lipids), leucoplasts (white plastids), etioplasts (plastids in the complete absence of light), and gerontoplasts (plastids found in senescing) (Keskitalo *et al.*, 2005). All plastid types can be interconverted, except the proplastids (Wise, 2006).

The chloroplasts are the most common form of plastids in plants (Figure 3.1). They are able to convert light into chemical energy through a process called photosynthesis. Chloroplasts are not just involved in photosynthesis, but they also play an essential role in plant metabolism (Neuhaus and Emes, 2000). Plastids produce fatty acids, aromatic and non-aromatic amino acids, purine and pyrimidine bases, isoprenoids (carotenoids and sterols) and tetrapyrroles (chlorophyll) (Neuhaus and Emes, 2000; Lopez-Juez and Pyke, 2005; Waters and Langdale, 2009).

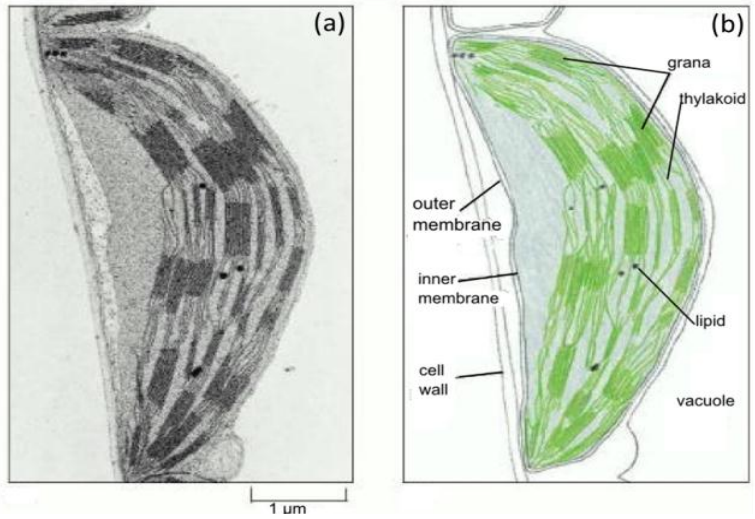


Figure 3.1 Chloroplast. **(a)** Electron micrograph of a chloroplast in a leaf of grass, showing the highly folded system of internal membranes containing the chlorophyll molecules by which light is absorbed. **(b)** An illustration of **(a)** Modified from Alberts *et al.*, 2002.

3.1.1 Plastid division

Plastid proliferation occurs by binary fission of pre-existing plastids. Plastids can only be transmitted to daughter cells through cell division; *de novo* synthesis of plastids is not known (Maple and Møller, 2007).

Plastid division starts with the formation of a ring like structure in the middle of the plastid, known as plastid division (PD) ring. This structure can be easily observed with the light microscope and two PD rings could be observed using transmission electron microscopy (TEM) (Figure 3.2). One PD ring is located on the stromal face of the chloroplast's inner membrane (bacterial origin) and the other one is located on the cytosolic face of the outer membrane (cytosolic division machinery). A number of proteins involved in plastid division have been identified and characterized, for example FtsZ, MinD, MinE and ARTEMIS. However, the way of how all these proteins interact with each other to coordinate plastid division remains unknown (Fulgosi *et al.*, 2002; López-Juez, 2007; Maple and Møller, 2007).

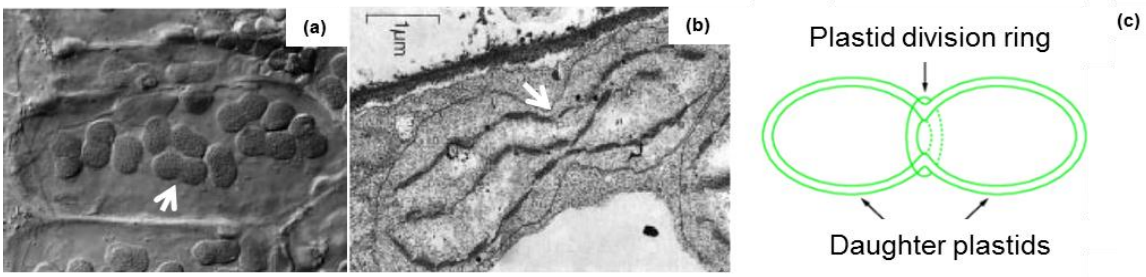


Figure 3.2 Dividing plastids **(a)** Nomarski optic micrographs showing accumulation of shaped pre-division plastids caused by keeping spinach leaf discs under low light intensity for 5 d. **(b)** Transmission electron micrograph showing constriction prior to division (scale bar = 1 µm). **(c)** Diagram of constricting doublet ring visible as densely stained areas inside and outside double plastid membrane. From: http://plantsinaction.science.uq.edu.au

3.1.2 Plastid inheritance

The inheritance of genes from organelles like plastids and mitochondria in plants, unlike the nucleus, does not follow Mendel´s laws (Birky, 2001) (Figure 3.3). Plastid inheritance can be studied in naturally variegated plants; initial evidences of an extra nuclear non-Mendelian inheritance of variegation were described in *Mirabilis jalapa* (Correns, 1909) and *Pelargonium zonale* (Baur, 1909).

The plastid inheritance theory and the random sorting out of plastids was proposed by Erwin Baur using the term "chromatophores" for plastids (Baur, 1909; Hagemann, 2000; 2010).

Plastid inheritance in higher plants can be either uniparental or biparental. While paternal plastid inheritance can be found in conifers (Szmidt *et al.*, 1987), biparental inheritance could be described only in some species like *Pelargonium*, *Oenothera* and *Hypericum* (Hagemann and Schröder, 1989).

In angiosperms, plastids are mainly maternally inherited (Figure 3.3), including *Beta*, *Mirabilis, Nicotiana*, *Zea*, *Arabidopsis thaliana* and many others. In *Arabidopsis thaliana* and *Nicotiana tabacum* a low frequency of paternal plastids in the seeds progeny have been reported (Hagemann and Schröder, 1989; Huang *et al.*, 2003; Azhagiri and Maliga, 2007).

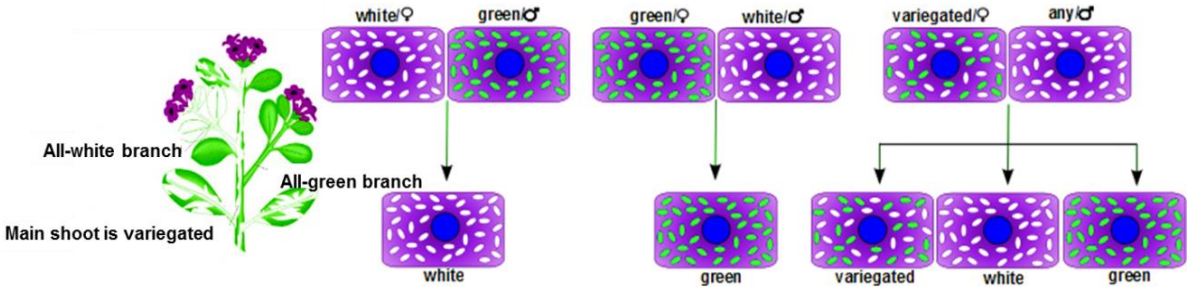


Figure 3.3 Maternal inheritance of plastids. In reciprocal crosses between green and white plants (or branches) of *Mirabilis jalapa,* the plastids of the mother plant determines the type of progeny. The cross white (female) x green (male) gives only white Fl-seedlings; the cross green (female) x white (male) gives only green F1- seedlings. A variegated mother plant (crossed with a green or white mate) can produce variegated, white and green seedling. Modified from Griffiths *et al.*, 1999.

Using different cytological and molecular methods, the routes for maternal inheritance could be analyzed. For example: physical exclusion of the plastids during pollen mitosis, elimination of plastids by formation of enucleated cytoplasmic bodies and autophagic degradation of male plastids in different stages (Nagata, 2010).

The sorting out of plastomes is due to a segregation process upon organelle division as well as random organelle segregation upon cell division. The first model of plastid inheritance was developed using the plant *Epilobium*. This inheritance model of partitioning uses the mathematical model of sampling

without replacement and the hypergeometric probability distribution (Michaelis, 1962a). Although the model is not exactly applicable, it helped to predict that vegetative segregation can be completed within one plant generation depending on the number of observed proplastids in the plant embryo cell (Michaelis, 1962b; Birky, 2001).

3.1.3 Plastid genome

The genetic information in plants is distributed among three cellular compartments: the nucleus, the mitochondria and the plastids. The plastids have their own genome, known as plastome. Plastomes from land plants are circular molecules of double stranded DNA, between 120 and 180 kb in size (Sugiura, 2003). However, different evidences described structural plasticity of the plastome in *Arabidopsis thaliana* and *Nicotiana tabacum,* which are characterized by the presence of circular, linear, bubble D/loop, lasso-like and unclassified plastome structures (Bendich and Smith, 1990; Lilly *et al.*, 2001; Bendich, 2004; Scharff and Koop, 2006).

Interestingly, plastids are highly polyploid and their plastomes are organized as nucleoids which are attached to the inner membrane (Sato *et al.*, 1997). A single leaf cell may contain hundreds of chloroplasts, each containing nucleoids with several copies of the plastome (Thomas and Rose, 1983). In average the number of copies of plastomes per leaf cell ranges from 1000 to 1700 in *Arabidopsis thaliana* and reaches up to 50,000 in wheat (Bendich, 1987; Zoschke *et al.*, 2007). Generally, the plastome of higher plants is conserved in size, organization and sequence. Plastome contains an inverted repeat region A and B (IRA and IRB respectively) and two single unique regions known as small single copy (SSC) and large single copy (LSC) regions (Sato *et al.*, 1999; Sugiura, 2003). Exceptions to this rule are some legumes and conifers (Wakasugi *et al.*, 2001). During the last 15 years, more than 130 complete plastomes have been sequenced and deposited in the GenBank Organelle Genome Resource (Yang *et al.*, 2010).

Chloroplast DNA is replicated transcribed and translated independently within the cell. However, chloroplasts strongly depend on imported proteins that are

encoded in the nucleus and imported into the organelle. For *Arabidopsis thaliana* it has been proposed that the nucleus encodes for about 2100 chloroplast proteins. In contrast, the whole chloroplast genome encodes for 117 proteins as well as tRNAs and rRNAs (Richly and Leister, 2004; López-Juez, 2007).

3.1.4 Plastid gene expression

Chloroplast gene organization, transcription and translation is similar to that of eubacteria. Chloroplast genes are organized mainly in operons and can be expressed as polycistronic units (Sugiura, 1992).

Transcription in plastids is mainly executed by two or three DNA-dependent RNA polymerases. The plastid genome encodes for one RNA polymerase (PEP) consisting of four core subunits *rpoA*, *rpoB*, *rpoC1* and *rpoC2*. Interestingly, PEP recognizes similar promoters to the *E. coli* sigma^{70} –type, including the -10 (TATAAAT) and the -35 (TTGACA) consensus region (Hager and Bock, 2000). Several RNA polymerases (NEP) like RPOTp and RPOTpm are imported from the nucleus into plastids (Krause *et al.*, 2000; Zerges, 2000; Courtois *et al.*, 2007).

Once a gene or polycistron has been transcribed, it may be modified post-transcriptionally, including RNA editing (e.g. base change from C to U) (Bock, 1998), RNA splicing (Bock and Maliga, 1995), 5´and 3´-end maturation by ribonucleases (Monde *et al.*, 2000b) and cleavage of the polycistronic RNA chains. Different evidence showed that the 5´and 3´untranslated region (UTRs) of plastid RNA´s are important for the stability of mRNA (Eibl, 1999; Monde *et al.*, 2000a; Zou *et al.*, 2003).

In the same way as in bacteria, chloroplast translation utilizes 70S ribosomes which do not require 5´caps or 3´poly (A) tails. However, the Shine-Dalgarno (SD)-like sequence required for ribosomal recognition and translation in bacteria, is not found in many chloroplasts´ mRNAs (Sugiura *et al.*, 1998).

The regulatory mechanisms of gene expression in plastids at the translational and posttranslational level remain unknown. Recently, Apel *et al.*, suggested that the major determinants for the protein stability in plastids are located in the N-

terminal region. Using mutated N-terminal regions they showed that a GFP-protein harboring glutamate or methionine in the second position was strongly accumulated in plastids (Apel *et al.*, 2010).

In summary, the level of protein expression in plastids not only depends on the transcription level and mRNA-stability, it also depends on protein stability.

3.2 Genetic transformation of plastids in higher plants

Due to the high level of gene expression, plastids are an attractive compartment for the expression of recombinant proteins. Recently, the expression level of a phage-lytic protein in chloroplasts reached 70 % of the plant total soluble protein fraction (Oey *et al.*, 2008). Another advantage is the expression of several genes in a single operon in chloroplasts (Lossl *et al.*, 2003; Quesada-Vargas *et al.*, 2005). Additionally, gene silencing and position effects on transgenes cannot be observed in plastids. From a biosafety perspective, plastid transformation significantly increases transgene containment because plastids are maternally inherited in most plants (Figure 3.3) (Bogorad, 2000; Maliga, 2003; Hagemann, 2010). Taken all together, these are the major advantages of this technology.

Stable plastid transformation was first achieved in the alga *Chlamydomonas reinhardtii* (Boynton *et al.*, 1988; Blowers *et al.*, 1989). Almost 20 years ago, it was also possible to introduce DNA into tobacco chloroplasts for the first time through the particle gun method (Svab *et al.*, 1990). Currently, plastid transformation represents a highly active research area which is documented in more than 25 reviews (e.g. Bogorad, 2000; Bock, 2001; Daniell, 2002; Bock and Khan, 2004; Maliga, 2004; Koop *et al.*, 2007; Maliga and Bock, 2011).

3.2.1 Gene transfer methods and transformation vectors

In general, there are two DNA delivery methods for a stable introduction of foreign DNA into plastids. The first one consists in bombarding of tissue or cells with DNA coated particles (Daniell *et al.*, 1990; Svab *et al.*, 1990). The second method treats isolated protoplasts (plant cells without cell wall) with polyethylene

glycol (PEG). PEG induces pores in cell membranes allowing the entrance of DNA into plastids, however, the exact mechanism is still unknown (Golds *et al.*, 1993; O'Neill *et al.*, 1993).

A prerequisite for the expression of heterologous proteins in chloroplasts is that the gene of interest must be flanked by plastid regulatory elements: a promoter with a 5´ untranslated region (UTR) and a ribosome binding sequence (e.g. Shine-Dalgarno sequence). A frequent combination used a plasmid containing the strong constitutive plastid 16 S ribosomal RNA promoter (Prrn) in combination with the viral T7g10-5´UTR (Staub *et al.*, 2000) or the psbA control elements (promoter, 5´-UTR) (Fernandez-San Millan *et al.*, 2003). A 3´ UTR region generally includes a stem-loop structure to stabilize the transcript. The most often used 3´UTRs are derived from the *psbA*, *rbcL* and *rps16* genes (Koop *et al.*, 2007).

The genetic transformation of plastids follows the principles of homologous recombination. Therefore, the transgene has to be flanked on either side by plastome sequences ensuring the integration of the transgene into a specific region within the plastome (Svab *et al.*, 1990) (Figure 3.4). Both sequences are required to allow the integration of foreign genes within the plastome. In average homologous flanks have a size of 1 kpb (Maliga, 2004). Stable integration of the transgene has been achieved in several regions of the plastome. Therefore intergenic regions are often chosen: *rbcL-aacD* (Svab and Maliga, 1993); *trnV-rps12/7* (McBride *et al.*, 1995); *trnI-trnA* (Kang *et al.*, 2003); *trnN-trnR* (Zou *et al.*, 2003) and *trnfM-trnG* (Zhou *et al.*, 2007). It is also recommended that only species specific vectors should be used for plastid transformation (Verma and Daniell, 2007).

3.2.2 Selection markers

Successful plastid transformation requires selection markers. The first selection marker described for plastid transformation in higher plants was a mutated plastid 16 S ribosomal RNA gene. This mutation confers resistance to spectinomycin (Svab *et al.*, 1990; Staub and Maliga, 1992). The most common selection marker used for plastid transformation is the bacterial spectinomycin

resistance gene *aadA* encoding for a 3´aminoglycoside-adenyltransferase (AADA). This protein inactivates spectinomycin and streptomycin antibiotics (Svab and Maliga, 1993). Figure 3.4 shows the standard components of a plastid transformation vector.

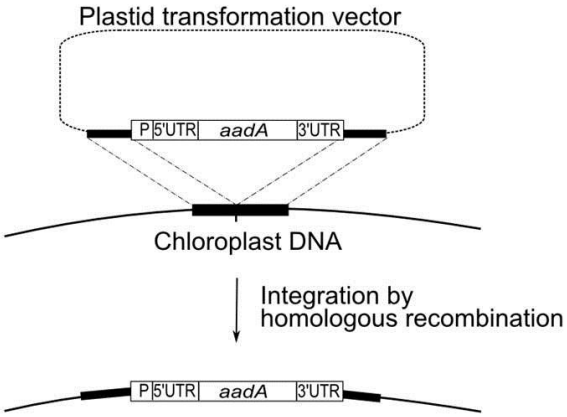


Figure 3.4 Targeting a transgene expression cassette into the chloroplast genome. The expression cassette consists of the *aadA* gene coding sequence fused to a chloroplast promoter (P) and 5´-untranslated region (5´UTR) on its 5´side and to a chloroplasts 3´-untranslated region (3´UTR) on its 3´side. Upon cell transformation with the plasmid, in which the expression cassette is flanked by chloroplast DNA sequences corresponding to the targeting region (indicated by a thick line), the cassette is inserted into the chloroplasts genome by homologous recombination. Modified from Rochaix, 1997.

Another selection marker is the neomycin phosphotransferase gene (*nptII*) which confers resistance to kanamycin. This gene is generally used in nuclear transformation because in plastid transformation it was not that efficient (Maliga, 2003). However, after the introduction of a Prrn promoter and a fusion of the first nucleotides of the *gfp* gene allowed a higher expression of NPTII in chloroplasts and thus the successful selection of transplastomic plants (Scharff and Koop, 2007). In 2002, the aminoglycoside phosphotransferase VI gene *aphA6* from *Acinetobacter baumannii* was described as a resistance marker for plastid transformation in tobacco. This *aphA6* gene encodes for kanamycin resistance (Bateman and Purton, 2000; Huang *et al.*, 2002).

Another version of selection markers combines aminoglycoside selection with secondary selection genes as *bar* and *pat* genes which encode for phosphinothricin acetyl transferases that confer resistance to the herbicides bialaphos and phosphinotricin. Additionally the *epsps* gene (5-enolpyruvylshikimate-3-

phosphate synthase) encodes for a resistance to glyphosate based herbicides (Iamtham and Day, 2000; Ye *et al.*, 2003).

After the bombardment of foreign DNA into the cell, it can be assumed that at the most one or a few plastomes are transformed. High selective pressure is needed to allow the elimination of the wild type plastomes and the amplification of transformed plastomes. Homoplasmic or homoplastomic cells (uniform population of transformed plastids) are obtained through several rounds of cell division and organelle segregation. Thus, homoplasmy can be achieved through several rounds of regeneration starting from leaves or tissue explants on selection medium (Svab and Maliga, 1993) (Figure 3.5).

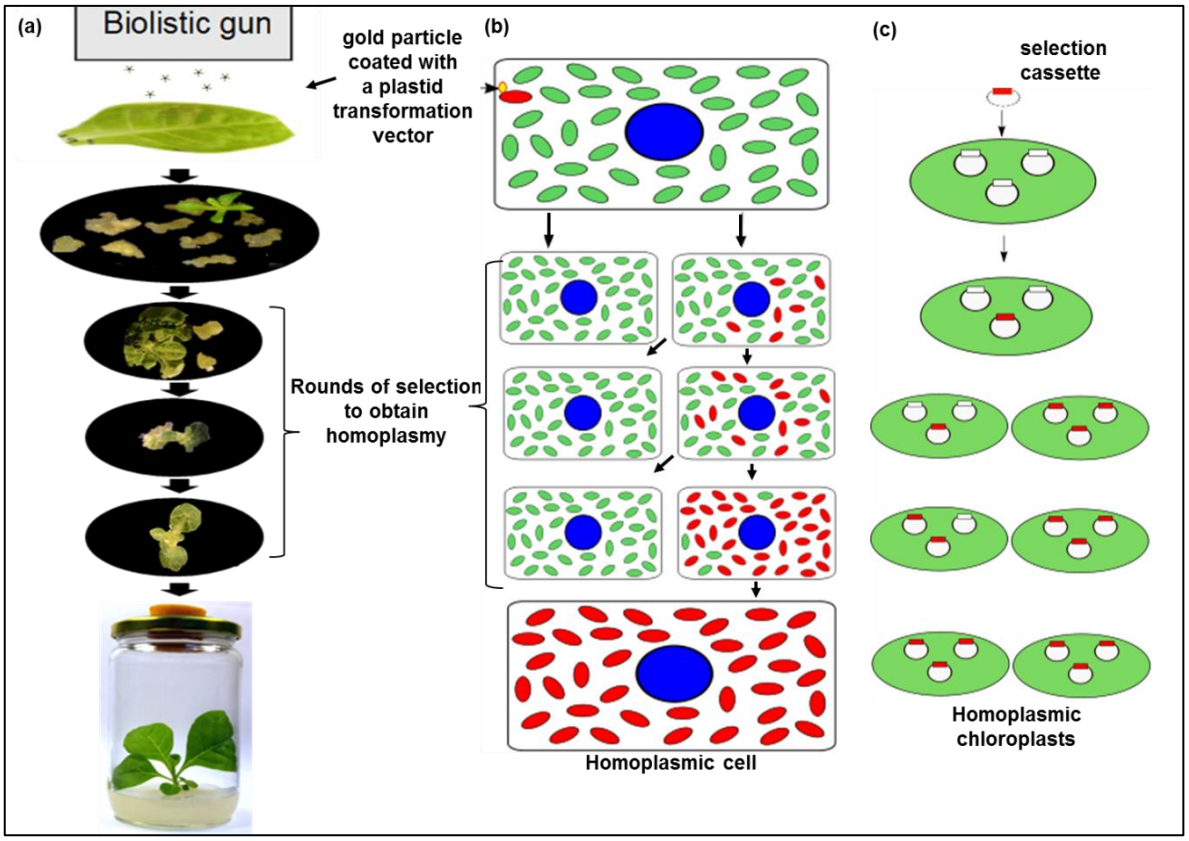


Figure 3.5 Plastid transformation. **(a)** Tobacco leafs are bombarded. Resistant green shoots appear on regeneration medium with antibiotic after 4-12 weeks. Shoots are usually heteroplasmic and at least three rounds of selection are needed to obtain homoplasmy (each round: 4 weeks). Homoplastomic lines are identified by Southern blotting then rooted in a free hormone medium. **(b)** Schematic description of sorting out of transformed chloroplasts. After bombardment just one or few chloroplasts are transformed (red color). Subsequent cell and organelle divisions in the presence of selective pressure favor the multiplication of transformed chloroplasts and the elimination of wild type cells. **(c)** Schematic description of the generation of homoplasmic chloroplasts. The target region is indicated as an open box. After the biolistic experiments, the resistance cassette (red) is integrated into the chloroplast genome. In the heteroplasmic state (left side) both wild type and transformed copies are present. After several rounds of selection, the homoplasmic chloroplasts are obtained.

3.2.3 Reporter genes

The use of reporter genes in plastid transformation allows the determination of transformation efficiency. One of the first reporter genes used in plastid transformation was the *gus* reporter gene (β-glucuronidase). Gus activity can be visualized by histochemical staining and fluorescence assays. Another reporter gene is *cat* (chloramphenicol acetyl transferase), first described in 1990, it took 20 years to be reported as a stable reporter gene (Daniell *et al.*, 1990; Zou *et al.*, 2003; Li *et al.*, 2010).

The use of fluorescent proteins such as GFP (green fluorescent protein) facilitates the selection of transformants in living cells as has been demonstrated for transient experiments (Hibberd *et al.*, 1998). Fusion between the *aadA* and *gfp* genes have been used as a visual and selection marker in *Nicotiana tabacum* (Khan and Maliga, 1999). Moreover, GFP has been also used as visual marker in lettuce, potato, *Lesquerella*, rice and poplar (Skarjinskaia *et al.*, 2003; Lelivelt *et al.*, 2005; Lee *et al.*, 2006; Okumura *et al.*, 2006; Valkov *et al.*, 2010). Last but not least, other fluorescent markers have been employed, such as the synthetic red shift variant of GFP (Reed *et al.*, 2001) or the YFP (Yellow fluorescent protein) (Buhot *et al.*, 2006; Zhou *et al.*, 2007). Recently, the bacterial luciferase operon, known as lux operon was expressed in tobacco plants. The light emission can be detected even with the naked eye in plastid transformed plants (Krichevsky *et al.*, 2010). Non-lethal visual markers represent a good alternative for the characterization of transformed plants.

3.2.4 Plastid transformation in *Nicotiana tabacum*

Tobacco is the best higher plant model for plastid transformation. The use of plastid transformation in tobacco helped to elucidate the function of genes as well as the transcription and translation machinery in plastids. For example, DNA replication, transcription and RNA processing, was further analyzed in this model including regulatory elements and the stability of proteins (Bock *et al.*, 1993; Bock and Maliga, 1995; Muhlbauer *et al.*, 2002; Zou *et al.*, 2003; Oey *et al.*, 2008).

Furthermore, more than 30 plastid genes have been inactivated in tobacco for functional analysis (Koop *et al.*, 2007). Additionally, more than 50 different recombinant proteins of biotechnological use have been expressed in tobacco chloroplasts. This includes plants which are resistant to herbicides, insects and diseases (McBride *et al.*, 1995; DeGray *et al.*, 2001; Lutz *et al.*, 2001). On top of that, several biopharmaceutical proteins and several vaccine antigens have been expressed in tobacco plastids (e.g. Daniell *et al.*, 2005; Bock, 2007; Koop *et al.*, 2007). A summary of proteins expressed in tobacco is shown in Table 3.1.

Table 3.1 Expression of several proteins in *Nicotiana tabacum*

Genes	Protein or final product	Reference
reporter genes		
uidA	β- Glucuronidase	(Staub and Maliga, 1993)
neo	neomycin phosphotransferase	(Carrer *et al.*, 1993)
cat	chloranphenicol acetyl transferase	(Li *et al.*, 2010)
gfp	green fluorescent protein	(Sidorov *et al.*, 1999)
e-yfp	e-yellow fluorescent protein	(Buhot *et al.*, 2006)
herbicide resistance		
bar	phosphinothricin acetyl transferase	(Lutz *et al.*, 2001)
epsps	5-enolpyruvylshikimate-3-phosphate synthase	(Daniell *et al.*, 1998)
insect resistance		
cry1Ac	*Bacillus turingencis* toxin	(McBride *et al.*, 1995)
***cry2Aa2* operon**	*Bacillus turingencis* toxin	(De Cosa *et al.*, 2001)
bioplastics		
egl12I	PBP (GVGVP) (Protein-based polymer)	(Guda *et al.*, 2000)
phbC-phbA-phbB	PHB (Polyhydroxybutyrate)	(Lossl *et al.*, 2005)
pathogen resistance		
msi-99	antimicrobial peptide	(DeGray *et al.*, 2001)
Protegrin-1	antimicrobial peptide	(Lee *et al.*, 2011)
drought or salt tolerance		
tpsI	threalose-6- phosphate synthase	(Lee *et al.*, 2003)
badh	betaine aldehyde dehydrogenase	(Daniell *et al.*, 2001)
biopharmaceuticals		
hgh	somatotropin	(Staub *et al.*, 2000)
igf	human insulin like growth factor	(Daniell *et al.*, 2009)
ifn-alpha2b	interferon alpha 2b	(Arlen *et al.*, 2007)
vaccine in plants		
tetC	Tetanus toxin C (*Clostridium tetani*)	(Tregoning *et al.*, 2003)
ltb	B subunit (*E. coli* enterotoxigenic)	(Kang *et al.*, 2003)
pag	PA protective antigen anthrax (*Bacillus anthracis*)	(Watson *et al.*, 2004)
caF1-lcrV	F1-V protein /Plague (*Yersinia pestis*)	(Arlen *et al.*, 2007)
ospA-histag	lipoprotein A/Lyme disease (*Borrelia burgdorferi*)	(Glenz *et al.*, 2006)
lecA	lectin A (*Entamoeba histolytica*)	(Chebola and Daniell, 2007)
p24	p24 antigen (Human Immunodeficiency Virus)	(McCabe *et al.*, 2008)
L1	L1 protein (Human papillomavirus)	(Lenzi *et al.*, 2008)

3.2.5 Plastid transformation in different species

Although plastid transformation in higher plants was established more than 20 years ago, stable transformants could only be obtained in relatively few species (Maliga, 2004). In order to obtain transplastomic crops, some critical points must be considered. One of them is the design of specific vectors for each species (Bock and Khan, 2004). Another critical point is the *in vitro* culture of crops, allowing a high regeneration capacity so that fertile plants can be recovered (Maliga, 2002; Koop *et al.*, 2007).

Currently, stable plastid transformation was successfully achieved for solanaceous crops using similar tissue culture conditions as for tobacco, potato, eggplant, tomato and petunia (Svab *et al.*, 1990; Sidorov *et al.*, 1999; Ruf *et al.*, 2001; Zubkot *et al.*, 2004; Singh *et al.*, 2010; Valkov *et al.*, 2010). Plastid transformation was also described in other species as soybean/*Glycine max* (Dufourmantel *et al.*, 2004); carrot/*Daucus carota* (Kumar *et al.*, 2004a); cotton/*Gossypium hirsutum* (Kumar *et al.*, 2004b); wheat/ *Triticum aestivum* (Cui *et al.*, 2011); lettuce/*Lactuca sativa* (Lelivelt *et al.*, 2005; Kanamoto *et al.*, 2006); rice/*Oryza sativa* (Lee *et al.*, 2006); sugar beet/*Beta vulgaris* (De Marchis *et al.*, 2008) and poplar/*Populus alba* (Okumura *et al.*, 2006). Finally there are some reports in the Brassicaceae family: *Lesquerella fendleri* (Skarjinskaia *et al.*, 2003); rapeseed/*Brassica napus* (Hou *et al.*, 2003; Cheng *et al.*, 2010), cabbage/(Liu *et al.*, 2007)and cauliflower/*Brassica oleracea* (Nugent *et al.*, 2005a) and *Arabidopsis thaliana* (Sikdar *et al.*, 1998). In *Arabidopsis thaliana* there is just one report which describes unfertile plants. However, stable transformation of chloroplast in *Arabidopsis thaliana* could not be reproduced in the last ten years (Sikdar *et al.*, 1998).

3.3 *Arabidopsis thaliana* as model organism

Arabidopsis thaliana is a member of the Brassicaceae family which also includes some cultivated species such as cauliflower, radish and mustard. Although *Arabidopsis thaliana* is not an agronomically important plant, several

advantages made *Arabidopsis thaliana* one of the most important model organisms in plant molecular biology (Bressan *et al.*, 2001; Koornneef and Meinke, 2010).

First, *Arabidopsis thaliana* has one of the smallest genomes in the plant kingdom with approximately 135 megabases. In addition, the nuclear, plastid and mitochondrial genomes have been sequenced (The Arabidopsis Genome Initiative 2000) (Ausubel, 2000; Koornneef and Meinke, 2010).

The nuclear genome of *Arabidopsis thaliana* consists of 5 chromosomes identified by Friedrich Laibach in 1907. Leibach is the founder of experimental research in *Arabidopsis thaliana* and in 1943, he proposed *Arabidopsis thaliana* as a model organism (Meyerowitz and Pruitt, 1985; Meyerowitz, 2001; Somerville and Koornneef, 2002). It can be easily cultivated and develops rapidly, taking about 6 weeks from seed germination to the production of a new crop of seeds. Moreover, *Arabidopsis thaliana* is a self-fertilizing diploid plant and can produce up to 50,000 seeds per plant.

The most common nuclear transformation method in *Arabidopsis thaliana* is the "dipping method". Here, the whole flowering plant is dipped into a solution containing *Agrobacterium tumefaciens* with a T-DNA plasmid. The T-DNA plasmid which contains the gene of interest is then introduced into cells by the *Agrobacterium tumefaciens* method. Once transformed cells develop into gametes, and the transgenic plants can be subsequently selected from the treated seeds (Chang *et al.*, 1994).

Mutations in *Arabidopsis thaliana* can be generated using x-ray irradiation, chemical mutagens, insertional mutagenesis via *Agrobacterium thumefaciens* or transposons (Meinke *et al.*, 1998). Rapid isolation of mutants together with the simple identification of the corresponding genes offers another advantage of *Arabidopsis thaliana*.

Due to these properties, *Arabidopsis thaliana* has become one of the most important model organism for the understanding of plant development, cell biology, biochemistry and physiology (Meyerowitz, 2001; Koornneef and Meinke, 2010).

3.3.1 Tissue culture in *Arabidopsis thaliana*

In most plants, tissue culture is critical for successful plastid transformation. *In vitro* regeneration of plants requires a suitable medium containing mineral salts, an energy source as sucrose, vitamins and phytohormones like cytokinins and auxins (Murashige and Skoog, 1962). Because plants possess totipotency in most of their cells, almost each individual somatic cell can regenerate a whole plant under controlled conditions (Vasil and Vasil, 1972).

In vitro regeneration of plants can be achieved through two pathways, either by somatic embryogenesis or by organogenesis. For somatic embryogenesis, plants can be regenerated directly from explants or indirectly after a callus phase (group of undifferentiated cells) (Figure 3.6).

In *Arabidopsis thaliana* somatic embryogenesis could be achieved from seed-derived calli (Huang and Yeoman, 1984) and from immature zygotic embryo-derived calli (Luo and Koop, 1997). Somatic embryos are bipolar, meaning that they contain both shoot and root apices. Starting from cotyledon derived callus, somatic embryogenesis can be induced by the addition of the synthetic auxin 2-4-dichlorophenoxyacetic acid (2-4-D) (Raghavan, 2004).

For *in vitro* organogenesis in *Arabidopsis thaliana,* a two-step procedure is required. First, to induce callus formation, the explants are cultured on an auxin-rich medium. Then, for the induction of shoots, they are transferred to a cytokinin-rich medium (Valvekens *et al.*, 1988). Regenerated shoots can be finally transferred to hormone-free medium or to a root induction medium to produce a complete plant.

De novo organogenesis involves three phases:

1. Acquisition of competence (dedifferentiation)
2. Induction
3. Morphological differentiation.

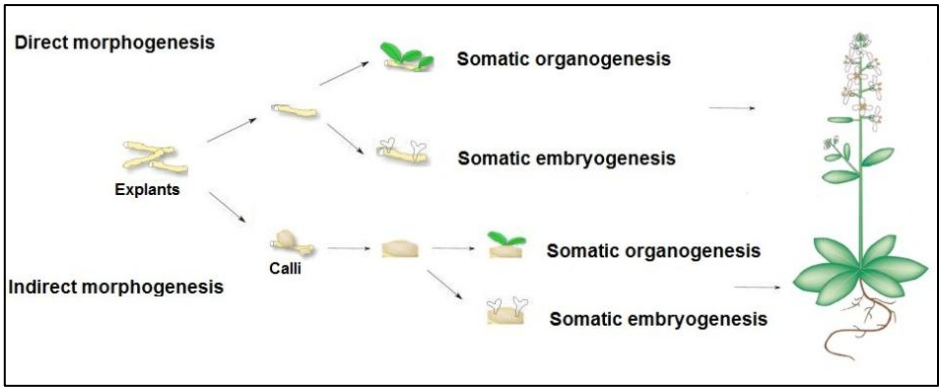


Figure 3.6 *In vitro* regeneration of *Arabidopsis thaliana*

Regeneration efficiency depends not only on tissue culture media, but it also depends on the explant source and plant ecotype. Successful regeneration of *Arabidopsis thaliana* has been achieved using different explants such as leaves (Feldmann and Marks, 1986; Schmidt and Willmitzer, 1988; Candela *et al.*, 2001), cotyledons (Patton and Meinke, 1988; Barghchi *et al.*, 1994; Zhao *et al.*, 2002), hypocotyls (Zhao *et al.*, 2002), seeds (Negrutiu *et al.*, 1975; Huang and Yeoman, 1984) and roots (Valvekens *et al.*, 1988; Atta *et al.*, 2009).

With regard to ecotypes, the highest regeneration efficiencies were reported in ecotypes C24, Wassilewskija (WS) and Landsberg erecta (Ler) when compared with other ecotypes as Columbia or RLD (Barghchi *et al.*, 1994; Luo and Koop, 1997; Candela *et al.*, 2001).

3.3.2 Protoplast culture in *Arabidopsis thaliana*

For protoplast isolation, the cell wall must be removed with fungal hydrolytic enzymes such as cellulase and macerozyme. In *Arabidopsis thaliana*, protoplasts have been isolated from several sources like leaves, cotyledons, roots, cell suspension and callus. Once the tissue has been incubated with suitable hydrolytic enzymes, protoplast isolation requires subsequent filtration, floatation and sedimentations steps.

Arabidopsis thaliana protoplasts have been used for the understanding of cellular processes including cell wall synthesis, cell division, photosynthesis activity, calcium signalling, stress and responses to plant hormones (Yoo *et al.*, 2007).

Nowadays, protoplast isolation and transient gene expression has become a standard tool in plant physiology. Long standing challenges in this research field thus far are high regeneration efficiencies of complete fertile plants from protoplasts (Dovzhenko *et al.*, 2003).

As for tissue culture, regeneration efficiency depends highly on the *Arabidopsis thaliana* ecotypes, for instance, ecotype C24 was successfully regenerated with the highest platting efficiencies of up to 30 % when compared with other ecotypes as Wassilewskija with 10 % and Columbia with 5 % (Dovzhenko *et al.*, 2003).

3.4 Rapid-Cycling *Brassica rapa,* a new model organism

Brassicaceae is a diverse group of crop plants economically important in agriculture and horticulture. Brassicaceae are related to *Arabidopsis thaliana* and includes vegetables as broccoli, cabbage, cauliflower, turnip and mustard condiments, from which several uses include, besides human consumption, animal feed and the production of industrial oil (Musgrave, 2000).

Brassicaceae include three diploid species, *Brassica rapa* or *campestris* (AA), *Brassica nigra* (BB) and *Brassica olaracea* (CC) and three naturally occurring allotetraploid species, *Brassica juncea* (AABB), *Brassica napus* (AACC) and *Brassica carinata* (BBCC). The genetic relationship between the *Brassica spp.* is described by the so-called "U´s triangle" proposed by Woo Jang-choon in 1935 (Hong *et al.*, 2008) (Figure 3.7).

Although Brassicas are a quite diverse and economically important group of plants, there is relatively little knowledge on their genetics and molecular physiology. This is due probably to its long reproductive cycles that last from 6 months up to a year for most of the horticultural types (Williams and Hill, 1986).

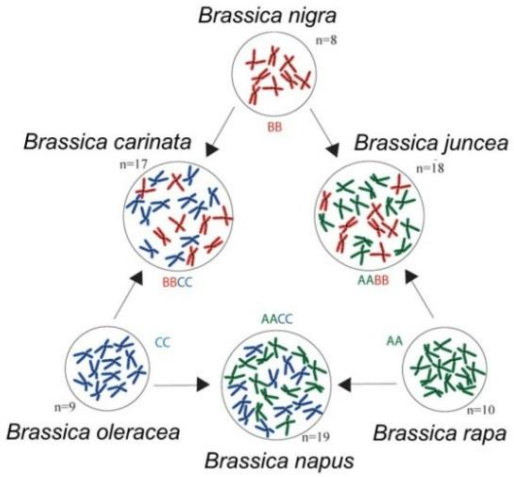


Figure 3.7 The genetic relationship between Brassica species of the "Triangle of U". Diploid species are indicated as AA (*Brassica rapa*), BB (*Brassica nigra*), CC (*Brassica olaraceae*). Chromosomes from each of the genomes A, B and C are represented by different colours. "n" represent the number of chromosomes in the haploid genomes. Figure from http://canseq.ca/

To solve this long reproductive cycle problem, Williams *et al*., worked with a seed bank of approximately 2000 accessions of Brassicas. They re-discovered *Brassica* seeds from a mountainous region of Nepal 20 years ago. Williams *et al*., bred this weedy *Brassica* until they were able to reduce its life cycle to 5 weeks under standard conditions (www.fastplants.org). Nowadays, Rapid-Cycling *Brassica rapa* (RCBr) known as Fast Plants® or Wisconsin plants are used in research and teaching (Musgrave, 2000).

Characteristics of RCBr are:

- Short time from planting to flowering (about 2 weeks)
- Rapid seed maturation with no seed dormancy (35-45 days)
- Small plant size (up to 20 cm)

RCBr is naturally self-incompatible for pollination and they represent a genetically diverse group with many different strains (Briggs and Goldman, 2006). All these characteristics made RCBr a very attractive model for teaching and also for learning genetics, ecology, botany and physiology (Wendell and Pickard, 2007). To date over 150 different lines have been described (www.fastplants.org).

4 Research aims

The main goal of this work was the establishment of protocols towards the plastid transformation in *Arabidopsis thaliana* and the Rapid-Cycling *Brassica rapa.* To achieve this goal, we focused on the generation of new vectors containing species specific sequences for the integration of transgenes into the chloroplast genome. Additionally, we concentrated on the standardization of tissue culture to obtain high regeneration efficiencies of fertile plants.

Plastid transformation is a well-established method in *Nicotiana tabacum*, for this reason tobacco plants were used as a positive control in the transformation experiments.

5 Materials and Methods

5.1 Chemicals and enzymes

2,4-dichlorophenoxy acetic acid (2,4-D)	Sigma-Aldrich, Taufkirchen, Germany
3-Indole acetic acid (IAA)*	Sigma-Aldrich, Taufkirchen, Germany
Acetone	Merck, Darmstadt, Germany
Agarose (Seakem® LE agarose)	Cambrex, Rockland ME, USA
Alginic acid	Sigma-Aldrich, Taufkirchen, Germany
Ammonium succinate	Sigma-Aldrich, Taufkirchen, Germany
Ammonium sulphate*	Duchefa, Haarlem, Netherlands
Ampicillin, sodium salt	AppliChem, Darmstadt, Germany
B5 salts	Duchefa, Haarlem, Netherlands
Bacto Agar	Difco, Detroit Michigan, USA
Benzylaminopurine (BAP)*	Sigma-Aldrich, Taufkirchen, Germany
Boric acid	USB, Cleveland, USA
Bromophenol blue	Merck, Darmstadt, Germany
β-Mercaptoethanol	Roth, Karlsruhe, Germany
Calf intestine phosphatase	Fermentas, St Leon-Rot, Germany
Calcium chloride dehydrate*	Merck, Darmstadt, Germany
Calcium pantothenate	Merck, Darmstadt, Germany
Casein hydrolysate*	ICN Biochemicals, USA
Cellullase R10	Duchefa, Haarlem, Netherlands
Cetyl trimethyl ammonium bromide (CTAB)	Serva, Heidelberg, Germany
Chloroform	Roth, Karlsruhe, Germany
D(+)-Biotin (vitamin B8)*	Duchefa, Haarlem, Netherlands
D-Glucose*	AppliChem, Darmstadt, Germany
Diaminoethanetetra acetic acid (EDTA)	Serva, Heidelberg, Germany
Dicamba*	Sigma-Aldrich, Taufkirchen, Germany
Dimanin C	Bayer Vital, Leverkusen, Germany
D-Mannose*	Sigma-Aldrich, Taufkirchen, Germany
DNA Dream Taq polymerase	Fermentas, St Leon-Rot, Germany
DNA Phusion polymerase	Finnzymes, Espoo (Finnland)
Ethidium bromide	Sigma-Aldrich, Taufkirchen, Germany
Folic acid*	Duchefa, Haarlem, Netherlands
Gelrite *	Duchefa, Haarlem, Netherlands
Glutamic acid	Fluka, Buchs, Switzerland
Glycerol	Roth, Karlsruhe, Germany
Glycine*	Merck, Darmstadt, Germany
Gold (0,6 µm)	Biorad, Munchen. Germany
Hydrochloric acid	Sigma-Aldrich, Taufkirchen, Germany
Indole butyric acid (IBA)*	Merck, Darmstadt, Germany
Isopropanol	Duchefa, Haarlem, Netherlands
Kanamycin	Duchefa, Haarlem, Netherlands
Kinetin	Sigma-Aldrich, Taufkirchen, Germany
L-arginin	Sigma-Aldrich, Taufkirchen, Germany
L-asparagin	Sigma-Aldrich, Taufkirchen, Germany
L-aspartic acid	Sigma-Aldrich, Taufkirchen, Germany
L-Glutamine	Sigma-Aldrich, Taufkirchen, Germany
L-proline*	Duchefa, Haarlem, Netherlands
Macerocyme R10	Duchefa, Haarlem, Netherlands
Magnesium chloride hexahydrate	AppliChem, Darmstadt, Germany

Magnesium sulphate heptahydrate	Roth, Karlsruhe, Germany
Magnesium sulphate monohydrate	Merck, Darmstadt, Germany
Manganese (II) chloride, tetrahydrate	Roth, Karlsruhe, Germany
Manganese sulphate monohydrate	Merck, Darmstadt, Germany
Mannitol	Duchefa, Haarlem, Netherlands
MES (2-(N-Morpholino)ethanol acid)	Sigma-Aldrich, Taufkirchen, Germany
Methanol (HPLC grade)	Merck, Darmstadt, Germany
MS salts	Duchefa, Haarlem, Netherlands
Myo-inositol *	Serva, Heidelberg, Germany
Naphthalene acetic acid (NAA)*	Sigma-Aldrich, Taufkirchen, Germany
Nicotinic acid	Sigma-Aldrich, Taufkirchen, Germany
Oligonucleotides	Metabion, Martinsried, Germany
Phyto agar*	Duchefa, Haarlem, Netherlands
Potassium acetate	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Potassium hydroxide	Merck, Darmstadt, Germany
Potassium iodide *	Merck, Darmstadt, Germany
Potassium nitrate	Merck, Darmstadt, Germany
Pyridoxine hydrochloride (vitamin B6)*	Duchefa, Haarlem, Netherlands
Riboflavin (vitamin B2)*	Duchefa, Haarlem, Netherlands
Rubidium chloride	AppliChem, Darmstadt, Germany
Silver nitrate*	Roth, Karlsruhe, Germany
Sodium acetate	Roth, Karlsruhe, Germany
Sodium chloride	Sigma-Aldrich, Taufkirchen, Germany
Sodium citrate	Sigma-Aldrich, Taufkirchen, Germany
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, Germany
Sodium EDTA	Roth, Karlsruhe, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium hypochlorite	AppliChem, Darmstadt, Germany
Spectinomycin	Duchefa, Haarlem, Netherlands
Spermidine	Duchefa, Haarlem, Netherlands
Sucrose*	Duchefa, Haarlem, Netherlands
Thiamine hydrochloride (vitamin B1)*	Merck, Darmstadt, Germany
Thidiazuron (1-Phenyl-3-(1,2,3-Thidizol)	Sigma-Aldrich, Taufkirchen, Germany
Tris(hydroxymethyl)-aminomethan (Tris)	Roth, Karlsruhe, Germany
Yeast extract	Duchefa, Haarlem, Netherlands

* For plant *in vitro*-culture media.

All the DNA markers, restriction and nucleic acids modifying enzymes were provided by MBI Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt Main, Germany) and Promega (Mannheim, Germany). Highly pure deionized distilled water was obtained using an "Ultra Clear" SG equipment (Barsbüttel, Germany).

5.2 Kits

NucleoSpin Plant Kit	Macherey-Nagel GmbH & Co., Düren, Germany
QIAquick Gel Extract Kit	Qiagen, Hilden, Germany
Plasmid Maxi Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit Qiagen	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
DIG High Prime DNA Labeling Starter Kit II	Roche Diagnostics GmbH, Mannheim, Germany

5.3 Disposable material

Centrifugation tubes polyprop. (15, 50 ml)	BD Falcon, Heidelberg, Germany
Filter paper Whatman 3MM	Whatman GmbH, Daasel, Germany
Microcentrifugation tubes (1, 2 ml)	Eppendorf AG, Hamburg, Germany
Nescofilm	Roth, Karlsruhe, Germany
N+-Nylonmembran	Amersham Biosciences, UK
PCR tubes 0.5 and 0.2 ml DNase/RNase-free	Peqlab GMBH, Erlangen, Germany
Petri dishes for bacteriology, steril (9 cm)	Renner, Dannstadt, Germany
Petri dishes for tissue culture, steril (6 cm)	Greiner, Solingen, Germany
Petri dishes for tissue culture, steril (9 cm)	Greiner, Solingen, Germany
Scalpels, sterile and disposable	Aesculap, Tuttlingen, Germany
Steril filter PES, 0.22µm	Serva, Heidelberg, Germany
Steril filter PES, 0.22µm, 250 ml	Serva, Heidelberg, Germany
Syringes BD Plastipak, 1, 5 and 10 ml	BD Biosciences Heidelberg, Germany
Polypropylene grids Scrynel PP2000	K.H.Büttner, Wasserburg, Germany

5.4 Equipment and instruments

Analytical balance AC 120 S	Sartorius, Göttingen, Germany
Analytical balance 2255 Sartorius	Sartorius, Göttingen, Germany
Autoclave Aesculap 420	Aesculap-Werke, Tuttilngen, Germany
Camera Canon EOS 400D +18-55/3,5-5,6 KIT	Canon, Tokio (Japan)
Centrifuge Universal 30 RF	Hettich, Tuttlingen
Centrifuge, refrigerated, model 5415	Eppendorf, Hamburg, Germany
Centrifuge, Z323K	Hermle, Wehingen, Germany
Electrophoresis power supply 2301	LKB Produkter, Bromma, Sweden
Electrophoresis chamber	Peqlab Biotechnologie GmbH, Germany
Fluorescence microscope „Axio Imager Z1"	Carl Zeiss Vision GmbH, Jena, Austria
Fluorescence microscope, Leica DM 1000	Leica Microsystems, Wetzlar, Germany
Fuorescence SteREO Lumar. V12	Carl Zeiss Vision GmbH, Jena, Austria
Forceps BD887R	Aesculap, Tuttlingen, Germany
Gel documentation system	MWG Biotech, Ebersberg, Germany
Growth cabinet Rumed Nr; 1200	Rubarth Apparate, Hannover, Germany
Haemocytometer	Fuchs-Rosenthal Friedrichsdorf, Germany
Osmometer O30	Gonotec GmbH, Berlin, Germany
Particle gun PDS/1000/He	Bio-Rad, München, Germany
PCR Express Thermocycler Hybaid	Hybaid, Ashford, UK
pH meter WTW pH522	WTW, Weilheim, Germany
Pipettes Pipetman 2,20,200, 1000 µl	Gilson, Middleton, Wisconsin, USAei
Ultraspec 3100 UV/Vis Spectrophotometer	GE Healthcare, UK

Sterile bench UVF6.12S BDK	BDK, Sonnenbühl-Genkingen, Germany
UV-Crosslinker	Amersham, Freiburg, Germany
Vacuum Concentrator	Bachofer, Reutlingen, Germany
Vortex mixer Genie 2 Modell 6560	Scientific Ind., Bohemia, NY, USA
Water bath Wagner	Wagner Munz, München, Germany

5.5 Software and internet tools

Function	Name	Reference
Sequence	Blast 2 Sequences (NCBI)	http://www.ncbi.nlm.nih.gov/BLAST/
Primer design	Primer3 Input 0.4.0	http://frodo.wi.mit.edu/
Vector design	Vector NTI Version 4.0.2	Invitrogen, Groningen, Netherlands
Gel analysis	OneDScan	MWG Biotech, Ebersberg, Germany
Pictures	Adobe Photoshop 6.0	Adobe Systems, San Jose (USA)
	AxioVision LE, Release 4.4	Carl Zeiss, Munich, Germany
Office Software	MS Office 2003-2007	Microsoft, Redmond (USA)

5.6 Biological material

5.6.1 Plant material

Nicotiana tabacum cv. Petit Havana was used as a control of plastid transformation. *Arabidopsis thaliana* and Rapid-Cycling *Brassica rapa* (Fast plants®) were used for standardization of tissue culture and towards plastid transformation experiments (Table 5.1).

Table 5.1 Plant material

Plant	Description	Abbreviation	Provided
Arabidopsis	*Arabidopsis thaliana* C24	C24	PD Dr. Jörg Meurer[a]
Brassica Fast plants®	Rapid-Cycling *Brassica rapa* C1	RCBr-C1	College of Agricultural & life sciences[b]
	Rapid-Cycling *Brassica rapa* B3	RCBr-B3	Assistent Scientist S. Woody[c]

[a] Botanic Institute, Ludwig-Maximillians-University, Munich, Germany
[b] Department of Plant Pathology , University of Wisconsin-Madison, USA
[c] Department of Biochemistry, University of Wisconsin-Madison, USA.

5.6.2 Medium for culture of bacteria

Medium, required for culture of *Escherichia coli*:

LB(Luria-Bertani) broth medium

Bacto-tryptone	10g
Bacto-yeast extract	5 g
NaCl	10g

pH to 7.0 with 1 N KOH, H_2O add 11. Sterilize by autoclaving for 20 minutes.

Escherichia coli, XL1-Blue (genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´*proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^{r})]), was used for cloning experiments. The bacteria were grown overnight on either in LB broth or in Petri dishes containing solidified LB (1.2 % Bacto agar) at 37 ^{o}C. Appropriate antibiotics at suitable concentration were used as described previously (Sambrook *et al.*, 2001).

5.7 Molecular methods

5.7.1 Isolation of genomic DNA of plants

Plant genomic DNA was extracted using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany) as described in the user protocol, or with the modified CTAB method (Murray and Thompson, 1980) using the following extraction buffer:

2x CTAB-Extraction buffer

Tris-HCl (pH 8)	200 mM
CTAB	2 % (w/v)
EDTA	20 mM
NaCl	1400 mM
PVP	1 % (w/v)
β-Mercaptoethanol	280 mM (before each use)

Plant tissue (100-200 mg leaf or calli) was ground in liquid nitrogen to a fine powder by using a mortar and a pestle. Plant material was homogenized in one volume of CTAB (2X) extraction buffer and incubated for one hour at 60^{o}C. For extraction, one volume of chloroform was added and tubes were incubated with orbital agitation for 30 min. All tubes were then centrifuged for 5 min, at 10 000 g. The upper layer was transferred to a sterile 2 ml Eppendorf tube and 5 µl RNAse A [10 µg/ml] were added. After incubation for 30 min at 42 ^{o}C, chloroform extraction was repeated. The upper layer was transferred again to a new Eppendorf tube and 0.7 volume of cold 2-Isopropanol (100 %) was added. The DNA samples were incubated at -20 ^{o}C for 30 min, and then centrifuged 10 min at 21 000 g. The supernatant was discarded; the DNA pellet was washed with cold 70 % EtOH solution, dried and dissolved in 50 µl of 10 mM Tris-HCl, pH 8.

5.7.2 Isolation of plasmid DNA

Plasmid DNA isolation was performed either on small or big-scale. The big-scale set up was required for transformation experiments with the particle gun method. Small-scale plasmid DNA preparations were used for cloning and sub-cloning experiments. DNA mini and maxi preparations were performed using the QIAprep Spin Miniprep Kit and the Plasmid Maxi Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer´s protocol.

As an alternative, the "TENS" method was used. The TENS method is a "rapid alkaline extraction" method (Birnboim and Doly, 1979) that has been modified according to (Eibl, 1999). First, 3 ml of LB medium with appropriate antibiotic concentration was inoculated with a single *E. coli* (XL1-Blue) colony and incubated on a shaker at 37 ^{o}C overnight. The next day, 2 ml of this culture were centrifuged at 15 000 rpm for 2 min and the pellet was resuspended in 300 µl of TENS buffer (50 mM Tris-HCl pH 7.5, 0.5 % SDS, 50 mM EDTA, 10 mM NaCl) by vortexing. Then, 150 µl of KoAc 3M (pH 5.2) were added and the samples were mixed by vortexing for 2-5 seconds. Cell debris and chromosomal DNA were pelleted by centrifugation at 15 000 rpm for 4 min. The supernatant was transferred into a new 1.5ml Eppendorf tube and 900 µl EtOH were added to precipitate the plasmid DNA, supported by incubating at –20 °C for 5 min. After centrifugation (15 000 rpm, 10 min), and washing with 70 % EtOH, the air-dried pellet was finally dissolved in 50 µl of water or in 10 mM Tris-HCl, pH 8.

5.7.3 Gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to their sizes. Agarose gels were prepared at a final concentration of 1 % in a buffer solution, either TAE 1X (40 mM Tris, 0.11 % acetic acid (v/v), 1 mM Na_2EDTA) or TBE 1X (90 mM Tris, 90 mM Boric acid, 2 mM EDTA) buffer. To dissolve the agarose, an agarose solution was boiled, and once this solution was cooled down, 2.5 µl of 10 mg/ml ethidium bromide (EtBr) stock solution was added to 50 ml. The melted gel was poured into a gel chamber with a comb. The

comb was removed after solidification and running buffer was added to the chamber. Before loading, samples were mixed with 6X loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerin, 0.12 M Na_2EDTA, pH 8.0). Electrophoresis was performed at 60-120 V. A 1kb DNA ladder or a λ/HindIII marker was included. DNA was indirectly visualized by UV light (254-366 nm). Ethidium bromide intercalates between the DNA base pairs, and it fluoresces under UV radiation (Sambrook *et al.*, 2001). Results were scanned using a gel documentary system (MWG, Ebersberg, Germany).

5.7.4 DNA purification and concentration

DNA purification was necessary after enzyme treatment of PCR amplification. After enzymatic digestions, DNA was loaded in a gel and separated according to size (see 5.7.3). Fragments of interest were excised and gel DNA extraction was performed using the QIAquick Gel Extract Kit (Qiagen, Hilden, Germany) according to manufacturers´ instructions. After a PCR or treatment of DNA with any modifying enzyme, the PCR Extract Kit (Qiagen, Hilden, Germany) was used as described in the user´s protocol.

DNA was subsequently concentrated by EtOH precipitation (Sambrook *et al.*, 2001). In this case, 2.5 volumes of 100 % of EtOH were added together with 0.1 volume of 3M sodium acetate (pH 4.8) followed by mixing. This solution was incubated for 1 to 2 hours at -20 ^{o}C and then centrifuged (12 000 rpm for 10 min). The pellet was once washed using 70 % EtOH and then air-dried and dissolved in 10mM Tris-HCl pH 8.0.

5.7.5 DNA quantification

For DNA quantification, agarose electrophoresis densitometry was chosen to estimate DNA integrity. Samples were loaded in a TBE 1X agarose gel (1 %) according to section 5.7.3. As standard, a λ/HindIII marker was used. 6 µl of this marker contains bands with the following known DNA amounts: 22, 24, 69, 100

and 240 ng. Pictures in "TIF" format from the gel were taken using a gel documentation system and analyzed using the OneDScan (MWG, Ebersberg, Germany) program.

5.7.6 DNA restriction and modification

For cloning experiments, analysis of vectors and transplastomic plants, restriction enzymes were used. The reaction conditions were used as suggested by the manufacturers´ following a standard protocol: 1 U of the enzyme for 1 µg DNA was supplemented with the appropriate buffer and incubated for 2 h. Reaction products were analyzed by gel electrophoresis (see 5.7.3).

Blunt ends can be generated by using the Klenow fragment (the large fragment of DNA polymerase I from *E. coli*) (Fermentas, St. Leon-Rot, Germany). The Klenow polymerase I exhibits 5'→3' polymerase activity and 3'→5' exonuclease (proofreading) activity but lack a 5'→3' exonuclease activity. After DNA digestions with endonucleases were heat inactivated according to the manufacturers´ instructions. To fille of the 5'-overhangs, 1 µl of a dNTP mix (2.5 mM each) and 2 U of Klenow fragment were added to the sample and incubated at 25 ^{o}C for 15 min. The enzyme was inactivated by adding 10 mM EDTA followed by heating at 75 ^{o}C for 20 min.

To minimize self-ligation, the termini of the blunted vectors were dephosphorylated with the enzyme SAP (Shrimp Alkaline Phosphatase) enzyme from Fermentas (St. Leon-Rot, Germany). Buffer conditions and temperature were used according to the manufacturers´ advice.

5.7.7 DNA ligation

Ligation of two double-stranded DNA fragments with cohesive or blunt termini was achieved using T4-DNA ligase from Fermentas (St. Leon-Rot, Germany). In most cases, 20 ng of vector were used with a 3:1 or 5:1 vector/insert ratio. The following formula was used to calculate this ratio: [(ng vector) x (kb size

of insert)]/[(kb size of vector)] x [molar ratio of (insert/vector)] = ng insert. Buffer and incubation temperature were used according to manufacturers´ instructions.

5.7.8 Preparation of competent cells

Heat-shock Rb^+ competent cells of *E. coli* XL-1Blue were prepared following a Hanahan D., (1983) modified method (Hanahan, 1983).

Solutions:

RF1	
100 mM	RbCl
50 mM	$MnCl_2 \cdot 6H_2O$
30 mM	KoAc
10 mM	$CaCl_2$
15 %	Glycerol (v/v)
pH 5.8	with CH_3COOH

RF2	
10 mM	RbCl
75 mM	$CaCl_2$
15 %	Glycerol (v/v)
pH 6.8	with NaOH
10 mM	MOPS (pH 6.5 with KOH)

A 5 ml LB starter culture was inoculated with 50 µl of a frozen stock of XL1-Blue cells. The culture was incubated overnight at 37 °C with constant shaking at 150 rpm. On the following day, a small aliquot equivalent to an OD_{600}= 0.1 was poured into 100 ml of fresh LB medium. Bacteria were grown until an OD_{600}= 0.45-0.6 was reached. Then, cells were incubated 2 h on ice and centrifuged for 10 min at 7000 rpm. The pellet was dissolved in 20 ml of RF1 solution and incubated again for 2 h on ice. Next, cells were centrifuged for 10 min at 7 000 rpm and the pellet was dissolved in 4 ml of RF2 solution. Finally, aliquots of 50 or 100 µl were frozen in liquid nitrogen and stored at -70 °C.

5.7.9 Heat shock transformation of *Escherichia coli*

An aliquot of 50 µl competent cells of *E. coli* XL-1Blue was placed on ice for 20 min. Afterwards, a suitable DNA concentration was added (< 10 µl) to the cells; the mixture was swirled and incubated on ice for 15 min. The mixture was heat-shocked without agitation at 42 °C for 60-90 seconds and placed on ice for 2-5 min. Then, 800 µl of LB medium were added to the tube and incubated at 37 °C, with

constant agitation at 200 rpm for 1 hour. Finally, 100 to 200 µl of the culture were spread on an LB plate with proper antibiotics and the plate was incubated at 37 °C ON.

5.7.10 PCR and sequencing

PCR was used to generation of plastid transformation vectors and for the characterization of transplastomic plants. To amplify DNA with high fidelity, the Phusion polymerase (Finnzymes, Espoo, Finnland) was used while the Dream Taq DNA polymerase from Fermentas (St. Leon-Rot, Germany) was used for standard PCR amplification according to the instruction manual. For the reaction mixture, a 25 mM dNTP mix was prepared from 100 mM dNTP´s set of Fermentas (St. Leon-Rot, Germany). Oligo primers were designed with the help of Primer 3 Input 0.4.0 software and synthesized by Metabion company (Martinsried, Germany). PCR amplifications were carried out in a Hybaid thermocycler (Heidelberg, Germany) with reaction settings and programming parameters as described in Tables 5.2 and 5.3. After amplification, 5 µl of each sample were characterized by gel electrophoresis.

Table 5.2 The reaction settings of PCR amplification

Components \ applications	DNA amplification with high fidelity	Identification of transplastomic plants
10 pmol/µl Primer (P1)	2.5 µl	1 µl
10 pmol/µl Primer (P2)	2.5 µl	1 µl
10 mM dNTP mixture	1 µl	1 µl
10 X or 5 X reaction buffer	10 µl	2.5 µl
Plant total DNA (50 ng~200 ng/µl)	2 µl	2 µl
Taq DNA polymerase (5 U/µl)	---	0.2 µl
Phusion DNA polymerase (3 U/µl)	0.5 µl	---
Distilled H_2O	31.5 µl	19.3 µl
Total volume	50 µl	25 µl

Table 5.3 The program settings of PCR amplification

Cycles	Applications	DNA amplification with high fidelity	Identification of transplastomic plants
1 cycle	Pre-denaturing	98 °C 4 m	94 °C 4 m
35 cycles	Denaturing	98 °C 30 s	94 °C 30 s
	Annealing	57 °C 30 s	55-60 °C 30 s
	Polymerization	72 °C 30 s	72 °C 30 s
1 cycle	Extension	72 °C 10 m	72 °C 10 s

DNA sequences were analysed, using the in-house sequencing facility (Martinsried, Germany).

5.7.11 Southern blot hybridization

Southern blots were carried out with the non-radioactive DIG method (DIG High Prime DNA Labeling and Detection Starter Kit II) from Roche (Mannheim, Germany), or with the standard radioactive method (Sambrook *et al.*, 2001). In either case 1-2 µg of probe were labeled according to the manufacturers´ instructions or to the Sambroock *et al*., (2001) instructions.

DNA was isolated from leaves of transplastomic plants growing in the culture chamber. About 2 µg of total DNA of each sample were completely digested with enzyme *Kpn*21 at 55 ^{o}C or *Nsi*I at 37 ^{o}C for 3-4 hours.

A 1 % agarose gel was prepared in TPE buffer (360 mM Tris, 300 mM Na_2HPO_4 H_2O, 70 mM EDTA) containing 0.5 mg/ml EtBr. The DNA digestion samples were loaded in a mixture with 6x DNA loading buffer in the gel slot and ran at 30-40 V overnight for optimal separation. The gel was documented and washed with distilled water. The gel was depurinated by submersion in 100 ml of 0.5 M HCl, in constant shaking at room temperature until the bromophenol blue marker changed from blue to yellow (10-15 min). The gel was then rinsed with double distilled water and denatured by incubating it twice with gentle shaking in a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 15 min at room temperature. Gels were immerged in a neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) two times, 15 min each, at room temperature with gentle shaking. Afterwards the gel was equilibrated for at least 10 min in 20 X SSC buffer (3 M NaCl, 0.3 M Na Citrate ($NaH_2C_6H_5O_7$), pH 7.0). For the transference of DNA from the gel to the membrane, a DNA blot unit (Figure 5.1), was prepared in the following way: about 8 pieces of Whatman 3 MM paper were soaked in 20 X SSC and used to form a "bridge" with one part of this paper in a shallow reservoir of 20 X SSC. The gel was placed on the top of the soaked sheets of Whatman 3 MM

paper. A sterile pipette was rolled over the gel to remove air bubbles. A piece of Hybond-N+ nylon membrane (Amersham, Braunschweig, Germany) was cut to the size of the gel and incubated for 5 minutes in 20 X SSC. The membrane was then placed above the gel and the pipette was used again to eliminate air bubbles as mentioned above. The blot assembly was completed by addition of a dry sheet of Whatman 3 MM paper, and a stack of paper towels, and finally a piece of 200 – 500 g weight on top. After 12-18 hours of overnight transfer, the Hybond-N+ nylon membrane was exposed to UV using the UV crossinglinker (Stratagene, Heidelberg, Germany) for DNA fixation.

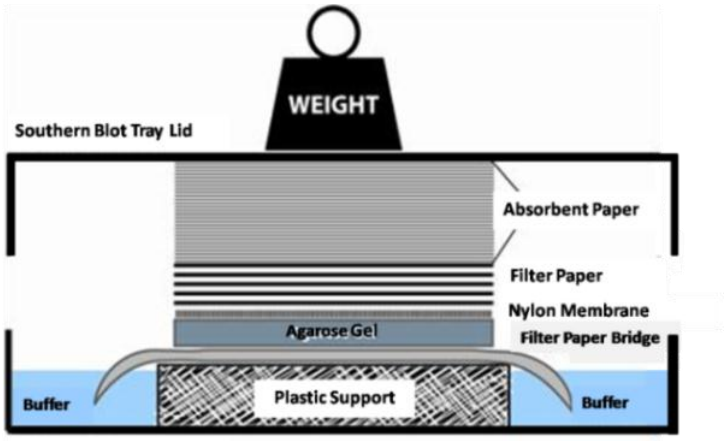


Figure 5.1 Southern blot tray (Image from www.gbiosciences.com)

The membrane was briefly rinsed in double distilled water. To prepare the hybridization buffer, 64 ml of sterile double distilled water were added to the Dig Easy Hyb granules (bottle 7) to dissolve them by stirring for 5 min at 37 ºC. Then, 30 ml of DIG-Easy Hyb buffer prewarmed to 45 ºC were added to the membrane and incubated at the same temperature for 1h in a rolling bottle incubator. The hybridization solution was prepared as follows: 20 µl of the labeled probe were incubated for 5 minutes in boiling water for denature and then the probe was chilled quickly on ice. Immediately, the labeled probe was added to a tube, containing 10 ml of prewarmed DIG-Easy Hyb buffer and mixed gentle by inversion. The DIG-Easy Hyp buffer was removed from the bottle, and 10 ml of

hybridization solution was added into the bottle containing the membrane. Hybridization was done at 45 °C overnight, in a rolling bottle incubator.

Detection:

Solutions:

Low Stringency buffer:	500 ml (2 X SSC + 0.1 % SDS): 50 ml 20 X SSC + 5 ml 10 % SDS
High Stringency buffer:	500 ml: (0.5 X SSC + 0.1 % SDS): 12.5 ml 20 X SSC + 5 ml 10 % SDS
Maleic acid buffer:	0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 with NaOH
Blocking solution:	12 ml 10 X Blocking solution + 108 ml Malein acid buffer.
Washing buffer:	0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 + 0.3 % Tween 20
Antibody solution:	Centrifuge Anti-Digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet 2 µl in 20 ml of Blocking solution 1 X (dilution 1:10 000 – prepare fresh 2h, 4 °C-).
Detection buffer:	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20 °C)

Detection was realized as follows

5.8 Plant tissue culture

Stock solutions for plant tissue culture were prepared with inorganic mineral elements (Table 5.4) and vitamins (Table 5.5).

Table 5.4 Composition of basal plant tissue culture media*

	Components in g/l	MS (Murashige and Skoog, 1962)	MS mod (Dovzhenko *et al.*, 1998)	B5 (Gamborg *et al.*, 1968)	B5 (G&E) (Gamborg and Eveleigh., 1967)	RS (Dovzhenko, 2001)	G & D (Gresshoff and Doy, 1972)
MACROELEMENTS 10 X	KNO_3	19	10.12	25		30	100
	$CaCl_2 \cdot 2H_2O$	4.4	4.4	1.5		0.15	
	$MgSO_4 \cdot 7H_2O$	3.7	3.7	2.5		12.33	1.7
	KH_2PO_4	1.7	1.7				
	$NaH_2PO_4 \cdot H_2O$			1.5			
	NH_4NO_3	16.5					100
	$(NH_4)_2SO_4$			1.34			
	NH_4PO_4						
	$Ca(NO_3)_2 \cdot H_2O$					0.708	23.6
	KCl						6.5
	KH_2PO_4					0.170	30
	$NaNO_3$					0.170	
MICROELEMENTS 100 X	NaEDTA	4		4			4
	KI	0.083		0.075			0.080
	H_3BO_3	0.620		0.300	0.300		0.03
	$MnSO_4 \cdot H_2O$	2.23		1	1		0.1
	$ZnSO_4 \cdot 7H_2O$	0.860		0.200	0.300		0.030
	$Na_2MoO_4 \cdot 2H_2O$	0.025		0.025	0.025		0.0025
	$CoCl_2 \cdot 6H_2O$	0.0025		0.0025	0.025		0.0025
	$CuSO_4 \cdot 5H_2O$	0.0025		0.0025	0.034		0.0025

* All components were prepared with distilled water and stored as 100 ml aliquots at -20 ºC.

Table 5.5 Amino acids and Vitamins

Components in g/l (100X)	MS (Murashige and Skoog, 1962)	B5 (Gamborg *et al.*, 1968)	PC (Glimelius *et al.*, 1986)	NT (Nagata and Takebe, 1971)	Cocktail 20 (Mere-Villanueva and Vazquez-Alejandro, 2003)	Ara (Márton and Browse, 1991)
Glycine	0.200				2.3	0.100
myo - Inositol	10	10	20	10	14.5	10
Nicotinic acid	0.050	0.100	0.200		0.150	0.050
Pyridoxine HCl	0.050	0.100	0.200		0.150	0.050
Thiamine HCl	0.010	1	0.100	0.100	0.300	0.500
Biotin			0.002		0.100	0.005
L - asparagine					1	
L - arginine					1	
L - aspartic acid					0.750	
Glutamine					0.60	
Glutamic acid					0.750	
Folic acid					0.100	
Riboflavine					0.010	
Urea					4.5	
$CaCl_2 \cdot 2H_2O$			20			
Ca-Pantothenate			0.200			

* All components were prepared with distilled water and stored as 100 ml aliquots at -20 ºC.

5.8.1 Shoot induction from seeds and cotyledons in *Arabidopsis thaliana*

Seeds from *Arabidopsis thaliana* C-24 were surface sterilized using the Cl gas sterilization method. About 30-40 mg of seeds were transferred into a 3 cm glass Petri dish and four Petri dishes were placed with their lid open in a desiccator under a fume hood. A beaker containing 100 ml of Calcium hypochlorite was placed in the desiccator next to the seeds. Then, 3-5 ml of concentrated HCl were added to the beaker to create chlorine gas. The desiccator jar was quickly sealed and in this way, the seeds were exposed to the chlorine gas for about 4 h. Finally, the Petri dishes containing seeds were transferred to a laminar flow hood and ventilated with the lid open for 1 h.

The composition of culture media used for plant regeneration from *Arabidopsis thaliana* is showed in Table 5.6. Induction and growth of calli from seeds was performed in a climate chamber with diurnal cycle of 16 h light (60 µmol/m^2s) at 25 °C followed by 8 h of darkness. Different PG callus induction media modified from (Negrutiu *et al.*, 1975) were used. To induce callus formation, PG1KN, PG1KD, PG20KN and PG20KD media were used as well as ARMI media (Márton and Browse, 1991). Upon incubating for four weeks, calli were transferred to the regeneration media SRA2N (Zhao *et al.*, 2002), SRAKN (Luo and Koop, 1997) and ARMIIc (Márton and Browse, 1991). Finally, for seed production, regenerated shoots were transferred to both root induction medium (RIM) (Luo, 1997) and seedling culture *Arabidopsis thaliana* medium (SCA) (Dovzhenko *et al.*, 2003).

The method of shoot regeneration from cotyledon explants has been modified from Zhao *et al.*, (Zhao *et al.*, 2002). Surface sterilized seeds were transferred into SCA medium and stored at 4 °C for 1-3 days. Germination was performed at a photoperiod of 16 h of light (130 µmol/m^2s) at 22 °C followed by 8 h of darkness at 20 °C. Cotyledons from 3, 6 and 8 days old seedlings were excised and transferred to callus induction medium (CIM). Explants were incubated for 2 days with 16 h light (100 µmol/m^2s) at 22 °C and 8 h dark. Cotyledons were transferred into a regeneration medium (SRAKN or SRA2N) under the same photoperiod

conditions. Finally, shoots were transferred to either RIM or to SCA medium for seed production.

Table 5.6 Germination and calli induction media in *Arabidopsis thaliana**

Components/l	SCA	PG1KN	PG1KD	PG20KN	PG20KD	CIM	ARMI
B5 salts	3.1 g						
MS salts						4.3 g	4.3 g
G&D Macro 10X		100 ml	100 ml	100 ml	100 ml		
B5 (G&E) Micro 100X		10 ml	10 ml	10 ml	10 ml		
B5 Vit 100X	10 ml						
MS Vit 100X						10 ml	
Ara Vit 100X		10 ml	10 ml				10 ml
Cocktail 20 100X				10 ml	10 ml		
$MgSO_4 \cdot 7H_2O$	1.14 g						
2,4 D (1mg/ml)			1ml		1ml		150 µl
Kin (1mg/ml)		50 µl	50 µl	50 µl	50 µl		
BA (1mg/ml)							600 µl
2ip (1mg/ml)						100 µl	300 µl
NAA (1mg/ml)		8 ml		8 ml		500 µl	
IAA (1 mg/ml)							3 ml
Sucrose	20 g						
Glucose		20 g	20 g	20 g	20 g		30 g
Agar		8 g	8 g	8 g	8 g	6 g	8 g
Gelrite	3 g						

*All media were adjusted to pH 5.8 and fill up to 1 l with distilled water, media were sterilized at 120 °C for 20 min.

Table 5.7 Shoots and roots induction media in *Arabidopsis thaliana**

Components/l	SRAKN	SRA2N	ARMIIc	RIMI	RIMII
MS salts	4.3 g	4.3 g	4.3 g	4.3 g	4.3 g
MS Vit 100X	10 ml	10 ml		10 ml	10 ml
Ara Vit 100X			10 ml		
Kin (1mg/ml)	2 ml				
2ip (1mg/ml)		900 µl	3 ml		
NAA (1mg/ml)	50 µl	100 µl	100 µl		1 ml
IBA (1mg/ml)				1 ml	500 µl
Sucrose	30 g	30 g	30 g	30 g	15 g
Agar	6 g	6 g	8 g	8 g	8 g

* All media were adjusted to pH 5.8 and fill up to 1 l with distilled water; media were sterilized at 120 °C for 20 min.

5.8.2 Protoplast isolation, embedding and culture in *Arabidopsis thaliana*

Protoplast culture was modified from Dovzhenko *et al.*, (2002). About 30-40 mg seeds were surface sterilized (see 5.8.1). Seeds were transferred to a 9 cm Petri dish containing SCA medium and stored at 4 ^{o}C for 1-3 days to break dormancy. Germination was performed with 16 h of light (130-150 µmol/m^{2}s) at 22 °C followed by 8 h dark at 22 °C, during 6-7 days. The seedlings were incubated at 4 ^{o}C in darkness during 1-3 days.

The media, employed for protoplast cultures are listed in Table 5.8. For pre-plasmolysis, cotyledons were excised and placed in a Petri dish containing 5 ml of MMC medium. After one hour incubation, cotyledons were cut into small pieces with a scalpel, transferred to a new Petri dish and washed once with 1-2 ml of MMC medium.

Subsequently, the MMC medium was removed and cotyledons were incubated in 5 ml of MMC supplemented with 50 mg/l cefotaxime, 0.5 % of cellulase R10 and 0.5 % of macerozyme R10 (10 % stock solutions of cellulase R10 and mecerozyme R10 were prepared with 13.4 % of sucrose in sterile distilled water, and sterilized by filtration) in the dark at 25 °C for 12-16 h (Figure 5.2).

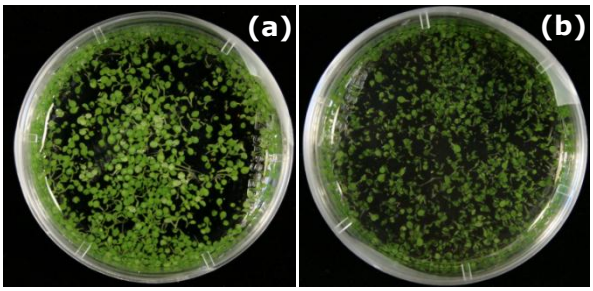


Figure 5.2 Cotyledons of *Arabidopsis thaliana* prepared for enzyme digestion. **(a)** Preincubation of cotyledons on a 6 cm Petri dish containing MMC medium. **(b)** Incubation of cotyledons on a Petri dish containing MMC medium with macerozyme R10 and cellulase R10.

Afterwards, the incubation mixture was passed through a stainless steel sieve (pore size, 100 µm). The filtrate was adjusted to 10 ml with MMC and centrifuged for 10 min at 50 g. The pellet was then resuspended in 10 ml of MSC medium, and 2 ml MMM medium were layered on top. After floatation (Figure 5.3), for 10 min

at 70 g, protoplasts were collected from the interface and transferred to a new centrifuge tube.

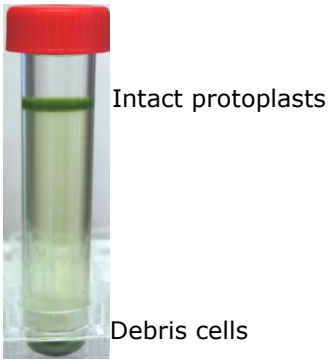


Figure 5.3 Protoplast floatation. Isolation of intact protoplast after 10 min centrifugation at 70 g

The volume was adjusted to 10 ml with MMM. From this suspension, 20 µl were transferred to a Haemocytometer (Fuchs-Rosenthal, Germany). Protoplasts on 5 big squares were counted and the average was calculated. To determine protoplast density, the average was multiplied with the dilution factor (10 ml= 50,000). Thereafter, protoplast suspension was pelleted for 10 min at 50 g, the supernatant was discarded and the protoplasts were resuspended in MMM and mixed with the same volume of F-Alginat at a density of 1-1.5 X 10^5 pp/grids. Protoplasts were immobilized in TAL (Thin Alginate Layers) as described by Luo and Koop (1997), 625 µl of protoplast alginate mixture were transferred to the surface of a Petri dish containing Ca-agar. A polypropylene grid of 10x10 meshes (Büttner, Wasserburg, Germany) was inserted into the protoplast-alginate mixture and incubated for 30 min.

After alginate solidification, grids with embedded protoplasts were placed upside down into Petri dishes containing 10 ml of *Arabidopsis thaliana* culture medium (PCA) for 30 min. Grids were subsequently transferred to new Petri dishes (6 cm) with 4 ml PCA medium. After formation of protoplast derived colonies, grids were placed on 9 cm Petri dishes containing SRAKN media. The regenerated shoots were rooted on RIM medium.

Table 5.8 Media to isolation and embedding of *Arabidopsis thaliana* protoplasts

Medium	Reference	Purpose	Basal medium	Compositions (mg or ml/l)
MMC	Luo, 1997	Protoplast floatation	---	1.952 g MES 1.47 g $CaCl_2 \cdot 2H_2O$ ≈ 85 g mannitol to 600 mOsm
MMM	Dovzhenko *et al.*, 1998	Protoplast embedding	---	1.952 g MES 1.020 g $MgCl_2 \cdot 6H_20$ 1.250 g $MgSO_4 \cdot 7H_2O$ ≈ 85 g mannitol to 600 mOsm
MSC	Luo, 1997	Protoplast floatation	---	1.952 g MES 1.470 g $CaCl_2 \cdot 2H_2O$ ≈ 135 g sucrose to 600 mOsm
F- Alginat	Luo, 1997	Protoplast embedding		1.370 g MES 2.500 g $MgSO_4 \cdot 7H2O$ 2.040 g $MgCl_2 \cdot 6H_20$ ≈ 80 g mannitol to 600 mOsm 2.800 g alginate
Ca-Agar	Luo, 1997	Protoplast embedding		2.940 g $CaCl_2 \cdot 2H_2O$ 10.00 g MES ≈ 85 g mannitol to 600 mOsm 10.00 g Agar
PCA	Modified from Dovzhenko *et al.*, 2003	Protoplast culture in *Arabidopsis*	B5 Salts (3.1g) B5 Vitamins 100 X (10 ml)	0.746 g $MgSO_4 \cdot 7H_2O$ 0.450 g $CaCl_2 \cdot 2H_2O$ 0.050 g L-glutamine 20.00 ml Coconut water 500 µl NAA (1mg/ml) 100 µl 2ip (1mg/ml) ≈ 85 g Glucose to 600 mOsm

All media were adjusted to pH 5.8. F-Alginat and Ca-agar were autoclaved, and the other media were sterile filtrated.

5.8.3 Plant tissue culture in Rapid-Cycling *Brassica rapa*

Seeds from Rapid-Cycling *Brassica rapa* (RCBr) C1 and B3 were surface sterilized with 70 % ethanol (v/v) for 1 min and then treated with 5 % (w/v) Dimanin C and one drop of Tween-20 for 10 min. After this treatment, seeds were washed three times with autoclaved distilled water for 10 min.

Media used for RCBr regeneration are described in Table 5.9. For each regeneration experiment, approximately 10 seeds were germinated on solid MS medium with 16 h of light (130 µmol/m^2s) at 22 ^oC followed by 8 h of darkness at 22 °C, or in RS medium with 16 h light (60 µmol/m^2s) at 25 ^oC followed by 8 h in the dark. Calli were induced from various explants. Cotyledons, hypocotyls, and roots were cut from 7 and 14 days old seedlings. For explant preparation, a stereoscope Stemi SR (Zeiss, Göttingen, Germany), was used. Cotyledon and root explants were prepared in a way that prevents presence of apical meristems

(Figure 5.4). Hypocotyls explants of approximately 2-4 mm size were cut at their sagittal axis to expose the vascular tissues.

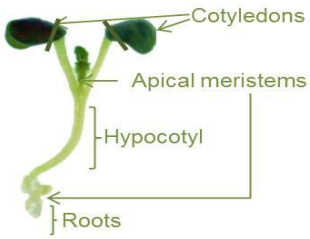


Figure 5.4 Seedling from 7 days old Rapid-Cycling *Brassica rapa*

For regeneration experiments, around 20 segments were transferred to a Petri dish containing MD1 or MDK media with 16 h of light (60 µmol/m²s) at 25 °C followed by 8 h of darkness. After 2-4 days, hypocotyl explants in MD1 medium were transferred to BD1 medium keeping the same growth conditions for calli induction. After 7 days on BD1 medium, explants were transferred to SIM medium for two weeks for the induction of shoots. For the induction of shoots, explants on MDK medium, were transferred to an MZI medium after two weeks and kept there for additional three weeks. For roots formation, shoots were transferred to RIM medium. Finally, after rooting, plants were transferred to RS medium to allow seeds formation or to the greenhouse with photoperiod of 16 h of light (60 µmol/m²s) at 25 °C followed by 8 h of darkness.

Table 5.9 Rapid-Cycling *Brassica rapa* tissue culture media

Components/l	**MS**	**RS**	**MDN** (Teo *et al.*, 1997)	**MD1**	**BD1**	**SIM** (Jonoubi *et al.*, 2005)	**MDK***	**MZI*** (Chen and Celio, 2000)
MS Salts	4.3 g		4.3 g	4.3 g			4.3 g	4.3 g
B5 Salts					3.1 g	3.1 g		
RS Macro 10 X		100 ml						
MS Micro 100 X		10 ml						
MS Vitamins 100 X	10 ml	10 ml	10 ml				10 ml	10 ml
B5 Vitamins100 X					10 ml	10 ml		
Myo inositol				100 mg				
Thiamine- HCl				1.3 mg				
KH_2PO_4				200 mg				
2,4 D (1mg/ml)				1 ml	1 ml		500 µl	
BA (1mg/ml)			4.5 ml			4.5 ml		
Kinetin (1mg/ml)							200 µl	
IAA (1mg/ml)								500 µl
Thidiazuron (1mg/ml)						300 µl		
Zeatin (1mg/ml)								2 ml
NAA (1mg/ml)			400 µl					
Sucrose	30 g	20 g		30 g	30 g	10 g	30 g	30 g
Agar	7 g			6 g	6 g	7g		
Phyto Agar							7 g	7 g
Gelrite		3 g						

*Media were prepared to pH 5.8 as a double concentrated stock and filter sterilized; the media were mixed 1:1 with double concentrated Agar (1.4%). The other media were adjusted to pH 5.8 and autoclaved.

5.8.4 Plant tissue and protoplast culture in *Nicotiana tabacum*

Seeds from *Nicotiana tabacum* cv. Petite Havana were surface sterilized with 70 % ethanol (v/v) for 1 min and then treated with 5 % (w/v) Dimanin C for 10 min. Seeds were washed three times for 10 min with autoclaved distilled water. Tobacco seeds were geminated on modified B5 medium (Table 5.10) at 25 ^{o}C with photoperiod 16 h light (60 μmol/m^{2}s) at 25 ^{o}C followed by 8 h dark. After one week, seedlings were isolated and growth during 3 weeks with the same growing condition.

Tobacco regeneration was obtained from leaves on RMOP medium (Svab *et al.*, 1990) or from protoplast derived colonies as described elsewhere (Dovzhenko *et al.*, 1998). For tobacco protoplast isolation, leaves from 2-3 weeks old plants were cut into approximately 1 mm stripes. For pre-plasmolysis, strips were incubated for one hour in a 9 cm Petri dish containing F-PIN medium (Table 5.10), for cell wall degradation, medium was discarded and changed for an F-PIN medium containing 0.25 % cellulase R-10 and 0.25 % macerozyme R-10 (see 5.8.2). Cells were further incubated in darkness at 25 °C for 14-16 h.

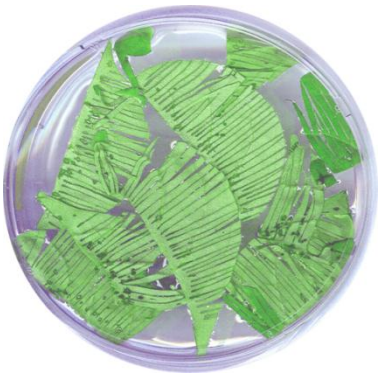


Figure 5.5 *Nicotiana tabacum* leaf strips in F-PIN medium

The incubation mixture was passed through a stainless steel sieve (pore size, 100 μm) and the filtrate was adjusted to 10 ml using F-PIN medium and then overlayered with 2 ml of MMM medium (Table 4.8, osmolarity was adjusted to 550 mOsm). Followed by centrifugation for 10 min at 50 g intact protoplasts were

removed and adjusted to 10 ml with MMM medium. Counting and embedding of protoplasts was realized as described before in section 5.8.2. Protoplasts were embedded at a density of 6 X 10^4 pp/grids. Grids with embedded protoplasts were placed upside down in a 6 cm Petri dish containing 10 ml of F-PCN medium (Table 5.10). After 30 min of incubation, grids were transferred into a new Petri dish (6 cm in diameter) with 2 ml of F-PCN medium. After protoplast derived colonies were formed, grids were placed on 9 cm Petri dishes containing solid RMOP medium. Regenerated shoots were rooted on hormone-free B5 mod medium.

Table 5.10 *Nicotiana tabacum* tissue culture media

Components / l	**B5 mod** (Dovzhenko, 1998)	**F-PIN** (Dovzhenko, 1998)	**F-PCN** (Dovzhenko, 1998)	**RMOP** (Svab *et al.*, 1990)
MS Basal medium				4.4 g
B5 Basal medium	3.1 g			
Macro MS mod 10X		100 ml	100 ml	
Micro MS 100 X		10 ml	10 ml	
NT Vitamins 100 X				10 ml
B5 Vitamins 100 X	10 ml			
PC Vitamins 100 X		10 ml	10 ml	
NH_4- Succinat (2M)[a]		10 ml	10 ml	
$MgSO_4 \cdot 7H_2O$	0.983 g			
MES		1.952 g	1.952 g	
BAP (1mg/ml)		1 ml	1 ml	1 ml
NAA (1mg/ml)		100 µl	100 µl	100 µl
Sucrose	20 g	≈130 g	20 g	30 g
Glucose			≈65 g	
Agar	8.0 g			6 g

All media were adjusted to pH 5.8. B5 mod and RMOP were autoclaved and the other media were adjusted to 550 mOsm and sterile filtrated with 0.2 µm-Filter. [a] 2 M Ammoniumsuccinat was prepared according to (Dovzhenko *et al.*, 1998): 23.6 g Succinic acid, 10.6 g NH_4Cl, approximately 22.4 g KOH to pH 5.8 adjusted to 100 ml and the solution was sterile filtrated.

5.9 Plant transformation

5.9.1 PEG treatment of protoplasts

The polyethylene glycol (PEG) method for nuclear or plastid transformation was performed as described previously (Koop *et al.*, 1996).

Solutions:

Transformation medium: 1l

$MgCl_2 \cdot 6H_2O$	3,045 g
MES	1.0 g
Manitol	~ 85 g to 550 mOsm
Distilled H_2O to 1 l	
pH 5.6 and sterile filtrated.	

40 % PEG solution: 26 ml

Manitol	1.275 g
$Ca(NO_3)_2 \cdot 4H_2O$	0.413 g
PEG-1500	10 g
Distilled H_2O to 26 ml mix to dissolve	
pH 9.75 and sterile filtrate d	

Protoplasts from *Arabidopsis thaliana* (see 5.8.2) or tobacco (see 5.8.4) were isolated, 1 X 10^6 pp were suspended in 100µl of Transformation medium and subsequently transferred to a 3 cm Petri dish made of glass. 50 µg of DNA, dissolved in 18 µl TE pH 5.6, were mixed with protoplasts and 7 µl of F-PCN (tobacco) or PCA (*Arabidopsis thaliana*). Immediately, 125 µl of PEG were added drop wise and incubated for 7.5 min. Afterwards, 125 µl of F-PCN or PCA were added and incubated for 2 min. Finally, 2,6 ml of F-PCN or PCA were added and protoplasts were incubated for 12 h at 25 ^oC in the dark. Protoplasts were embedded with alginate as described previously in 5.8.2 (*Arabidopsis thaliana*) or 5.8.4 (tobacco). After 7 days in liquid culture media, protoplast derived colonies were transferred to regeneration media with appropriate antibiotics for the selection of plants.

5.9.2 DNA transfer using the particle gun method

For particle gun experiments, a standard protocol was used. Sterilization of particle gun chamber, microcarrier launch assembly, rupture disc holder, target shelf, macrocarrier holder, macrocarriers and stopping screens was performed with 100 % EtOH. Rupture discs were sterilized individually by washing them in 75 % isopropanol for 5-10 s. Subsequently, all components were air dried in the flow bench. Then, 60 mg of Gold particles (0.6 µm particle size from Bio-Rad, USA) were suspended in 1 ml EtOH p.a. The gold particle suspension was vortexed for at least 1 min and then 35 µl were added immediately into a new sterile 1.5 ml Eppendorf tube. The gold was spun down at 13 000 rpm for 10 s and the supernatant was removed. Working on ice, 230 µl sterile dH_2O were mixed with the gold pellet. Because the gold needs to be resuspended, this is best

achieved by a combination of vortexing and scraping from the wall with a Gilson tip. After addition of 250 µl of 2.5 M $CaCl_2$ (stored in aliquots at -20 °C) and its subsequent vortexing, 25 µl of DNA (1.0 µg/µl in water) were added. Once again, the sample was mixed with a vortex for a few seconds, and 50 µl of 0.1 M spermidine (aliquots -70 °C) were added. After vortexing, the mixture was incubated 10 min on ice and vortexed again every 2 min. The gold was spun down for 1 min at 13 000 rpm, and the supernatant was removed. The gold was washed 2 times with 600 µl of 100 % EtOH p.a (between each step a centrifugation for 1 min was necessary). It is very important that the fine gold is removed from the wall and that the pellet is broken up. Finally, the gold was resuspended in 72 µl of 100 % EtOH p.a and placed on ice.

Shooting parameters:

Pressure of helium:	1100 psi (7584.17 Kilopascal)
Rupture discs:	900 psi (6205.2 Kilopascal)
Distance rupture disc-macrocarrier:	8-10 mm
Distance macrocarrier-stopping screen:	10 mm
Distance stopping plate-leaf:	7 cm
Vacuum:	27 – 28 inches Hg (91.4-94.8 Kilopascal)

5.4 µl of the DNA-coated gold suspension were applied to the middle of each macrocarrier and air-dried completely. The macrocarrier was placed into the macrocarrier holder. The rupture disc, the stopping screen and the macrocarrier holder were deployed correctly in the vacuum chamber. The Petri dish containing plant material was placed at the right position inside the chamber, the chamber door was closed and the vacuum was turned on until 26-27 inches Hg. The pressurized helium tank was open and bombardment was realized according to Bio-Rad instructions. Then, plant material was removed and transferred into a clime chamber for one or two days before selection.

5.10 Selection conditions

PEG in Arabidopsis and tobacco: After the initial 6-8 days of protoplast culture, 500 mg/l spectinomycin for tobacco or 20-40 mg/l spectinomycin for *Arabidopsis thaliana* were included in solid regeneration media for the selection of colonies.

After bombardment: Plant material was transferred to selection medium supplemented with antibiotic, 3 days after bombardment. Resistant colonies were collected after 4-5 weeks of culture and then transferred to same fresh media:

Selection	**Tobacco**	***Arabidopsis***	**RCBr**
Kanamycin		10 – 40 mg/l	
Spectinomycin	500 mg/l	10 – 40 mg/l	10-40 mgl/l
Phosphinothricin		1 – 7 mg/l	
Bialaphos		2 – 10 mg/l	

5.11 Microscopy

In order to characterize putative plastid transformed lines, epidermal peelings were prepared from young leaves using water. To analyze chlorophyll autofluorescence (excitation: 543 nm, emission: 580 nm) and AmCyan fluorescence (excitation: 543 nm, emission: 580 nm), samples were analyzed with a fluorescence microscope from Leica Microsystems (DM 1000). Finally, to acquire better quality pictures, a Leica TCS SP2 spectral laser-scanning confocal microscope (Leica microsystems, Wetzlar, Germany) was employed.

6 Results

The present work focuses on the establishment of a protocol for plastid transformation in plant model organisms. Therefore, regeneration protocols in *Arabidopsis thaliana,* Rapid-Cycling *Brassica rapa* and *Nicotiana tabacum* were established, plant specific vectors with different insertion sites were constructed and finally, a PEG (Polyethylene glycol) or biolistic transformation method was used for the transformation of the plants.

6.1 Towards plastid transformation in *Arabidopsis thaliana*

For plastid transformation experiments in *Arabidopsis thaliana* different experimental approaches were used: 1. A reliable regeneration protocol was established. 2. Plant specific vectors for plastid transformation were constructed. 3. Vectors with different fluorescence and selection markers were tested. 4. Two different transformation methods were performed.

6.1.1 Regeneration from cotyledons in *Arabidopsis thaliana*

For regeneration experiments, cotyledon explants from 3-9 days old *Arabidopsis thaliana* ecotype C-24 were used. As expected, shoot regeneration was reduced dramatically in old explants (Figure 6.1-a) as described by Zhao *et al*., (2002). The best regeneration was obtained with 3 days old cotyledons. However, 3 days old seedlings are not easy to manipulate and to use in particle gun experiments. In this work, 6-7 days old seedlings were used for the establishment of regeneration protocols. Preculture of cotyledons on callus induction medium (CIM) for 2-4 days (section 5.8.1) improved explant regeneration greatly in comparison with cotyledon shoot regeneration *Arabidopsis* medium (SRAKN). It almost doubled the regeneration efficiency from 45 % to 85 % (Figure 6.1-b).

Approximately 3 to 4 shoots were obtained from 6 days old explants after incubation of the cotyledons on CIM for two days and subsequent incubation in SRAKN medium for 3 weeks. Although silver nitrate is widely used to improve

regeneration efficiencies in Brassicaceae, in our experiments the addition of silver nitrate had a negative effect on regeneration. This effect was very severe in the case of CIM/SRAKN in which regeneration efficiency was reduced from 85 % to 28 % (Figure 6.1-b).

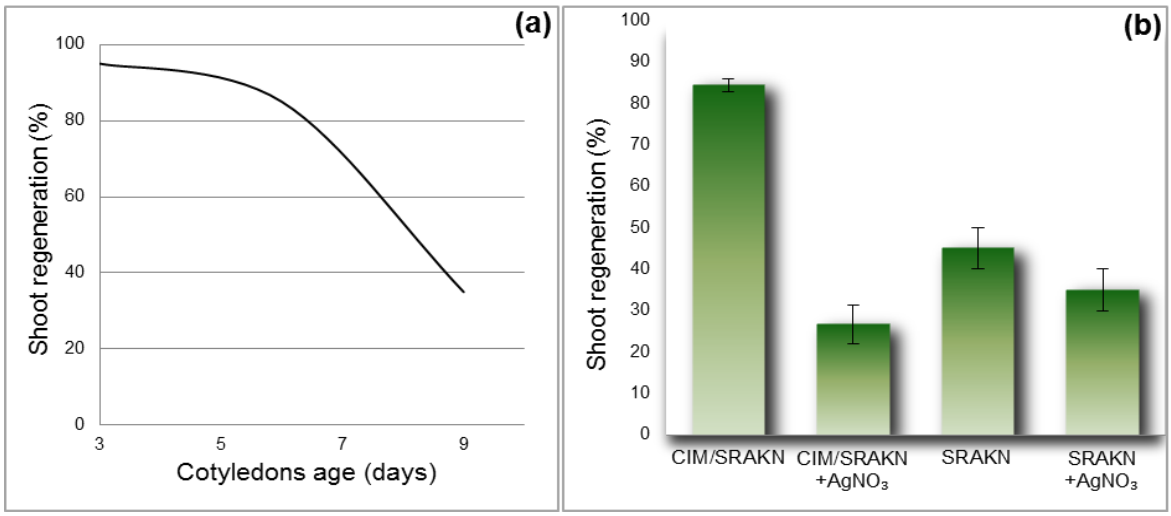


Figure 6.1 Regeneration from cotyledons in *Arabidopsis*. **(a)** Effect of explant age on regeneration efficiency. **(b) I**nfluence of different media on shoot regeneration from cotyledons in *Arabidopsis thaliana* C-24 after three weeks on the indicated medium.

Exposure of regenerated shoots on a root induction medium for 10 days increased root formation in greater than 90 %. These regenerated plants were able to mature into fertile plants. Figure 6.2 shows the complete regeneration cycle starting from cotyledons to fertile *Arabidopsis thaliana* C-24 plants.

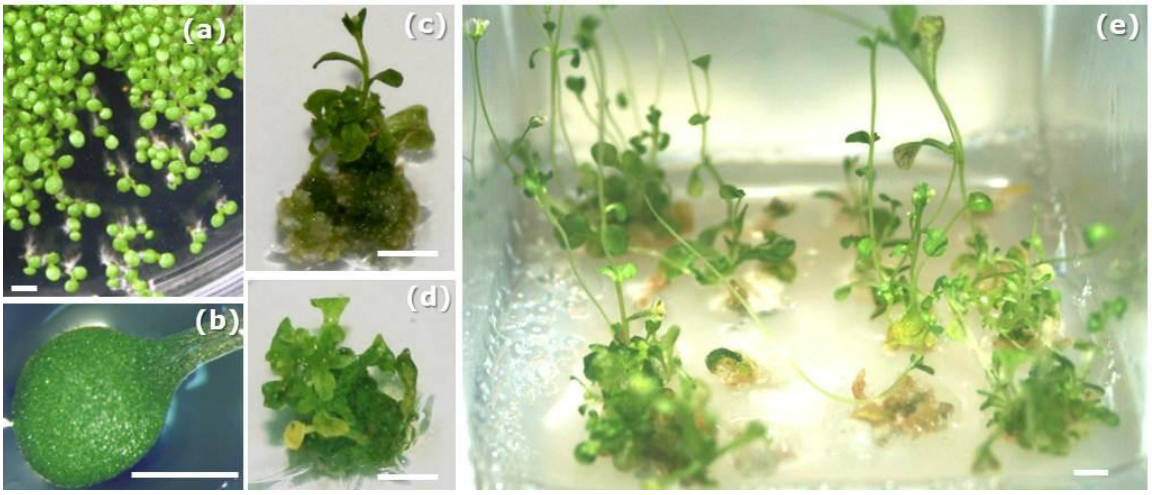


Figure 6.2 *Arabidopsis thaliana* C-24 regenerated from cotyledon explants. **(a)** Seedlings growing on SCA germination medium. **(b)** Cotyledon on CIM medium for callus induction. **(c)** Shoot formation was induced on CIM/SIM media after 20 days. **(d)** Shoot formation was induced on SIM medium after 20 days. **(e)** Mature plants on MB medium for seed production. Bars: 3 mm.

6.1.2 Regeneration of *Arabidopsis thaliana* from seed explants

Regeneration from *Arabidopsis thaliana* C-24 using seeds as explants was already described (Negrutiu *et al*., 1975), however, we improved the seeds regeneration method. Media components are described on section 5.8.1. In summary, two protocols were used, one protocol was based on Márton and Browse (1991) and the other one based on PG derived media from Negrutiu *et al*., (1975).

Based on Márton and Browse method: *Arabidopsis thaliana* C-24 seeds were germinated on ARMI medium (Table 5.6). After 10-12 days on ARMI medium, callus formation was analysed and two characteristic types of seedlings were observed. Seedlings were either short with abundant callus (Figure 6.3-a) or seedlings were longer with a small callus formation between hypocotyl and roots (Figure 6.3-b). After two weeks on ARMI medium, almost 90 % of seedlings produced calli. Those calli were transferred to fresh medium for additional two weeks. Calli from ARMI medium were abundant and compact as described before (Azhagiri and Maliga, 2007). For shoot induction, four weeks old calli were transferred to SRAKN, SRA2N and ARMIIc regeneration media (Table 5.7).

After 20 days on ARMIIc medium, just small shoots were obtained in approximately 50 % (Figure 6.3-c). Best results were obtained when calli were transferred to SRAKN medium with up to 83 % of regeneration (Table 6.1).

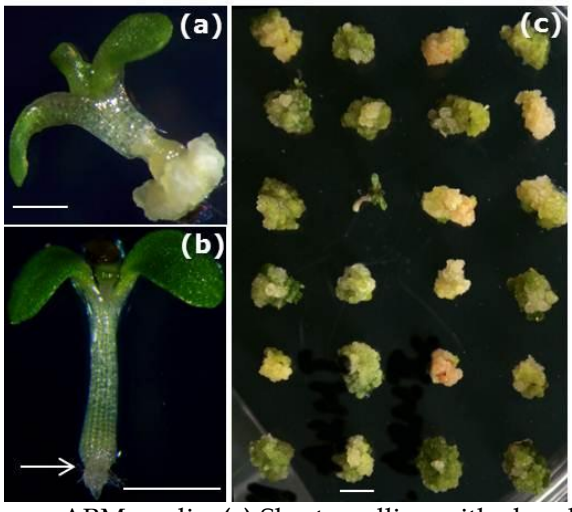


Figure 6.3 Callus induction on ARM media. **(a)** Short seedling with abundant calli, after 12 days on ARMI medium. **(b)** Long seedling with small calli (arrow) between roots and hypocotyl after 12 days on ARMI medium. **(c)** Abundant calli after 20 days on ARMIIc medium. Bars: 3 mm

Additionally to ARM media, *Arabidopsis thaliana* C-24 seeds were germinated on PG derived media (based on Negrutiu *et al*., 1975). Therefore, seeds were placed on PG1KD, PG20KD, PG1KN and PG20KN media (Table 5.6) either in the darkness or in the light. Seeds germinated to give small seedlings within 3-4 days. As described by Huang and Yeoman, (1985), callus was initiated from hypocotyl and from roots of the seedlings and became visible after 12-15 days (Figure 6.4-j). Around 80-95 % of the plated seeds produced calli on PG derived media as described in Table 5.1. Seedlings on PG derived media with Ara or B5 Vitamins, usually were translucent, however, in presence of Cocktail 20, seedlings were opaque (Figure 6.4). After 3-4 weeks on PG derived media, callus from seeds were almost 30 times bigger.

Seed derived callus could be induced on all media, without distinction between use of auxins or vitamins. Callus induced on PG derived media containing NAA (8 mg/l NAA) looked hairy, rather compact, and had root-like formations on its surface as described previously (Negrutiu *et al*., 1975).

After one month on callus induction media, calli were transferred to SRAKN, SRA2N and ARMIIc regeneration media (Table 5.7). One week on regeneration media was sufficient for shoot regeneration (Figure 6.4). Seeds which were germinated under light gave the best results. Shoots were quantified and results are presented in Table 6.1. In summary, best regeneration efficiency was achieved with the following combination of media: four weeks on PG20KN and subsequent transfer to SRA2N medium (up to 90 %) or four weeks on PG20KD and transfer to SRA2N medium with up to 93 % or SRAKN medium with up to 97 % regeneration (Table 6.1).

Table 6.1 Regeneration from seed derived callus

Calli media	Calli induction (%)	Shoot regeneration (%)		
		SRAKN	SRA2N	ARMIIc
PG1KN	85 ± 5	50 ± 5	50 ± 5	---
PG1KD	90 ± 5	81 ± 5	66 ± 1	87 ± 1
PG20KN	85 ± 5	20 ± 5	85 ± 5	---
PG20KD	90 ± 5	**97± 1**	92 ± 1	90 ± 2
ARMI	85 ± 5	83 ± 10	80 ± 7	50 ± 10

Regeneration obtained with the combination of PG1KN–SRAKN or SRA2N was not always reliable (Table 6.1). Low regeneration (approximately 20 %) was obtained with PG20KN–SRAKN combination media, meaning that many roots were formed (Figure 6.5-f). However when calli was transferred from PG20KN to SRA2N medium, approximately 85 % of all calli were able to produce shoots. With this combination (PG20KN-SRA2N) 2-4 shoots per explant were obtained (Figure 6.5-e).

Callus induced on media containing 2,4-D as auxin grow rapidly and presented a better friability. Regeneration between 3 to 7 shoots per explant were obtained either with cocktail 20 or Ara vitamins (Figure 6.4). Although, number of shoots was similar in all cases, best regeneration results were achieved with PG20KD-SRAKN with up to 97 %of regeneration. This PG20KD medium contains cocktail 20 a mixture of nitrogen compounds and vitamins (Mere-Villanueva and Vazquez-Alejandro, 2003). Addition of cocktail 20 in tissue cultures of carrot (*Daucus carota*) improved significantly the regeneration efficiencies (Rosales-Mendoza, 2007). In *Arabidopsis thaliana* up to 97 % of regeneration efficiencies was obtained. This represent the number of seed derived calli producing shoots. If the numbers of shoots per explant are included, we obtained regeneration efficiencies between 500 to 700 % (Figure 6.4).

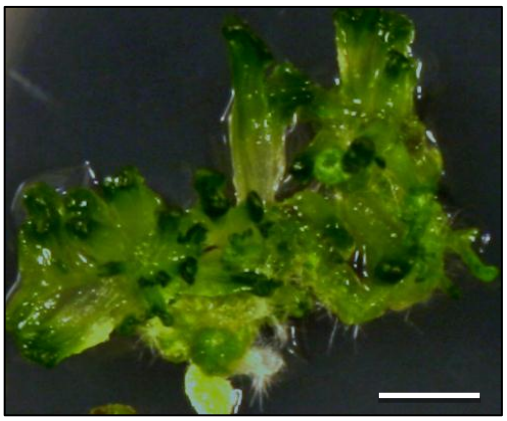


Figure 6.4 Indirect shoot organogenesis from seed derived calli in *Arabidopsis thaliana* C-24. Combination PG20KD/SRAKN media. Bar: 5 mm.

Shoots were transferred to a root induction media (Table 5.7) or to a hormone free medium (SCA, see table 5.6). Healthy fertile plants were obtained from all media combination. Figure 6.5-m shows an example from healthy fertile plant obtained from seeds derived calli.

Regeneration capacity from seed derived calli remained stable for at least 6 weeks old calli (data not shown).

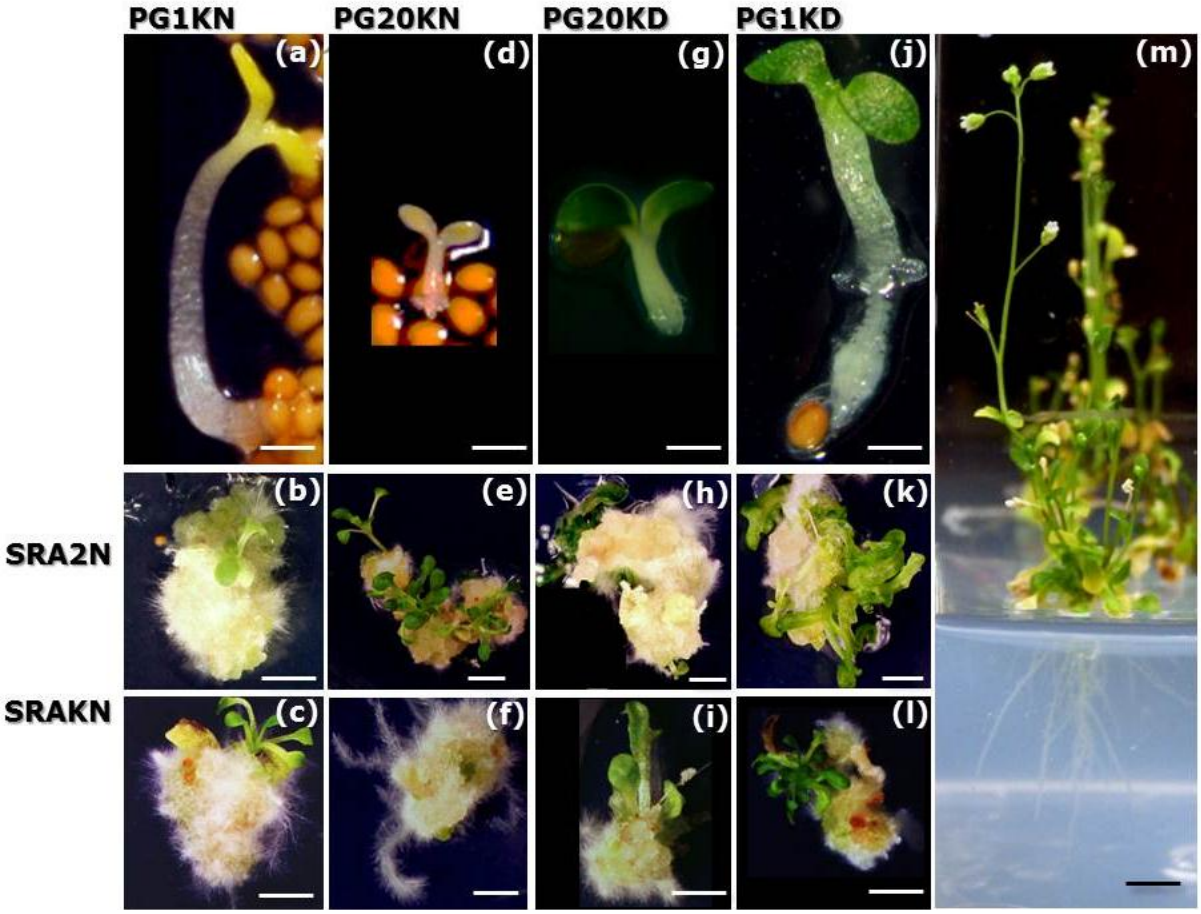


Figure 6.5 Shoot regeneration from seed derived callus of *Arabidopsis thaliana* C-24. **(a, d, g, j)** 10 days old seedlings on PG1KN, PG20KN, PG20KD and PG1KD respectively. **(b, e, h, k)** regeneration from one month old calli, after 12 days on SRA2N and **(c, f, i, l)** on SRAKN medium (**m**) Mature plant growing on SCA medium after 3 weeks. Bars: 1 mm

6.1.3 *Arabidopsis thaliana* protoplast culture and regeneration

After establishing a protocol for the regeneration of plants from cotyledons and seeds the next step was to regenerate plants from cotyledon protoplasts.

Regeneration from *Arabidopsis* cotyledon protoplasts was reliable using a modified method from our group (Dovzhenko *et al.*, 2003). The main modifications consisted in seeds sterilisation with Cl_2 gas, germination with intensive light (130-150 µmol/m^2s^1), modification of Protoplast culture medium (PCA) and finally addition of Cefotaxime (50 mg/l) on germination and on PCA medium to avoid contamination. Dovzhenko *et al.*, used Dicamba and NAA as phytohormones in the presence of casein. In this work presence of casein was avoided and Cytokinin 2-ip was used instead auxin Dicamba. Platting efficiencies were increased up to 40-60 %, almost twice as reported by Dovzhenko *et al.*, (2003).

In the present work 2,4-D (2,4-Dichlorophenoxyacetic acid) (1mg/l), a synthetic auxin which supports dedifferentiation and cell division in plant tissues was tested in PCA medium. This also increased platting efficiencies, however, protoplast derived colonies were not able to produce many shoots on SRAKN or SRA2N media (data not shown). Normally, first cell divisions were found after 1 or 2 days on PCA medium (Figure 6.6).

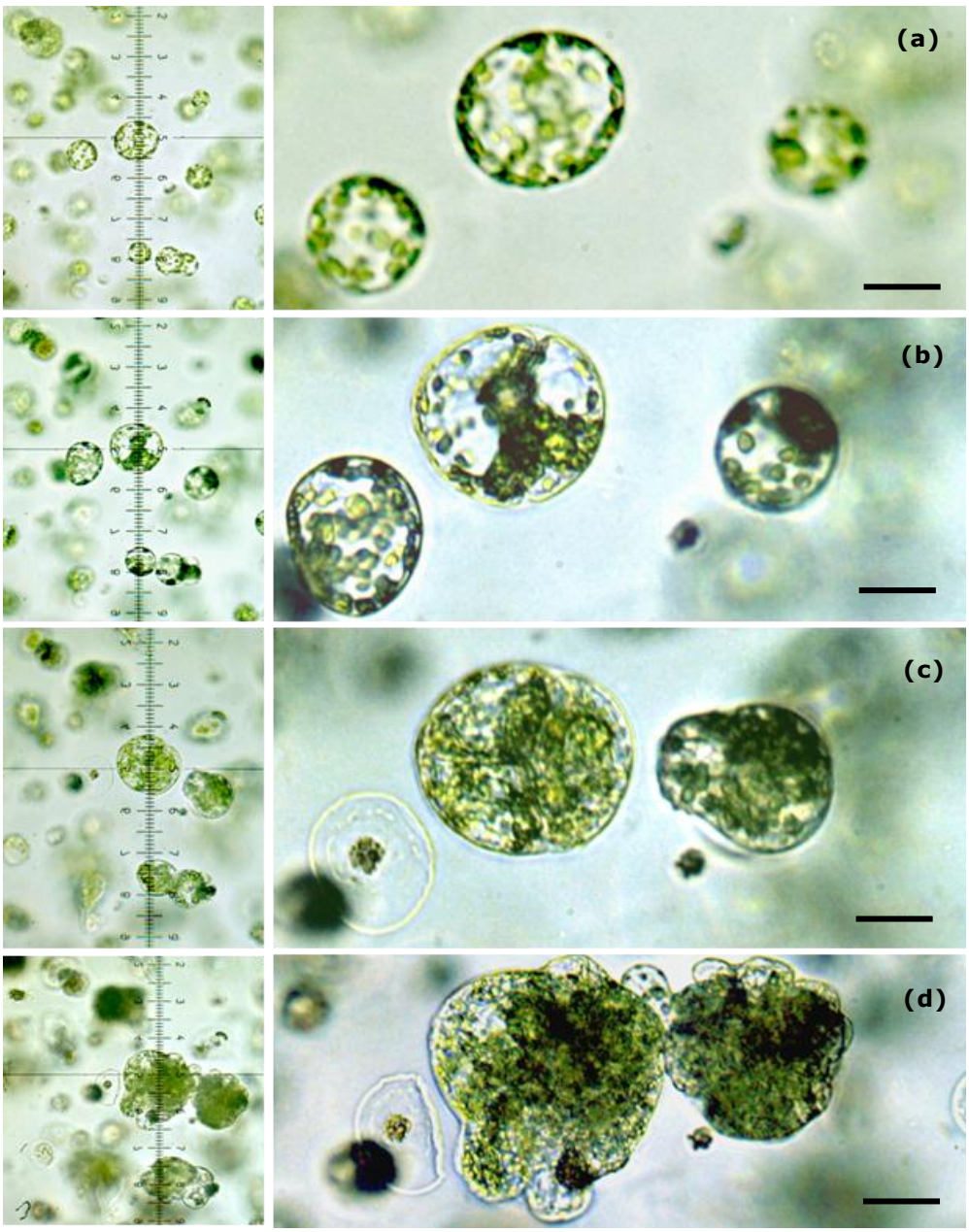


Figure 6.6 *Arabidopsis thaliana* C-24 cotyledon protoplasts **(a)** Cotyledon protoplasts, one day after isolation. Cotyledon protoplasts cell divisions three **(b)**, five **(c)**, seven **(d)** days after protoplast isolation. Bars: 10 µm

5-7 days after protoplast isolation, protoplast derived colonies became visible with the naked eye (Figure 6.7). Grids containing 7-8 days old protoplast derived colonies were transferred to shoot regeneration medium. The best regeneration medium contains Kinetin and NAA as phytohormons (SRAKN). First shoots were observed after 5-6 days (Figure 6.7 b-c). After 15-20 days on SRAKN medium, more than 120 shoots per grid were obtained (Figure 6.7-f).

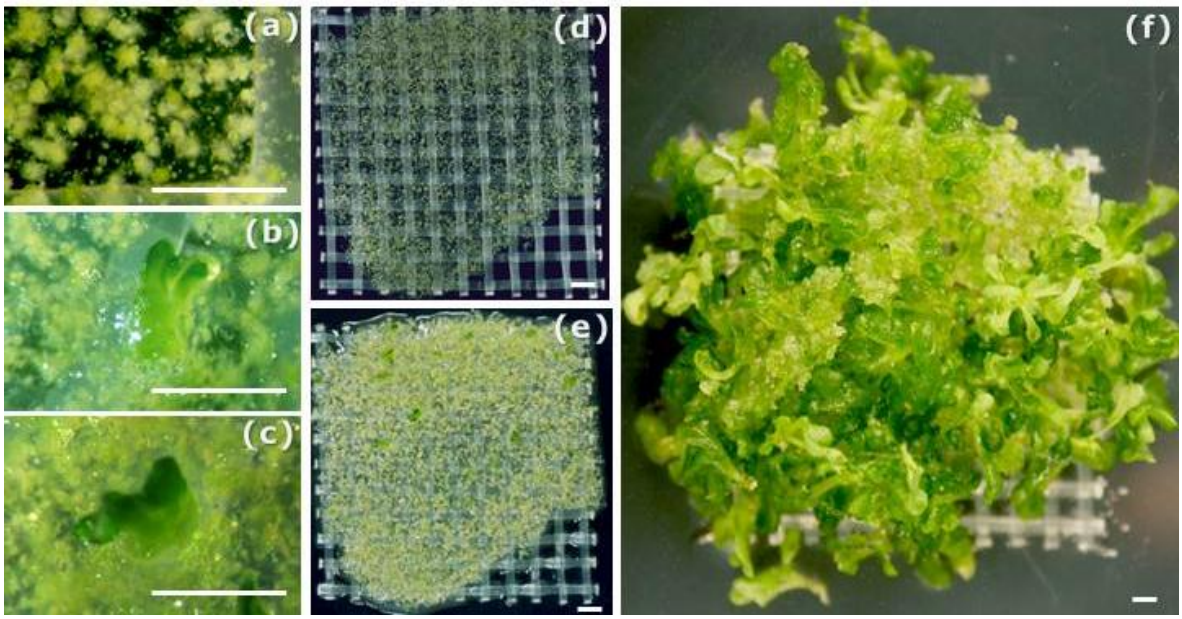


Figure 6.7 Shoot formation from protoplast derived colonies in *Arabidopsis thaliana* C-24. **(a, d)** 9 days old protoplast derived colonies on Regeneration media. **(b, c, e)** First shoots from protoplast derived colonies after 5 days on SRAKN medium. **(f)** Grid containing shoots from protoplast derived colonies after 20 days. Bars: 2 mm

SRAKN medium revealed the best regeneration efficiencies; however, regeneration was extremely fast (just five days). To support the selection of transformed cells, a regeneration protocol with a prolonged callus phase might be required. Therefore, SRAKN medium was modified by the addition of 0.25, 0.5 mg/l or 1 mg/l of 2,4-D. SRA2N medium was used at same time and 7 days old protoplast derived colonies were transferred to those media. Results are shown on Figure 6.8. As expected, addition of 2,4-D to SRAKN medium allowed the induction of calli from protoplast derived colonies after two weeks. Addition of 0.25 mg/l of 2,4-D was sufficient to produce calli as shown on Figure 6.8 (a).

SRA2N and SRAKN + 0.25 m/l 2,4-D were the best option to produce calli from protoplast derived colonies, because competence to produce shoots was maintained and fertile plants were obtained.

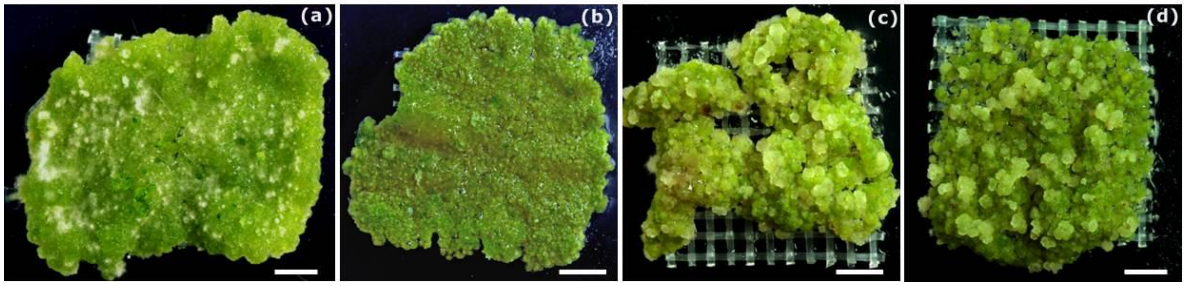


Figure 6.8 Callus induction from *Arabidopsis thaliana* C-24 protoplast derived colonies. **(a)** Grid containing calli and shoots from protoplast derived colonies after three weeks on SRA2N medium. **(b, c, d)** Grids containing calli from protoplast derived colonies after three weeks on SRAKN media containing 0.25 mg/l; 0.5 mg/l and 1 mg/l 2,4-D respectively. Bars: 5 mm

Once shoots from protoplast derived colonies were obtained, those shoots were transferred to phytohormone-free medium or to root induction medium supplemented with auxins (Table 5.7). Shoots from protoplast derived colonies were competent to form roots (80-90 %) in almost all media, including medium free of phytohormones (Figure 6.9). To produce seeds, the plants were either cultured on phytohormone-free medium, root induction medium or transferred to soil and were grown in the greenhouse.

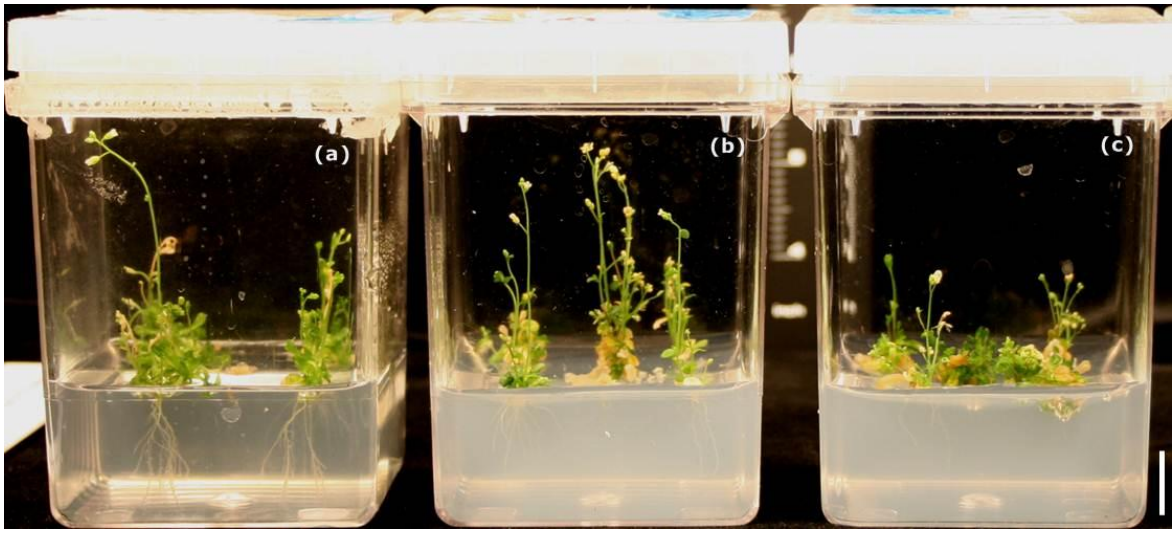


Figure 6.9 Root induction from shoots of protoplast derived colonies in *Arabidopsis thaliana* C-24. **(a)** Root induction after two weeks on ½MS medium free from phytohormones. **(b)** Root induction on medium RIMI containing IBA as auxin. **(c)** Root induction on medium RIMII containing IBA and NAA as auxins. Bar: 1 cm

6.1.4 Evaluation of the sensitivity of *Arabidopsis thaliana* protoplast derived colonies to selection agents

The next step on the road to stable plastid transformation was the optimization of selective conditions. In this work, kanamycin, spectinomycin, bialaphos and phosphinothricin selection conditions for *Arabidopsis thaliana* plastid transformation, were tested. Four different genes were used as selection marker: *nptII* gene encodes neomycin phosphotransferase II (Carrer *et al.*, 1993), the *aphA6* gene encodes a 3´aminoglycoside phosphotransferase type VI (Huang *et al.*, 2002) both genes confer a resistance to Kanamycin; the *aadA* gene encodes aminoglycoside 3´transferase (AADA) (Svab and Maliga, 1993) conferring resistance to spectinomycin and streptomycin and finally the *bar* gene encodes a phosphinothricin acetyl transferase (PAT) enzyme (Lutz *et al.*, 2001) and confers resistance to the herbicides phosphinothricin and bialaphphos. To determine the optimal antibiotic or herbicide concentration for the inhibition of callus growth and shoot induction, grids containing protoplast derived colonies were transferred to regeneration medium containing kanamycin (0, 20, 25, 30 or 40 mg/l), spectinomycin (0, 5, 10, 15 or 20 mg/l) or phosphinothricin (0, 2.5, 5, 7, 10 mg/l) (Figures 6.10-6-11).

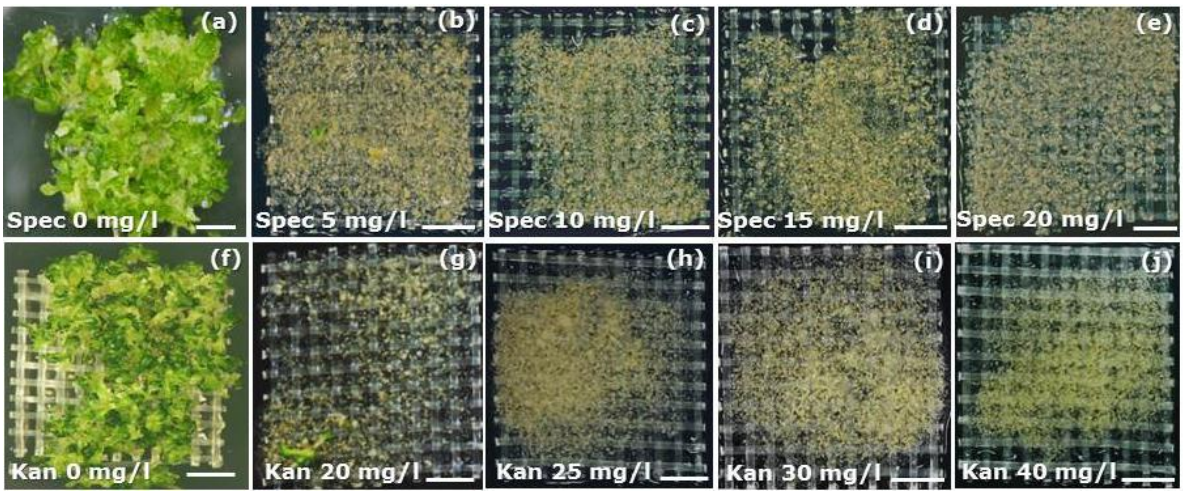


Figure 6.10 Effect of spectinomycin (Spec), kanamycin (Kan) on shoot regeneration from protoplast derived colonies in *Arabidopsis thaliana* C24. Protoplast derived colonies after 20 days on SRAKN media containing spectinomycin 0 **(a)**, 5 **(b)**, 10 **(c)**, 15 **(d)** and 20 **€** mg/l. Protoplast derived colonies after 20 days on SRAKN media containing Kan 0 **(f)**, 20 **(g)**, 25 **(h)**, 30 **(i)** and 40 **(j)** mg/l. Shoots were detected in media containing 20 mg/l of Kan with inhibition of regeneration on media containing Kan 25 mg/l. Bars: 5 mm

Addition of 10 mg/l of spectinomycin to SRAKN medium was sufficient for inhibition of shoot regeneration. On SRAKN medium containing 5 mg/l spectinomycin, two shoots were obtained after 20 days. After 25 days on SRAKN containing 15 mg/l spectinomycin, shoots were obtained, indicating that spectinomycin was not stable and that for successful selection fresh antibiotic is required. Addition of 25 mg/l of kanamycin to SRAKN media was necessary to ensure inhibition of the regeneration from protoplast derived colonies.

The inhibition of regeneration strongly depends on the presence of the herbicide phosphinothricin (PPT). In the presence of 2.5 mg/l PPT in SRAKN medium 10 to 15 protoplast derived calli were formed, however this number could be decreased to one or two calli per grid by the addition of 5 – 10 mg/l PPT (Figure 6.11). Higher concentrations of PPT (15 mg/l) were highly toxic for protoplast derived colonies. Therefore, addition of 2.5 or 3 mg/l of PPT on SRAKN medium seem be sufficient for selection of protoplast derived colonies, in plastid transformation experiments.

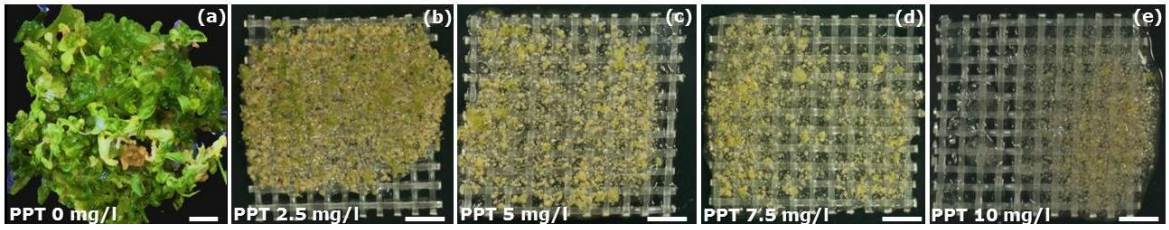


Figure 6.11 Effect of phosphinothricin (PPT) on shoot regeneration from protoplast derived colonies in *Arabidopsis* Protoplast derived colonies after 20 on SRAKN medium containing PPT 0 **(a)**, 2.5 **(b)**, 5 **(c)**, 7.5 **(d)** and 10 **(e)** mg/l. Bars: 5 mm

6.1.5 Construction of *Arabidopsis thaliana* plastid transformation vectors

To allow stable integration into the *Arabidopsis* plastome, vectors with markers flanked by *Arabidopsis* plastid sequences were required. Because insertion of sequences in plastid transformation occurs by homologous recombination (Staub, *et al*., 1992), it was necessary to choose *Arabidopsis* plastome sequences flanking the selected integration site.

Homologous fragments (INSL and INSR) flanking the targeted insertion point between *rbcL* (Nucleotide positions in the NCBI database: 54958-56397) and *accD* (Nucleotide positions in the NCBI database: 57075-58541) genes were amplified using total *Arabidopsis thaliana* C24 DNA as template and the PCR primers AraRub-XbaI (ACGTCTAGAGGATCTGCGAATC) and AraccD-BamHI (AATT TAATGGGATCCGCAGAG). The 2.5 Kb PCR product was digested with *Spe*I-*BamH*I enzymes and this fragment was ligated into pUC18 vector using *Xba*I-*BamH*I sites. This new vector containing *rbcL* and *accD* from *Arabidopsis thaliana* C24 was named pC24 vector. pC24 vector was sequenced and a substitution C-G was detected in the *accD* gene. A C-G substitution represents a change from Threonine to Serine in the *accD* gene (Appendix 1).

Additional vectors containing other recombination regions in the *Arabidopsis thaliana* plastome were also used in this work. pSTS4 vector with flanks for insertion between *trnfM* and *trnG* genes was obtained from Prof. Ralph Bock (Max-Planck-Institute of Molecular Plant Physiology). pBA vector with flanks for insertion between *trnV-rps12/7* was obtained from Christian Stelljes. The insertion sites from the vectors in *Arabidopsis thaliana* plastome are shown on Figure 6.12.

In total three different insertion sites were chosen to allow homologous recombination in the *Arabidopsis thaliana* plastome (Figure 6.12)

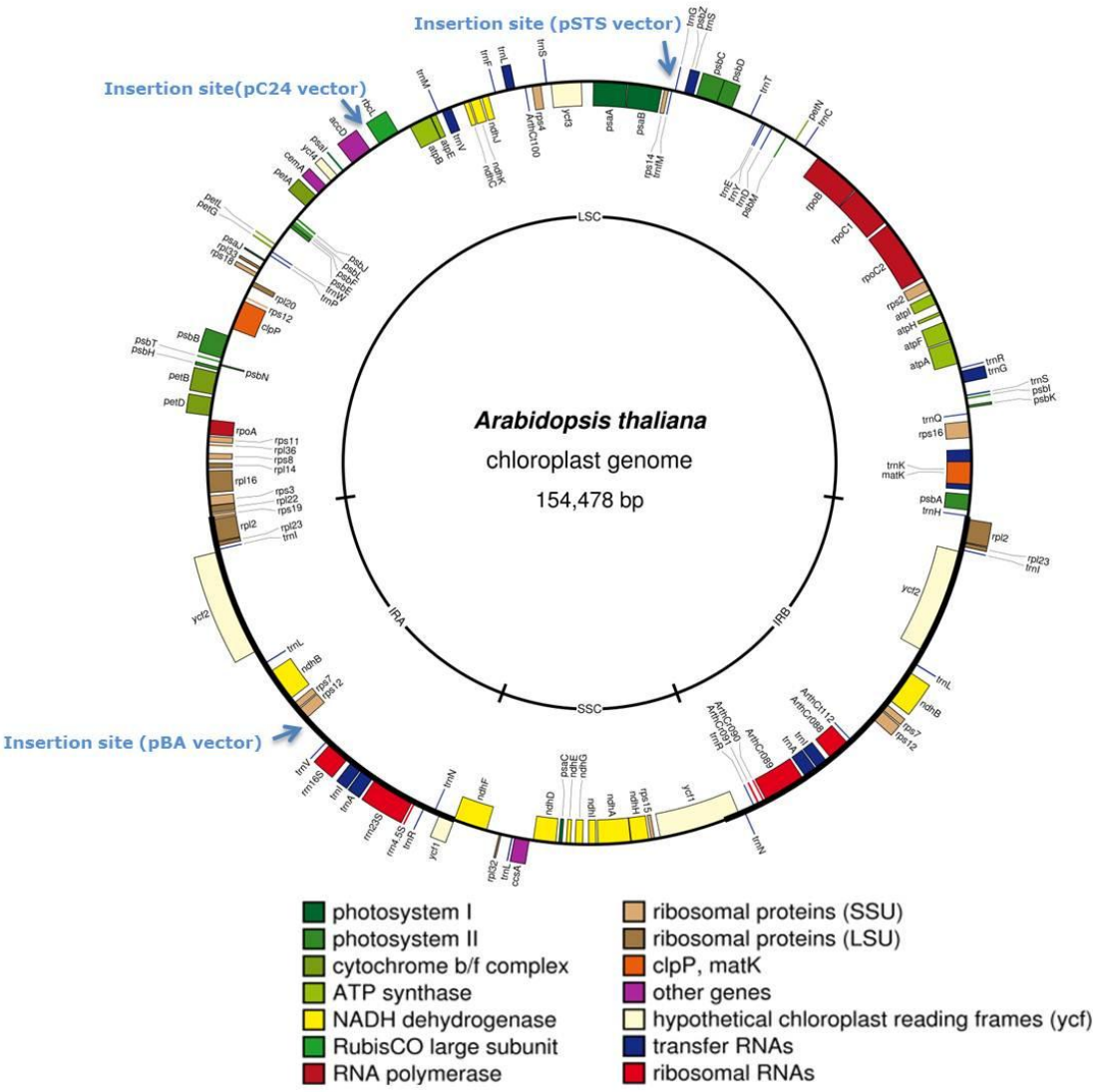


Figure 6.12 Gen map of *Arabidopsis thaliana* chloroplast. Genes shown on the inner circle are transcribed clockwise, and those on the outside counter-clockwise. Genes with related functions are shown with the same color. The genome shows two copies of the inverted repeat region (IR) separating the large (LSC) and small (SSC) single copy regions. Arrows show insertion position for integration of foreign genes with the name of the vector containing those regions. The map was created using "OrganellarGenomeDRAW" software developed by M. Lohse and O. Drechsel in the Bock laboratory.

In the present work visual and selective markers were chosen, as visual fluorescence markers, three markers were used: DsRed (Red Fluorescent Protein), GFP (Green Fluorescent Protein) and AmCyan (Cyan Fluorescent Protein). With the help of visual markers transformed tissue can easily be selected (Khan and Maliga, 1999). As selection marker four different genes were used: *aadA*, *nptII*, *aphA6* and *bar* genes (section 6.1.4). The mode of how fluorescence or selection markers (with insertion sites) were combined is presented in Figure 6.13

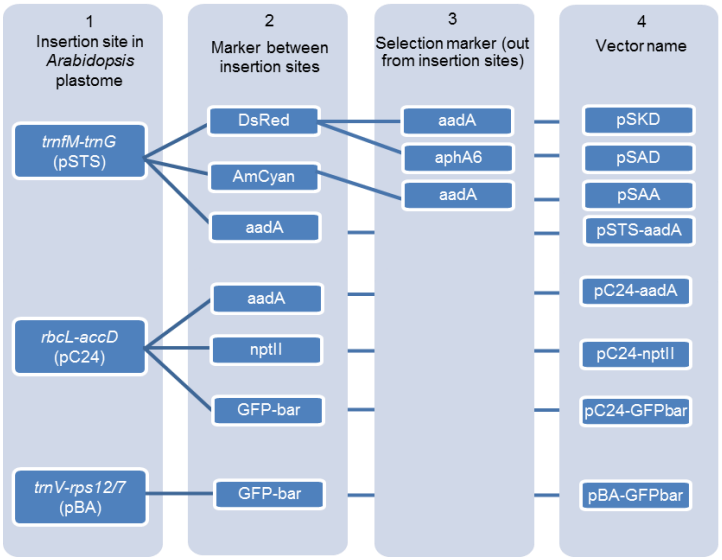


Figure 6.13 Plastid transformation vectors in *Arabidopsis thaliana*. 1. Homologous flanks in *Arabidopsis* plastome, vector name is indicated under bracket. 2. Selection or fluorescence marker was cloned between homologous flanks. 3. Selection marker was cloned outside of homologous flanks. 4. Name of plastid transformation vector is indicated.

Once vectors with insertion sites in the *Arabidopsis* plastome were obtained, cassettes containing selection or fluorescence markers were constructed. In order to achieve high expression in plastids, the Prrn tobacco 16S-rRNA (Svab and Maliga, 1993), in addition to the 5'UTR of gene 10 from bacteriophage T7 (T7g10) and MASIS sequences (Herz *et al.*, 2005) were cloned in front of the *AmCyan* and *DsRed* genes. The 3´UTR Tprl32 was cloned at the end of fluorescence markers.

A new vector containing DsRed was cloned in the following way: the *DsRed* gene was cut out with *Nco*I-*Xba*I from plasmid pGJ1425, kindly provided by Angela Dietzmann (Jach *et al.*, 2001) and was ligated to the 3´ UTR regulatory region (Trpl32) of the p546 vector. The cassette *DsRed*::Trpl32 was cut out with *Nco*I-*Xho*I and was ligated with pUC18-Prrn vector and restricted with the same enzymes. The new pUC18-DsRed vector (Figure 6.14-a) was used in the next steps. A Petri dish with *E. coli* XL1 Blue colonies (transformed with pUC18-DsRed vector), were observed under Fluorescence SteREO Lumar. V12 (Fig 6.14-c).

For the creation of plastid transformation vectors containing AmCyan fluorescence marker, the *AmCyan* gene from pAmCyan vector (Clontech®) was cut out with *Xba*I, and the fragment was further partially digested with *Nco*I. This

fragment was ligated with plasmid pUC18-DsRed, which has been digested with the same restriction enzymes combination. The new pUC18-AmCyan vector was sequenced and the expression of AmCyan was detected in *E. coli* XL1 Blue colonies under Fluorescence SteREO Lumar. V12 (Fig 6.14-d).

The vector pC24-GFPbar with the *Arabidopsis* rbcL-accD flanks containing PrrnT7g10-MASIS::*gfpbar*::Trpl32 cassette was kindly provided by Peter Wehmann (AG Koop, LMU). Expression of GFP was also detected under UV (Figure 6.14-e).

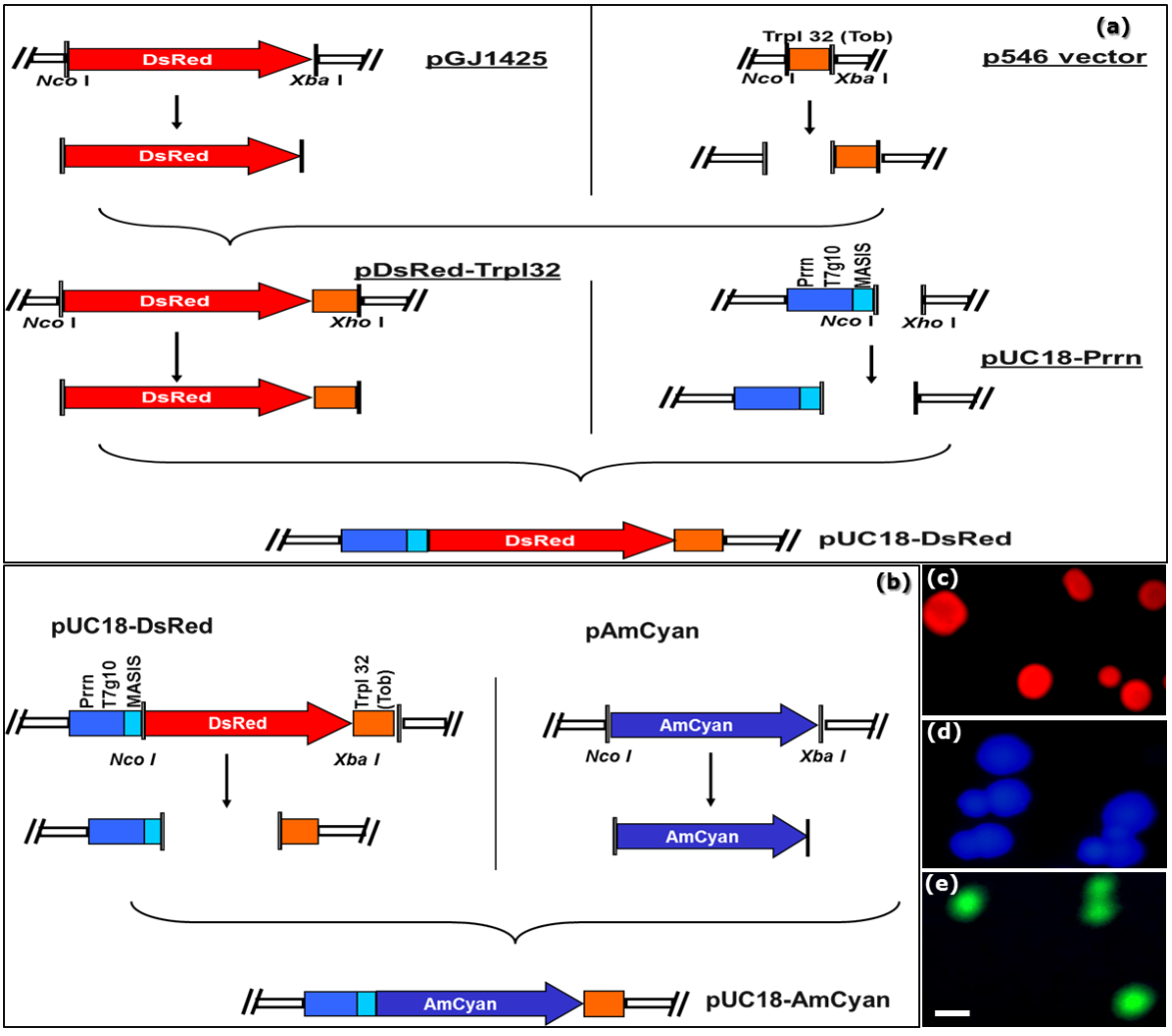


Figure 6.14 Fluorescence markers for plastid transformation. **(a)** Schematic presentation of cloning steps of a DsRed vector for plastid transformation. **(b)** Schematic presentation of cloning steps of an AmCyan vector for plastid transformation. Restrictions sites for cloning steps are shown. **(c, d)** Colonies from *E. coli* XL1 Blue showing DsRed and AmCyan fluorescence, colonies were observed under Fluorescence SteREO Lumar. V12. **(e)** Colonies from *E. coli* XL1 Blue expressing GFPbar, observed under UV lamp. Bars: 2 mm.

pSTS-aadA vector was generated by ligating Prrn::*aadA*::TrbcL (*Not*I-*Xba*I) from pKCZ-aadA vector into the *Not*I-*Xba*I of pSTS4 vector (Figure 6.15)

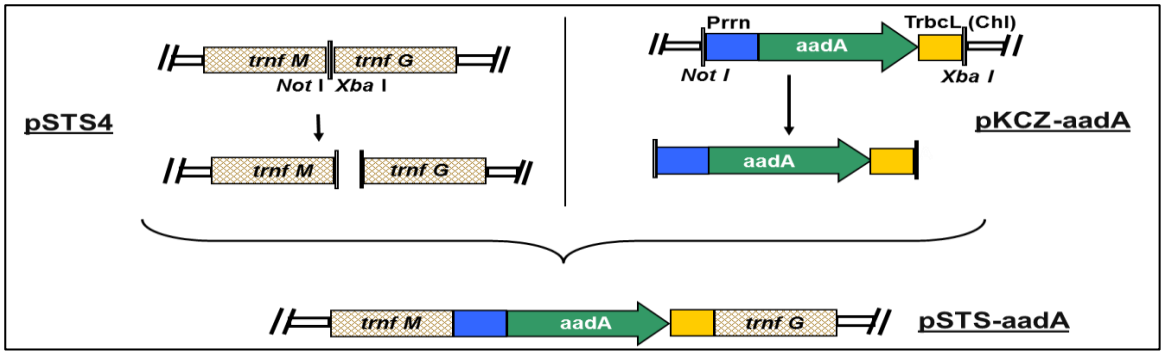


Figure 6.15 Schematic presentation of cloning step of a pSTS-aadA vector. Restriction sites for cloning steps are shown. Hatched boxes represent *Arabidopsis* homologous flanks.

pSKD (pSTS-DsRed-aphA6) vector was cloned by ligating the cassette Prrn-T7g10-MASIS::DsRed::Trpl32 (*Sal*I-*Xho*I) from pUC18-DsRed into the *Sal* I site of pSTS4 vector. An *aphA6* cassette in the backbone of pSTS-DsRed vector was cloned by digestion with *Avi*II and once vector was linearized, it was ligated to an PpsbA::*aphA6*::TrbcL (*Sma*I) cassette obtained from pGEMaphA6 vector (kindly provided by Dr. Lars Scharff AG Koop, LMU). Kanamycin resistant pink colonies were chosen for further characterization (Figure 6.16).

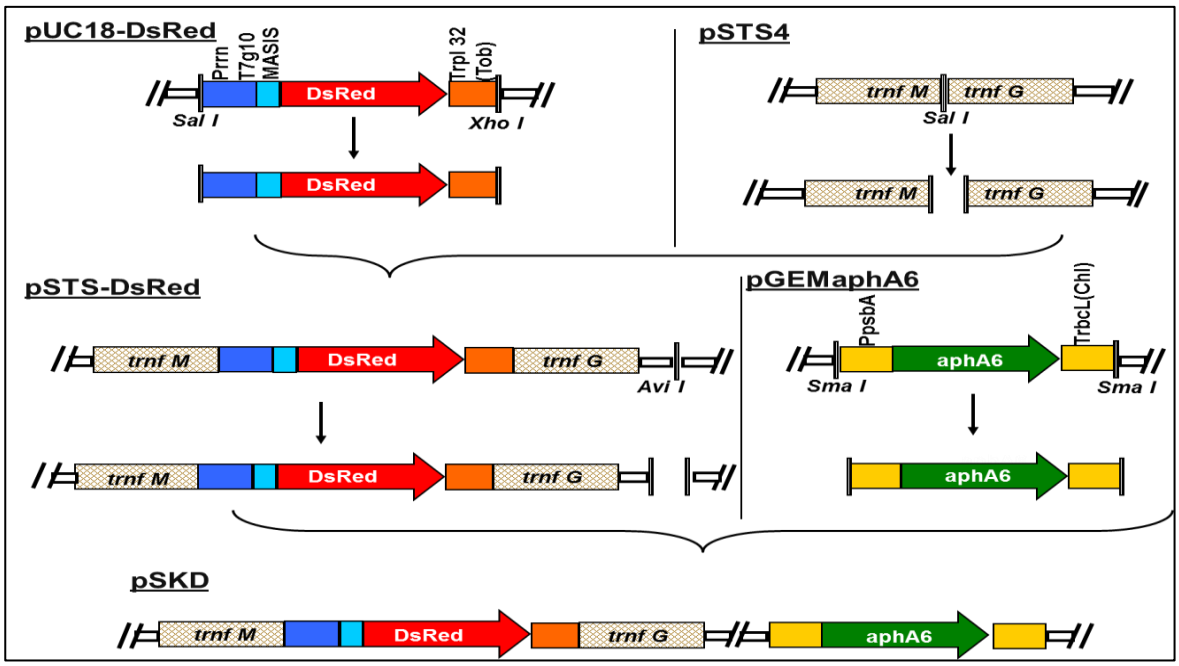


Figure 6.16 Schematic presentation of cloning steps of a pSKD vector. Restrictions sites for cloning steps are shown. Hatched boxes represent *Arabidopsis thaliana* homologous flanks

pSAD (pSTS-DsRed-aadA) vector was obtained by replacing *aphA6* cassette in pSKD vector for *aadA* cassette, pSKD vector was digested with *Nsb*I-*Mlu*I enzyms, vector was purified and ligated to an PpsbA::*aadA*::TrbcL cassette from pUC18-aadA vector digested with *Mlu*I-*Nsb*I enzyms. Ligation of those fragments was transformed and spectinomycin resistant pink colonies were characterized and sequenced (Figure 6.17).

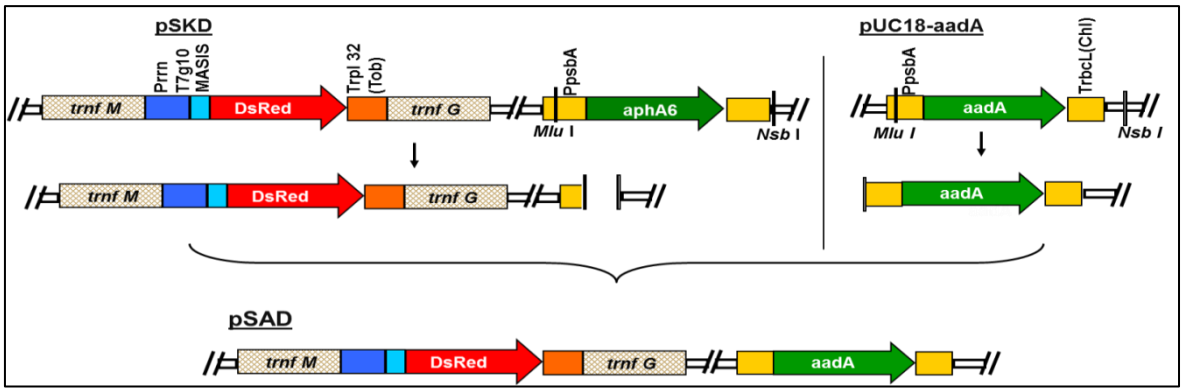


Figure 6.17 Schematic presentation of cloning steps of a pSAD vector. Restriction sites for cloning steps are shown. Hatched boxes represent *Arabidopsis* homologous flanks

pSAA (pSTS-AmCyan-aadA) vector was generated in two steps, first Prrn-T7g10-MASIS::*AmCyan*::Trpl32 cassette (*Pst*I-*Xho*I) from pUC18-aadA vector was cloned into *Pst*I-*Xho*I sites of pSTS4 vector. An Prrn-T7g10-MASIS::*AmCyan*::Trpl32 (*BsrG*I-*Xho*I) cassette from pSTS-AmCyan vector was cloned in *BsrG*I-*Xho*I sites of pSAD vector. Resistant blue colonies were further characterized and the vector was sequenced (Figure 6.18).

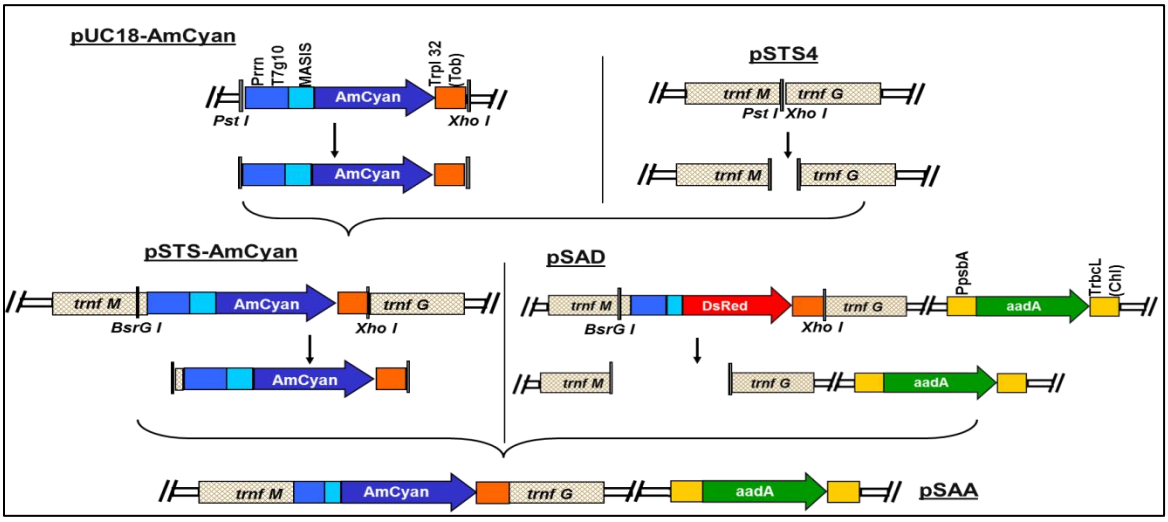


Figure 6.18 Schematic presentation of cloning steps of a pSAA vector. Restrictions sites for cloning steps are shown. Hatched boxes represent *Arabidopsis* homologous flanks.

pC24-aadA vector was obtained by ligating Prrn::*aadA*::TrbcL (*Sma*I-*Eco47*III) cassette from pKCZ-aadA vector into the *Bst1107*I site of pC24 vector. pC24 vector contains *rbcL* and *accD* flanks from *Arabidopsis thaliana* C-24. (Figure 6.19)

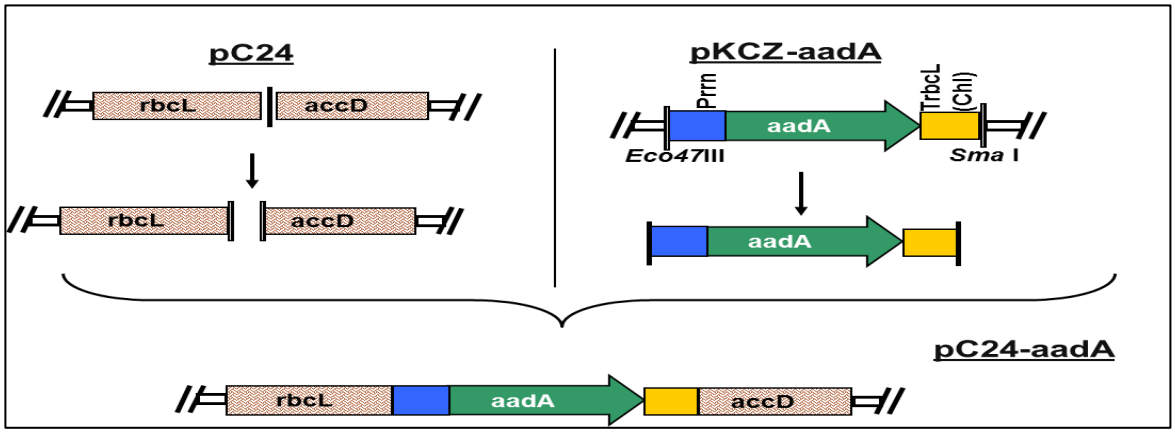


Figure 6.19 Schematic presentation of cloning step of a pC24-aadA vector. Restrictions sites for cloning steps are shown. Hatched boxes represent *Arabidopsis thaliana* homologous flanks.

pC24-nptII vector was obtained by replacing aadA (*Xho*I-*Spe*I) cassette from pC24-aadA vector for PNG1014::*nptII*::TrbcL (*Xho*I-*Spe*I) from pKCZ-nptII vector. Kanamycin resistant colonies were characterized and sequenced (Figure 6.20).

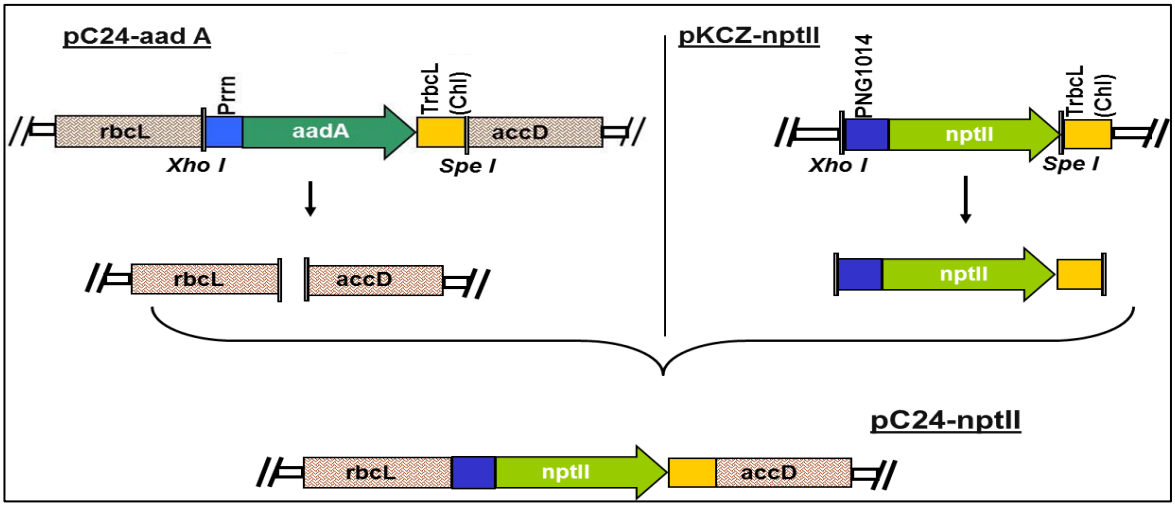


Figure 6.20 Schematic presentation of cloning step of a pC24-nptII vector. Restrictions sites for cloning steps are shown. Hatched boxes represent *Arabidopsis thaliana* homologous flanks.

6.1.6 Particle gun and PEG-based plastid transformation experiments in *Arabidopsis thaliana*

After the establishment of regeneration protocols in *Arabidopsis thaliana* and the generation of appropriate vectors particle gun and PEG mediated plastid transformation were realized with *Arabidopsis thaliana* plastome specific vectors (Figure 5.9). Gold particles (0.6µ) covered with desirable DNA were used for particle gun experiments as described in section 5.9.2. Grids containing 7-10 days old protoplast derived colonies or 20 days old seed derived calli from *Arabidopsis thaliana* C-24 were used as plant material. Around 6 to 9 grids were bombarded with one vector each experiment. Two days after bombardment, plant material was transferred to a selective regeneration medium as described on 6.1.4. In total, 43 independent particle gun experiments with 338 single bombardments were executed with vectors containing aminoglycoside resistance genes (Table 6.2). However, only some putative transplastomic lines could be obtained.

Particle gun experiments were performed with vectors containing DsRed as fluorescence marker and *aadA* or *aphA6* as selection marker in the backbone of the vector (pSKD and pSAD vectors). After two transformation experiments with pSKD vector, 4 putative transformant lines were obtained, with pSAD vector 8 putative lines were obtained. To detect DsRed expression and the presence of the *aphA6* gene, fluorescence microscopy and PCR were used, respectively. All putative analysed lines were negative for the *aphA6* gene and for the DsRed fluorescent protein (Figure 6.21).

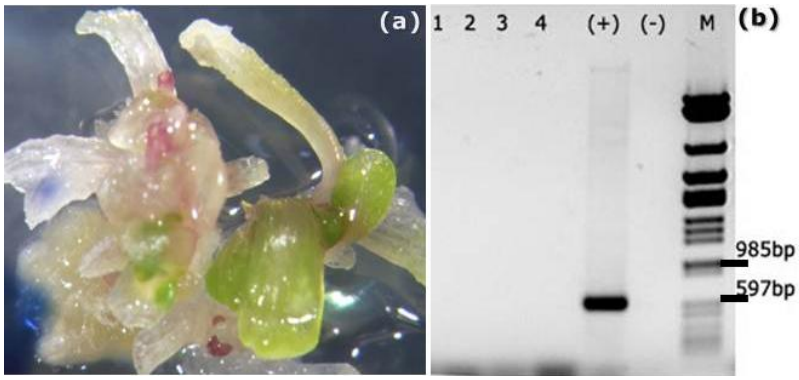
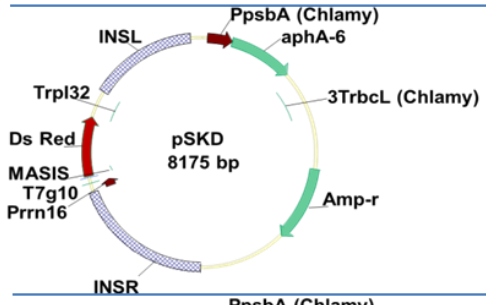
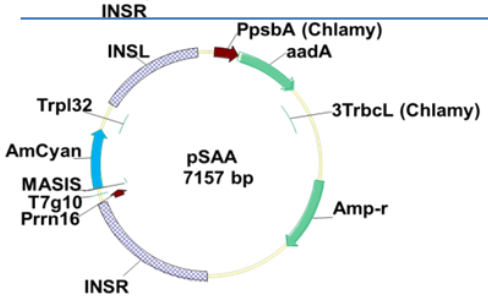
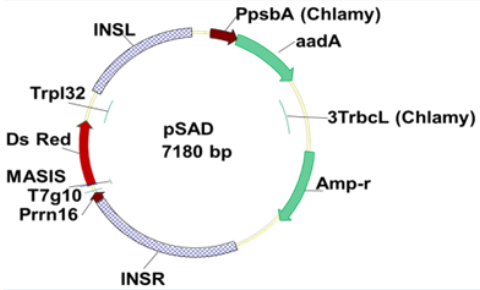
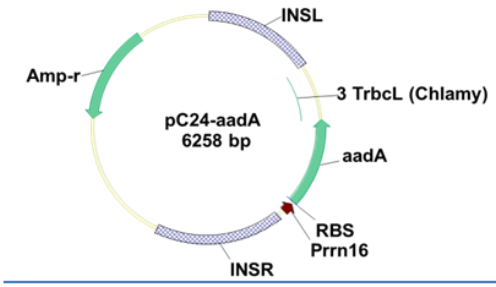
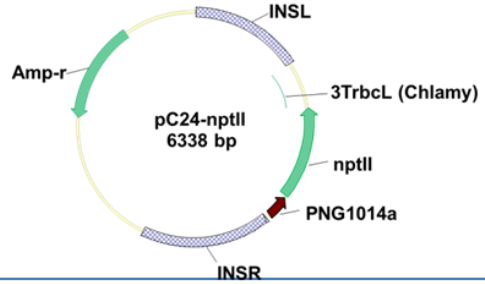


Figure 6.21 *Arabidopsis thaliana* plastid transformation experiments with pSKD vector. (a) Putative SKD line growing on SRAKN medium containing 20 mg/l kanamycin. (b) Results from putative lines analysed by PCR to detect the presence of the *aphA6* gene (658bp). pSKD vector was used as postive control (+) and water as negative control (-) Lambda Eco 47I was used as a marker (M). Agarose gel 1 %. Bar: 5 mm

Table 6.2 Particle gun experiments with aminoglycoside resistance genes

***Arabidopsis thaliana* plastid transformation vectors**	**Description**
	Insertion: *trnfM-trnG* Selection: PpsbA::*aphA6*::TrbcL (*Chlamydomonas*) Fluorescence marker: Prrn-T7g10-MASIS::*DsRed*::Trpl32 **Number of plates = 13**
	Insertion: *trnfM-trnG* Selection: PpsbA::*aadA*::TrbcL (*Chlamydomonas*) Fluorescence marker: Prrn-T7g10-MASIS::*DsRed*::Trpl32 **Number of plates = 48**
	Insertion: *trnfM-trnG* Selection: PpsbA::*aadA*::TrbcL (*Chlamydomonas*) Fluorescence marker: Prrn-T7g10-MASIS::*AmCyan*::Trpl32 **Number of plates = 74**
	Insertion: *trnfM-trnG* Selection: Prrn::*aadA*::TrbcL (*Chlamydomonas*) **Number of plates = 80**
	Insertion: rbcL-accD Selection: Prrn ::*aadA*::TrbcL(*Chlamydomonas*) **Number of plates = 23**
	Insertion: rbcL-accD Selection: Prrn-T7g10-14ASGFP::*nptII*::TrbcL (*Chlamydomonas*) **Number of plates = 100**

DsRed gene was replaced with AmCyan gene as fluorescence marker for plastid transformation experiments with pSAA vector introducing a new fluorescence marker for plastid transformation (Wenck *et al.*, 2003). The pSAA vector contains an AmCyan cassette between *Arabidopsis thaliana* flanks and an *aadA* cassette on the backbone of the vector (Table 6.2).

In general 74 grids containing protoplast derived colonies from *Arabidopsis* were bombarded in 8 independent particle gun experiments with pSAA vector.

pSAA vector brought a higher number of putative transformants. In one experiment around of 65 spectinomycin resistant shoots were obtained. However, most putative lines (40 shoots) were obtained from two grids which were transferred to SRAKN medium (Table 5.7) without spectinomycin after 20 days. Figure 6.22 shows a putative line. Shoots from candidate lines were separated and approximately 20 shoots were transferred to regeneration medium containing 40 mg/l of spectinomycin. Those lines became white and died after three weeks under selective conditions. The rest of the candidate lines were further grown on antibiotic free media, and healthy fertile plants were obtained (Figure 6.22-e). Putative lines were analysed by fluorescence microscopy to detect AmCyan expression and by PCR to amplify the *AmCyan* or the *aadA* genes. PCR was negative in all analysed lines and no fluorescent line could be detected.

To further analyse the putative lines, T1 plants were germinated on SCA medium containing 40 mg/l of spectinomycin, however, they only produced white seedlings (data not shown).

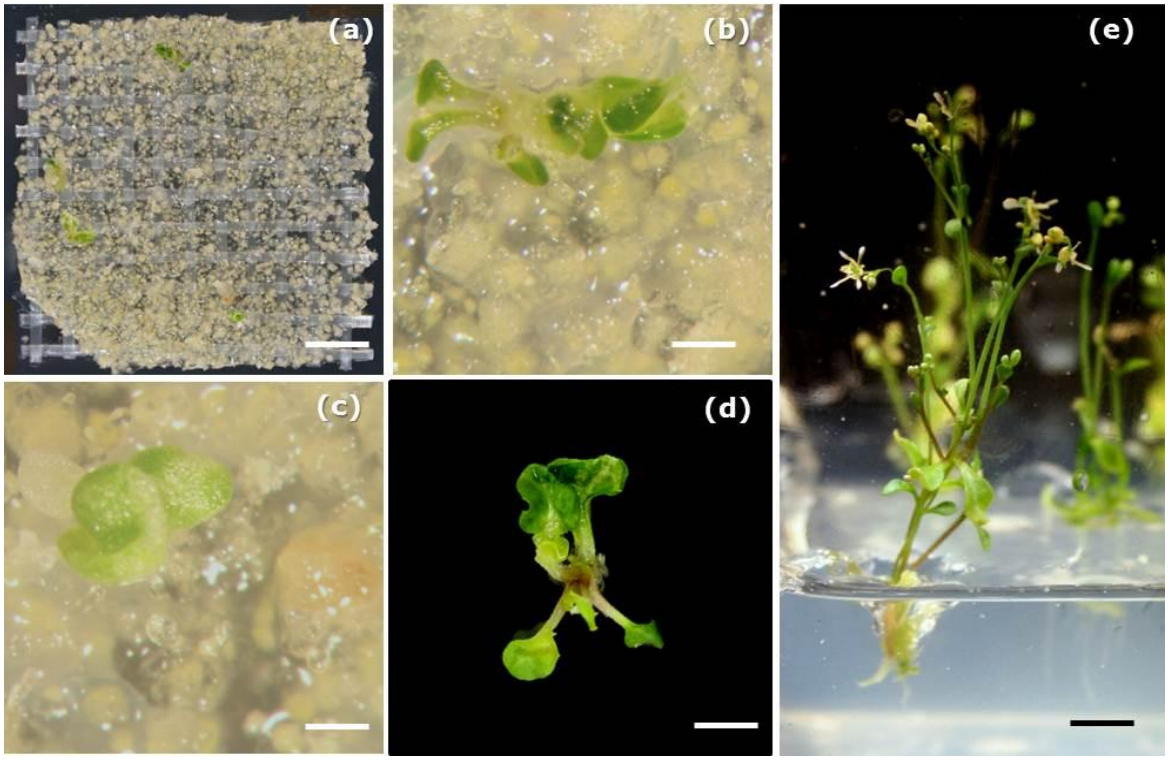


Figure 6.22 *Arabidopsis thaliana* plastid transformation experiments with pSAA vector. **(a)** Grid containing protoplast derived colonies bombarded with pSAA vector. First shoots were obtained after 12 days on SRAKN medium containing 20 mg/l spectinomycin. **(b-c)** Close-up from a spectinomycin resistant shoot. **(d)** SAA derived line growing on free antibiotic SRAKN medium after two weeks. **€** SAA derived lines growing on SCA medium production seeds. Bars: 5 mm

In summary, 203 plastid transformation experiments were performed with vectors containing different selection markers in the backbone of the plasmid (pSAD, pSKD and pSAA). Although, the number of putative transformants was huge, all analysed lines were negative. In order to produce a callus phase before regeneration, medium SRA2N (Table 5.7) was used. In addition vectors containing selection markers between insertions flanks were used for bombardment experiments. A summary of particle gun based plastid transformation experiments with aminoglycoside selection markers and herbicide markers is shown on Table 6.3.

Table 6.3 Summary of particle gun based plastid transformation experiments in *A. thaliana* C-24.

Vector	Insertion in Arabidopsis	Selection Marker	Fluorescence Marker	Particle gun experiments	Putative lines	PCR
pSAD	*trnfM-trnG*	aadA	DsRed	48	8	(-)
pSKD	*trnfM-trnG*	aphA6	DsRed	13	4	(-)
pSAA	*trnfM-trnG*	aadA	AmCyan	74	89	(-)
pSTS-aadA	*trnfM-trnG*	aadA	---	80	---	---
pC24-aadA	*rbcL-accD*	aadA	---	23	30	(-)
pC24-nptII	*rbcL-accD*	nptII	---	100	29	(-)
pC24-GFPbar	*rbcL-accD*	bar	GFP	70	--	--
pBA-GFPbar	*rps12/7-trnV*	bar	GFP	95	--	--
Total				**503**	**126**	

As an alternative approach to the particle gun experiments, the PEG mediated transformation was used in parallel. The PEG transformation method is well established in chloroplast transformation and has been widely used in transient nuclear transformation in *Arabidopsis thaliana* protoplasts (Golds *et al.*, 1993). However, most protoplasts died after PEG treatment and thus cannot be used for regeneration experiments. To establish a PEG transformation protocol which allows the stable transformation of protoplasts, the PEG concentration was reduced and protoplasts were incubated in PCA medium. In the present work protoplasts were treated either with 20 % or with 40 % PEG1500 in the presence of pGFP vector (kindly provided by PD Dr. Cordelia Bolle) (50 µg) as described in section 5.9.1. GFP gene is under the 35S promoter (Promoter from cauliflower mosaic virus (CaMV) and the nos terminator (nopaline synthase). GFP is expressed in cytoplasm from protoplasts.

Two days after PEG transformation, transient GFP expression was detected in the cytoplasm by fluorescence microscopy meaning that the PEG method is working (Figure 6.23). Subsequently, protoplast derived colonies were cultured and regenerated on non-selective PCA and SRAKN medium.

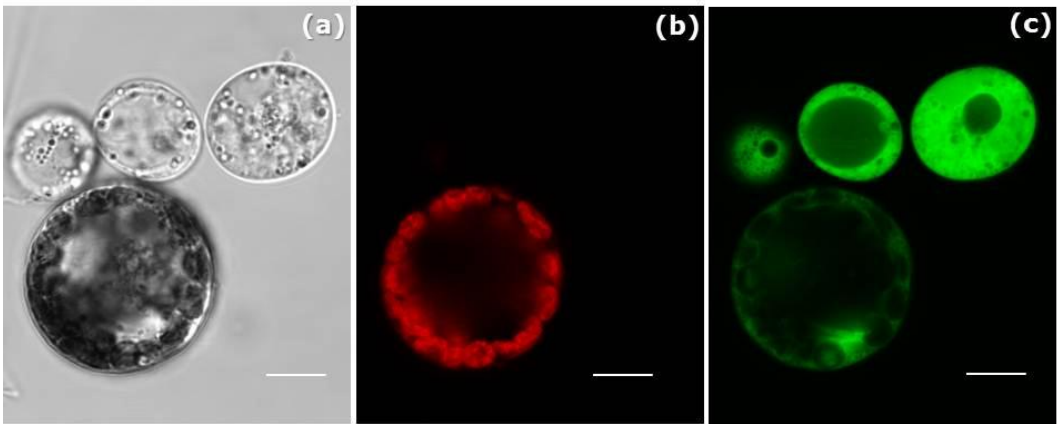


Figure 6.23 PEG transformation from *Arabidopsis thaliana* C-24 with a pGFP vector. (a) Protoplast under DIC. (b) Chlorophyll (c) GFP filter. Protoplast were analyzed by confocal laser scanning microscopy. Bars: 10 µm

Two plastid transformation experiments with pC24-nptII and pSTS-aadA vectors were realized, but all protoplast derived colonies died under selective conditions.

In addition ten PEG mediate plastid transformation experiments using the pC24-gfpbar vector were realized. In all cases no resistant plants were obtained.

6.2 Towards plastid transformation in Rapid-Cycling *Brassica rapa*

Rapid-Cycling *Brassica rapa* (RCBr) represents a new model system in plant biology (Williams and Hill, 1986; Musgrave, 2000). RCBr are small in size (approximately 20 cm), require a minimum time from seed to flowering (14-17 days), are highly female fertile and life cycle is short (28 to 35 days). We first established a RCBr plant regeneration protocol focusing on lines C1 and B3. B3 lines are wild type RCBr and C1 lines are a anthocyanin less mutant, *anl/anl* and require cross pollination. Then, plant specific vectors for plastid transformation with AmCyan fluorescence marker and the *aadA* gen which confers resistance to spectinomycin and streptomycin antibiotics, were constructed and RCBr was bombarded with the help of a particle gun.

6.2.1 Plant regeneration from Rapid-Cycling *Brassica rapa*

To develop a suitable regeneration protocol different media with two different explants were used (Table 6.3). First, two protocols with RCBr cotyledons as explants and two different media were utilized for plant regeneration (Teo *et al.*, 1997; Cogbill *et al.*, 2010). However, no shoots could be obtained (Figure 6.24) (data for Cogbill *et al.*, 2010 method, are not shown).

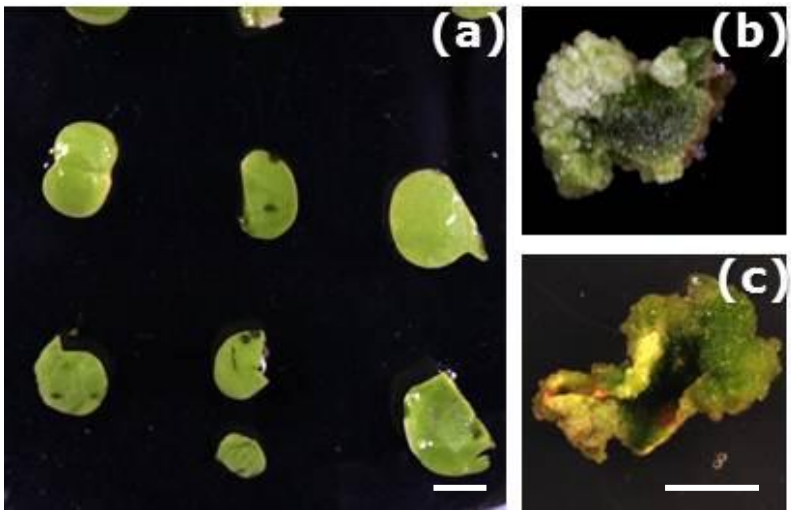


Figure 6.24 Callus production from cotyledons of Rapid-Cycling *Brassica rapa* (RCBr)- C1(a) 7 days old cotyledons from RCBr-C1 on MDN medium. (b) Cotyledon derived calli from RCBr-C1 after three weeks on MDN medium. Bars: 5 mm

Table 6.4 Summary from regeneration experiments in Rapid-Cycling *Brassica rapa*

Protocol	α (Chen and Celio, 2000)	β (Teo *et al.*, 1997)	γ (Jonoubi *et al.*, 2005)
Explant	Hypocotyls	Cotyledons	Hypocotyls
Preculture	----	----	2 days on MD1 medium
Calli	3 weeks on MDI medium	----	7 days on BD1 medium
Regeneration	3 weeks on MZI medium	3 weeks on	3 weeks on SIM medium
Regeneration	3 weeks on MZI medium	MDN medium	3 weeks on SIM medium
Efficiences C1	**20-50 %**	**0**	**50-80 %**
Efficiences B3	**20-40 %**	**0**	**20-50 %**

In a second set of experiments, hycototyls were utilized as explants. Protocols were originally described in *Brassica napus* (in this work named as "α" Chen and Celio, 2000and "γ" (Jonoubi *et al*., 2005)). Regeneration efficiency was about 20-50 % in protocol "α". However, protocol "γ" was even better with efficiencies of 50 -70 % (Figure 6.25). In all cases, shoots and fertile plants could be achieved.

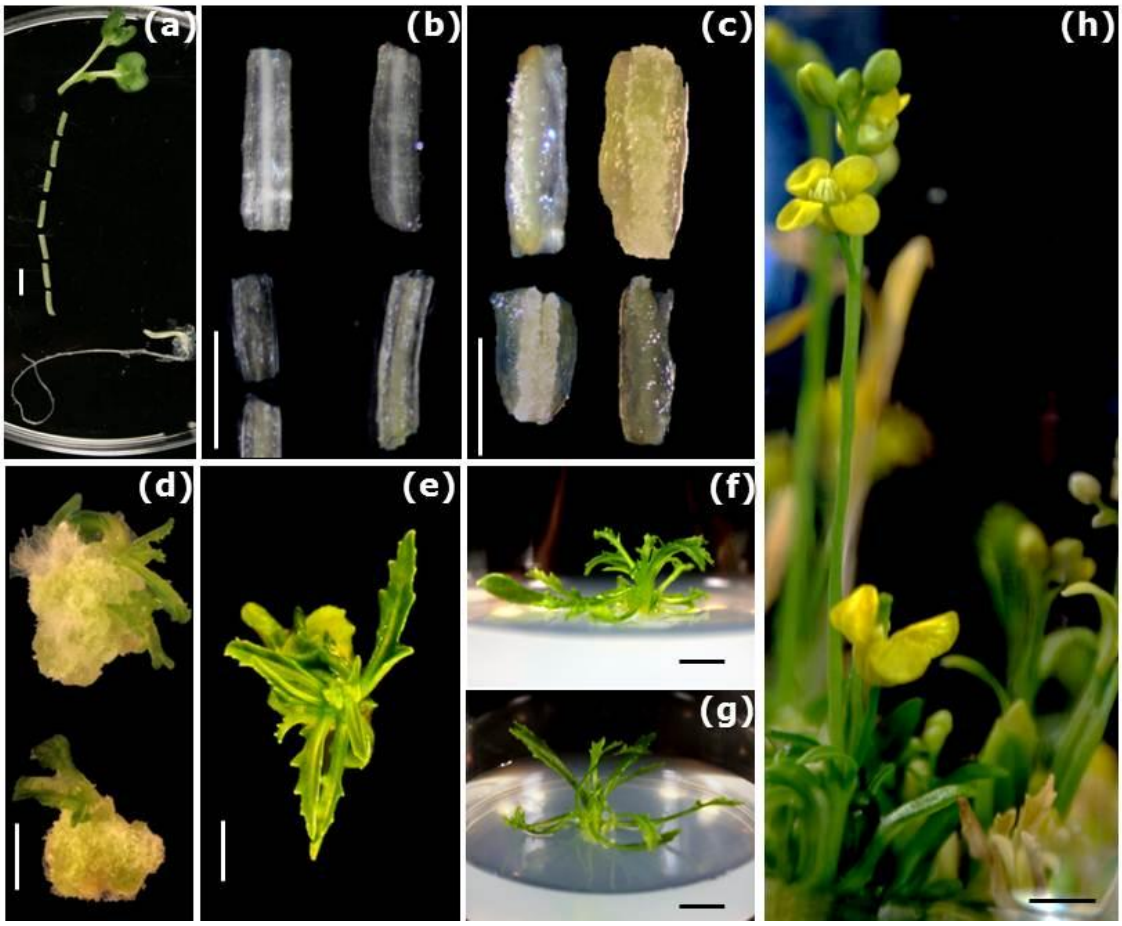


Figure 6.25 Regeneration from Rapid-Cycling *Brassica rapa* C1. **(a)** 7 days old cut seedling from Rapic-Cycling *Brassica rapa* **(b)** Hypocotyl segments on MD1 preculture medium. **(c)** Hypocotyl segments on BD1 callus induction medium. **(d)** First shoots on hypocotyl segments after four weeks on SIM medium to induce shoot production. **(e)** Shoot after one week on hormone-free medium. **(f, g)** Shoots after two weeks on root induction medium. **(h)** Mature plants on RS medium for seed production. Bars: 4 mm

6.2.2 Particle gun experiments for plastid transformation in Rapid-Cycling *Brassica rapa*

For plastid transformation in Rapid-Cycling *Brassica rapa* a vector was constructed, with insertion sites between the *trnV-rps12/7* genes. Fragments were amplified using Rapid-Cycling *Brassica rapa* (B3) total DNA as template and the PCR primers BrFw (AATTGAGCTCGTTCTATTCG) and BrRw (ATTGTCGACTTAC TGCCACT). The PCR product from RCBr was cloned into pJET® vector. One out of four clones was sequenced and no errors were detected. The PCR product was 100 % identical in sequence to *Brassica rapa* subsp. Pekinensis chloroplast sequence (NCBI: DQ231548.1/GI:85816402). *Brassica rapa* sequence was subcloned (*Sac*I-*Sal*I) in pUC19 vector.

pUC19-Br vector containing RCBr insertion sequence was linearized with *Eco R*V enzyme and dephosphorylated with SAP enzyme. The linearized fragment was ligated with an AmCyan cassette (PpsbA::AmCyan::Trlp32) which came from a digestion of pICF-BB-AmCyan vector (Torabi, 2009) with *Xho*I-*Sma*I. The *Xho*I site was removed by filling-in the site with the Klenow fragment of DNA polymerase. XL1 Blue competent cells were transformed with the ligation mixture. Positive, blue fluorescing colonies were characterized (Figure 6.26).

In order to obtain a vector containing an *aadA* cassette, pUC19-Br-AmCyan vector was digested with *Sca*I-*Pst*I and the fragment containing AmCyan cassette was cloned into the *Sca*I-*Pst*I sites from pUC18 vector. pUC18-Br-AmCyan vector was digested with *Kpn*I-*Sal*I and ligated to a *Kpn*I-*Xho*I fragment containing an *aadA* cassette (Prrn::*aadA*::TrbcL) from pC24-aadA vector. XL1 Blue competent cells were transformed with this ligation mixture and colonies growing on LB-Spec 100mg/l medium were obtained. pBr-AmCyan-aadA vector was characterized and purified vector was used to particle gun experiments on RCBr plants.

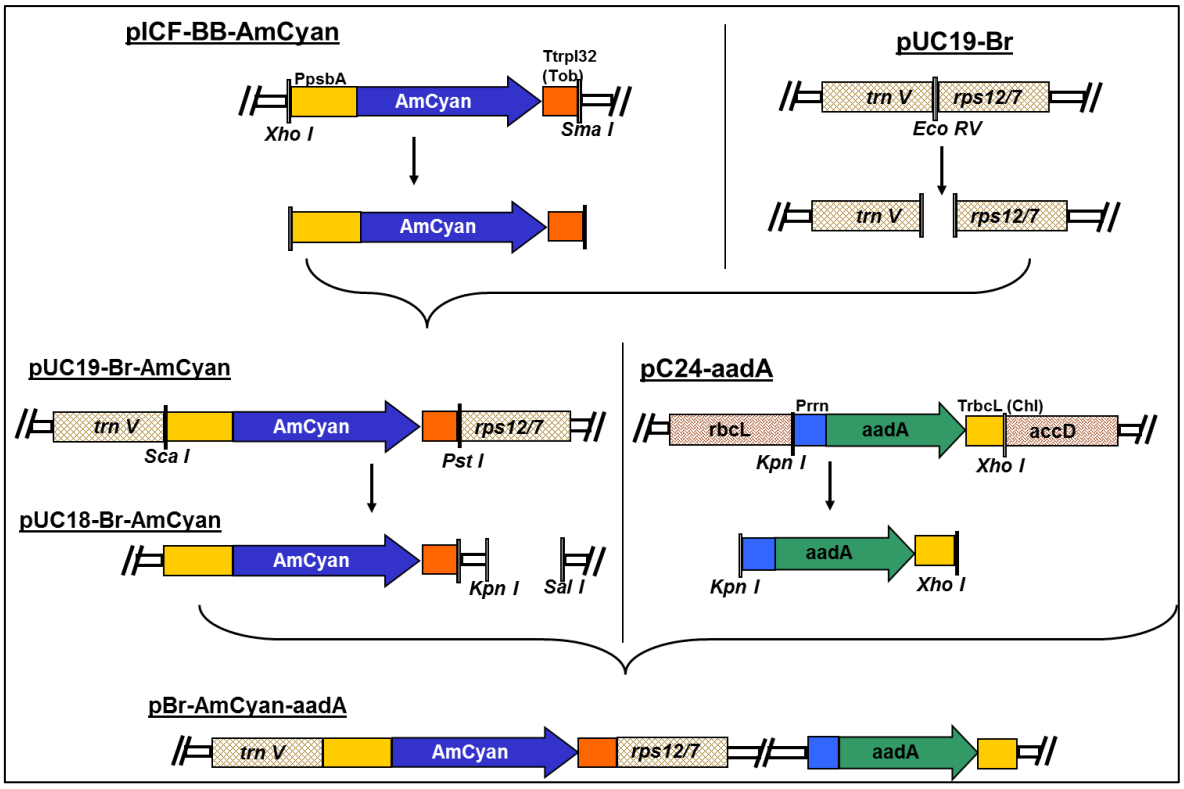


Figure 6.26 Schematic presentation of cloning step of a pBr-AmCyan vector. Restriction sites for cloning steps are shown. Hatched boxes represent Rapid-Cycling *Brassica rapa* homologous flanks.

After the development of a plant regeneration protocol and the construction of a specific plastid transformation vector, particle gun experiments were performed. Hypocotyls from RCBr were cut from around 70-80 plants and transferred to a preculture medium two days before bombardment experiments.

Two days after particle gun bombardment, hypocotyls were transferred to a BD1 callus induction medium (Table 5.9) containing 20 mg/l spectinomycin, for 7 days and subsequently were transferred to a SIM medium containing 20 mg/l spectinomycin. Figure 6.27 shows hypocotyls prepared for particle gun experiments and the effect of spectinomycin on shoot regeneration in RCBr hypocotyl explants. In summary 15 mg/l spectinomycin was sufficient to inhibit shoot regeneration.

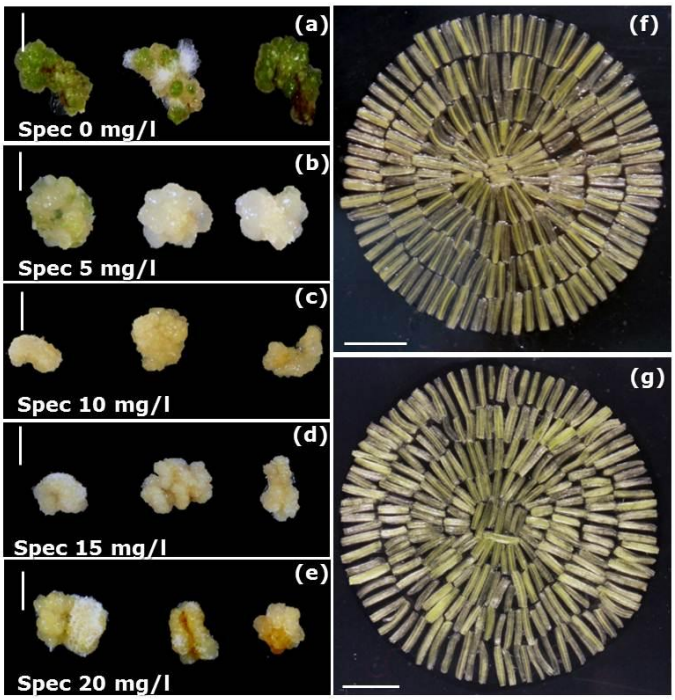


Figure 6.27 Effect of spectinomycin (Spec) on shoot regeneration from hypocotyls in RCBr. Hypocotyls on SIM medium containing Spec 0 **(a)** 5 **(b),** 10 **(c)**, 15 **(d)**, 20 **(e)** mg/l. Hypocotyls on MD1 medium for particle gun experiments. Bars: 3 mm.

In total, 12 particle gun experiments were done, using hypocotyls from RCBr as explants. Eight experiments were realized using pBr-AmCyan-aadA vector. In addition, four experiments were performed with pC24-AmCyan-aadA (with *Arabidopsis thaliana* flanks). From approximately 9,000 explants only one green callus was obtained. However, this green callus bleached after 15 days on SIM containing Spec 15 mg/l.

6.3 AmCyan as visual marker for plastid transformation in *Nicotiana tabacum*

AmCyan fluorescent protein (from *Anemona majano*) represents a new alternative as visual marker in plant biotechnology (Wenck *et al.*, 2003). One advantage of AmCyan is its brightness relative to eGFP fluorescent protein and another one is its resistance to photobleaching. So far, however, only nuclear transformation has been achieved. In the present work an AmCyan gene with regulatory elements for plastid transformation was used in *Arabidopsis thaliana* and RCBr. To determine whether the AmCyan cassette can be transcribed and expressed in plastids, *Nicotiana tabacum* cv. Petit Havana was transformed as a control. A tobacco pKCZ (Zou *et al.*, 2003) derived vector, containing an AmCyan cassette (PrrnT7g10-MASIS::*AmCyan*::Trpl32) followed by an *aadA* cassette (PpsbA::*aadA*::TrbcL (Chlamy) was constructed (Figure 6.28).

We used the usual protocol for plastid transformation in tobacco. Thus, either 7 days old protoplast derived colonies or three weeks old leaves from tobacco were bombarded with gold particles covered with a pKCZ-AmCyan-aadA vector. Two days after bombardment, protoplast derived colonies or leaf pieces were transferred to selective RMOP medium (spectinomycin 500 mg/l) for 20 days. Afterwards, medium was changed and plant material was incubated for additional 20 days on RMOP medium (spectinomycin 500 mg/l). In order to obtain transplastomic plants, several rounds of selection and regeneration were carried out. Table 6.5 shows a summary of particle gun experiments in tobacco. One experiment was performed with nine leafs, the other one with seven grids containing protoplast derived colonies. Approximately, 5 putative lines were gained per bombarded leaf and 2 putative lines were gained per each grid containing protoplast derived colonies.

Table 6.5 Summary of particle gun plastid transformation experiments in *Nicotiana tobacum*

Experiment	Method	Plant material	Putative lines	Analyzed lines
KAA-1	Particle gun	Leaves	48/9	15
KAA-2	Particle gun	Protoplast derived colonies	15/7	2/15

Putative lines were further analysed by PCR, fluorescence microscopy and finally by southern blotting. Some results are presented in figure 6.28, showing the chloroplast transformation vector (pKCZ-AmCyan-aadA), pieces of leaves prior – and after selection and amplification of the *aadA* gene by PCR from lines: 121, 151, 171, 191, 194 and 194-2. PCR amplification was positive (see arrow).

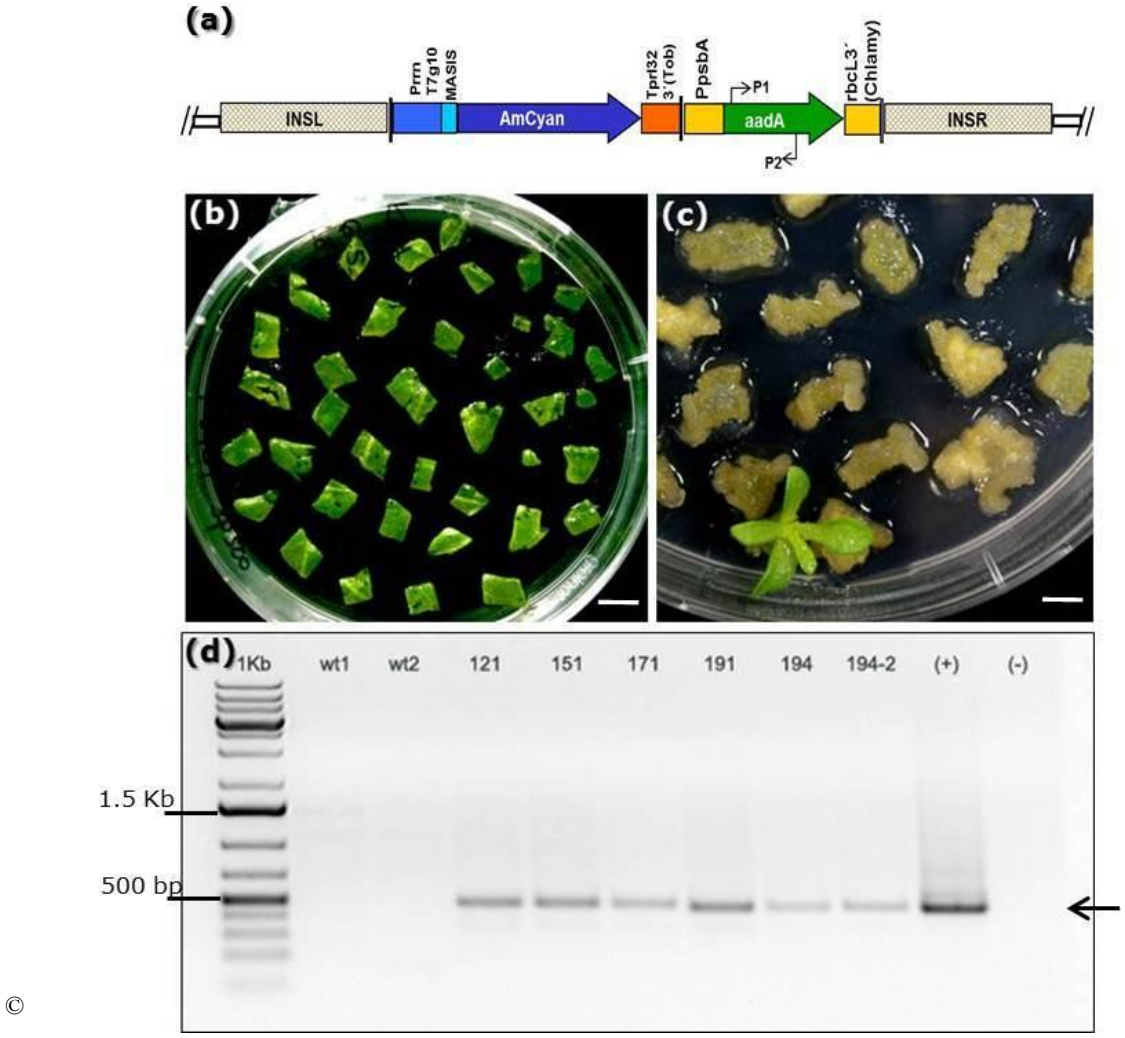


Figure 6.28 Plastid transformation in *N. tabacum* with the AmCyan gene. **(a)** Schematic presentations of the transformation vector containing AmCyan and aadA cassettes. Hatched boxes, homologous flanks, between trnR-ACG and trnN-GUU. Arrows indicate P1 and P2 for PCR amplification of the aadA gene. **(b)** Tobacco leaf pieces under selective conditions, two days after bombardment (RMOP-Spec 500 mg/l). **(c)** Three weeks after bombardment, showing a resistant shoot. **(d)** aadA amplifications from six lines independent lines. As positive control pKCZ-AmCyan-aadA vector was used, water was used as negative control and 1 Kb Plus was used as marker. Agarose TAE gel 1 %. Bars: 5 mm

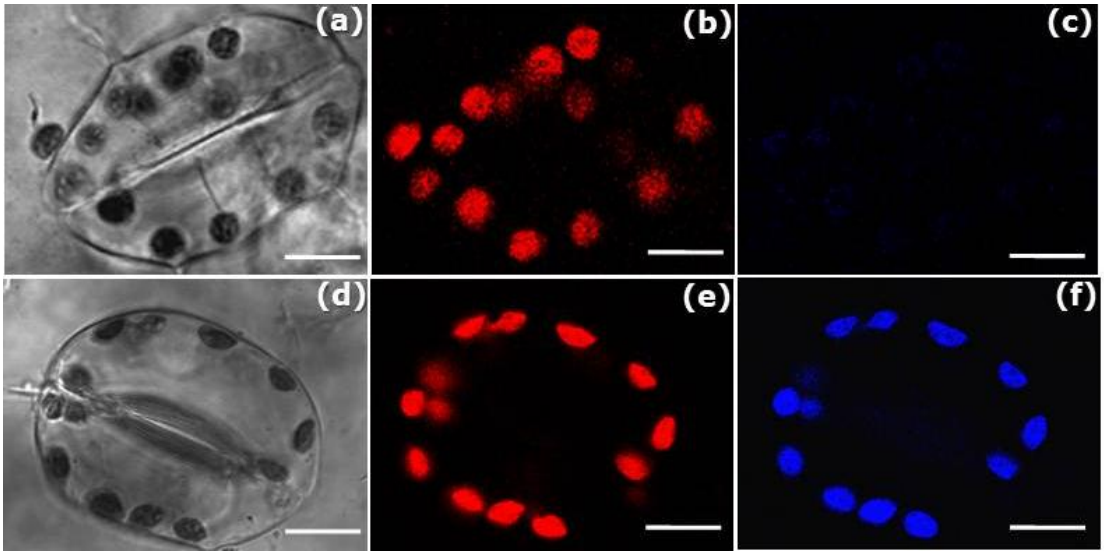


Figure 6.29 AmCyan accumulation in tobacco transplastomic chloroplast. **(a)** Stoma showing two guard cells under white light from a wild type plant. **(b)** Chlorophyll autofluorescence from a wild type plant. **(c)** Guard cells from wild type plant showing absence of AmCyan fluorescence. **(d)** Stoma showing two guard cells under white light from KAA194 line. **(e)** Chlorophyll autofluorescence from KAA194 line. **(f)** Guard cells from KAA194 line showing AmCyan fluorescence. Tobacco leaves were analyzed by confocal laser scanning microscopy. Bars: 10 µm

Positive lines were further characterized for *AmCyan* gene expression. All chloroplasts in the analyzed lines expressed AmCyan (Figure 6.29). Fertile plants were grown in the greenhouse to get seeds and sufficient plant material from T0 and T1 plants for Southern Blotting. Also, no difference in the phenotype between tobacco wild type and AmCyan lines could be observed (Figure 6.30)

Southern Blot analysis allows us to check the correct integration of the AmCyan gene in the plastome. Figure 6.30 displays the Southern Blot analysis and the comparison of WT plants with one representative KAA line. Column 2 from Southern blot shows the WT signal (5,701bp), in contrast, the analyzed lines in the columns T1-T11 which display a DNA fragment signal from 8,561bp, indicating the integration of *AmCyan* and *aadA* genes into the tobacco plastome. Line T8 shows a signal of circa 9,000 bp which can be explained by an intermolecular recombination (Huang *et al.*, 2002).

A weak wild type signal is observed in all positive lines, even after ten cycles under selection (each cycle means regeneration induced during three weeks on

RMOP containing 500 mg/l spectinomycin). This weak signal suggests that copies from the wild type genome might still be present in all T1 analyzed lines.

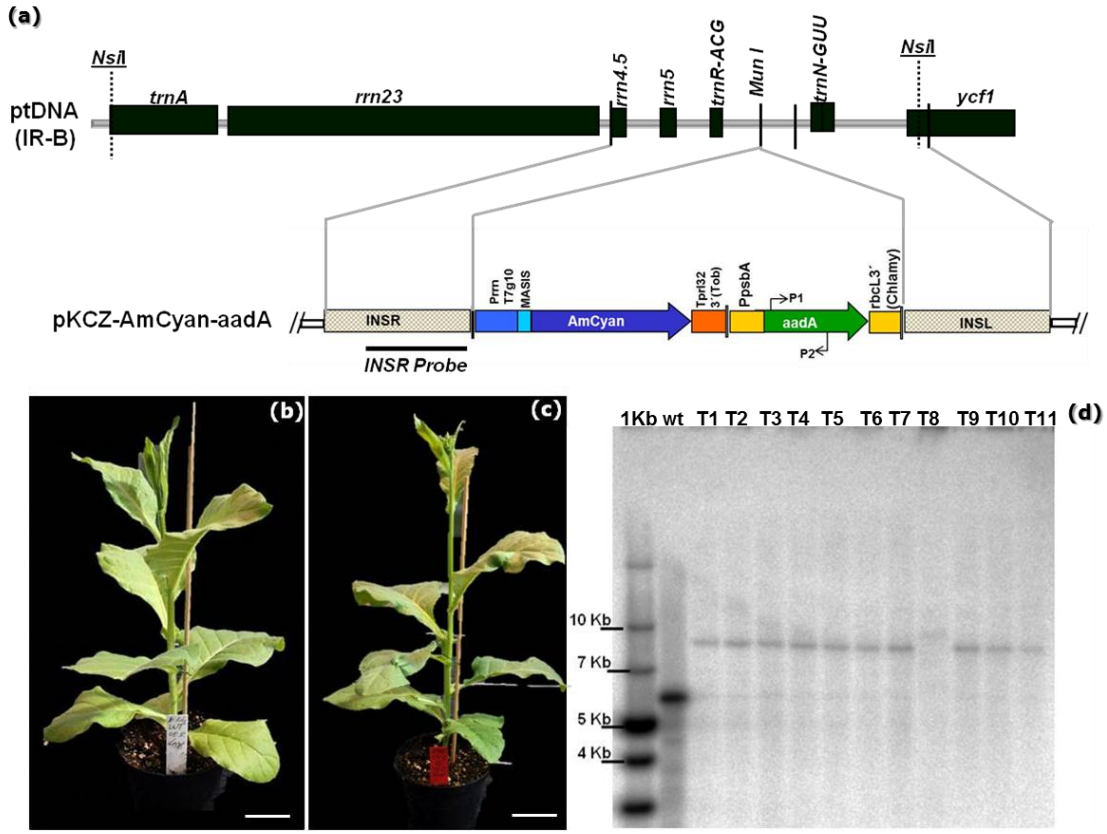


Figure 6.30 Southern blot analysis of 10 transplastomic plants generated with a vector containing AmCyan fluorescence marker. **(a)** Schematic presentation of insertion sites in the tobacco plastome (ptDNA), hatched boxes: homologous flanks. Insertion occurred at the *Mun*I site. The probe used for Southern blot analysis is indicated. **(b)** Eight weeks old wild type tobacco plant growing in the greenhouse **(c)** Eight weeks old tobacco KAA line growing in the greenhouse. **(d)** Southern blot analysis of 10 lines and one wild type control (wt). Total DNA was digested with *Nsi*I and transferred to a nylon membrane. The blot was hybridized with the INSR probe (855 bp). Wild type resulted on a 5,701 bp fragment and transplastomic lines resulted on 8,561 bp fragment. Bars: 10 cm

To ensure the absence or presence of wild type plastome copies in those KAA transplastomic lines, we performed reciprocal crosses of two independent tranplastomic lines with wild type plants. This allows the screening of the progeny for the presence or absence of a spectinomycin resistance gene in the plastome by germination of seeds on selective medium. This method was proposed by Svab and Maliga (1990) (Figure 6.31).

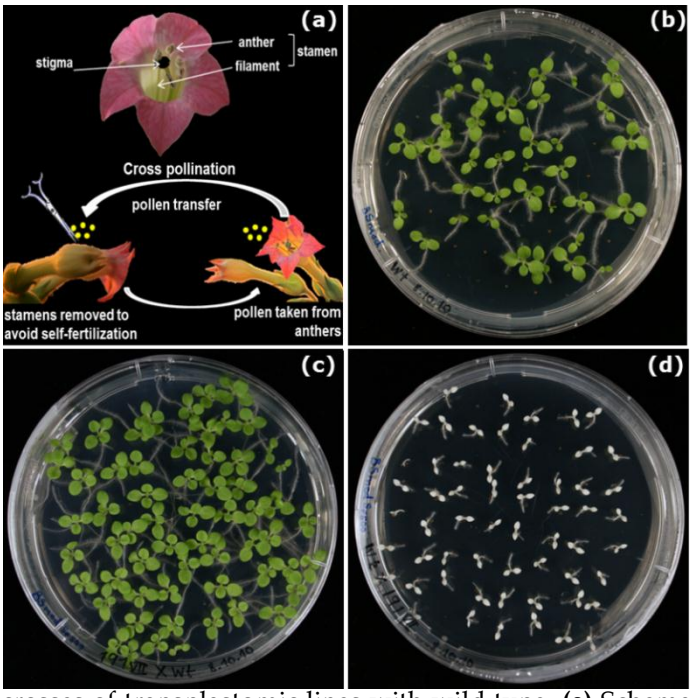


Figure 6.31 Reciprocal crosses of transplastomic lines with wild type. **(a)** Schematic representation of reciprocal crosses between wild type and transplastomic plants. Pollen of transplastomic lines was used to pollinize wild-type (first cross), and pollen of wild-type plants was used to pollinize transplastomic lines (reciprocal crosses). **(b)** As a control, seedlings from self-crossed wild type tobacco plants were germinated on antibiotic-free medium. Seedlings develop normal. **(c)** Crosses between a transplastomic line as the maternal parent with pollen from the wild type tobacco plant as donor. Seedlings develop normal in the presence of spectinomycin, showing the maternal inheritance of the resistance cassette (green). **(d)** Crosses between wild type as the maternal parent with pollen from transplastomic line as donor. Maternal inheritance is shown by the antibiotic-sensitive (white) seedlings growing on spectinomycin-containing medium. Pollen of transplastomic plants does not transmit the transgene to the progeny.

Wild type and KAA lines containing an *AmCyan* and an *aadA* gene were transferred to soil and grown to maturity. Seed pods were collected from selfed plants and from reciprocal crosses. Surface sterilized seeds were germinated on B5 medium containing spectinomycin (500 mg/l). Self-pollinized transplastomic plants and crosses with a transplastomic line as the maternal parent gave rise to resistant (green) plants. Seeds collected from wild type plants yield antibiotic sensitive (white) seedlings (data no shown) crosses with a wild type as the maternal parent gave rise to sensitive plants. Importantly, pollen from transplastomic lines does not transmit the transgenes (either *aadA* or *AmCyan* genes) (Figure 6.31). Taken together, we could successfully introduce AmCyan, a new fluorescence marker into chloroplast genome of *Nicotiana tabacum.*

7 Discussion

7.1 Towards plastid transformation in *Arabidopsis thaliana*

Because *Arabidopsis thaliana* is a well characterized plant model organism with thousands of available mutants, plastid transformation would be a helpful tool for the elucidation of many aspects in plant biology. Thus, the establishment of a reproducible plastid transformation method in *Arabidopsis thaliana* was considered highly desirable for more than ten years (Sikdar *et al.*, 1998; Maliga, 2004; Dhingra and Daniell, 2006).

From the biotechnological point of view, other alternatives as plant viral vector systems represent a better option for transient expression of transgenes and large scale production of biopharmaceutical proteins (Gleba *et al.*, 2007). Nowadays, plastid transformation is well established in tobacco and there it represents a tool both, in basic and applied research (Koop *et al.*, 2007; Maliga and Bock, 2011).

In this study, we focused on the most critical steps to achieve plastid transformation in *Arabidopsis thaliana* and RCBr. To allow the generation of fertile plants out of transformed cells, first, a reliable regeneration protocol must be established. Then, for the transformation and specific introduction of genes into plastids, an appropriate vector that ensures site specific integration and the selection of the transformed cell is required. For selection, the vector often contains an antibiotic resistance gene to detoxify antibiotics and a visual marker as GFP. Finally, a plant transformation method such as particle gun or PEG mediated transformation method must be established.

7.1.1 Regeneration protocols in *Arabidopsis thaliana*

For successful plastid transformation, a method which allows the regeneration of fertile plants out of a single transformed cell is needed. Therefore, in this study different protocols have been described using different cells and tissue sources. On one hand tissues as cotyledons and seeds are used and on the

other hand isolated plant cells without a cell wall (protoplasts) can be used for the regeneration of fertile plants.

In *Arabidopsis thaliana* a major obstacle for plastid transformation was the lack of a reproducible and efficient regeneration protocol (Acedo, 1986; Feldmann and Marks, 1986). In this study we established reliable tissue and cell culture procedures allowing high regeneration efficiencies.

Tissue culture

Most regeneration protocols using cotyledons or seeds in *Arabidopsis thaliana* follow a two-step procedure. First, explants are dedifferentiated with the help of a callus induction medium (intermediate auxin : cytokinin ratio) and subsequently, the callus is transferred to a shoot induction medium (low auxin : high cytokinin) (Valvekens *et al.*, 1988).

Regeneration efficiencies in *Arabidopsis thaliana* also depend on ecotype and tissue type. Between all the analysed ecotypes, C24 has one of the highest organogenesis and embryogenesis capacities (Schmidt and Willmitzer, 1988; Chaudhury and Signer, 1989; O'Neill and Mathias, 1993; Barghchi *et al.*, 1994). Recently our group confirmed these finding in *Arabidopsis thaliana* ecotype C24 (Luo and Koop, 1997; Dovzhenko *et al.*, 2003).

On the basis of these results, we used several explants of the ecotype C24 in this study. Furthermore, by the combination of several previously reported protocols, the regeneration rate was improved.

The use of cotyledons as explants has been previously described. In this thesis, a callus induction medium was applied according to Zhao *et al.*, (2002) and a regeneration medium as described by Luo *et al.*, (1999). Shoots could be regenerated with efficiencies of up to 85 % using 6 days old cotyledons. This is almost 4 times more than previously reported by Zhao *et al.*, (2002).

Successful establishment of a regeneration protocol also depends on the age and the origin of the explants. A reduction in the regeneration capacity was observed

when two weeks old leaves were used (personal communication with Salar Torabi). Other explant sources have been used in the regeneration of shoots in *Arabidopsis thaliana* including hypocotyls, leaves (Schmidt and Willmitzer, 1988), roots (Valvekens *et al.*, 1988) and seeds (Negrutiu *et al.*, 1975).

Regeneration of plants using seeds as explants for the induction of organogenesis or embryogenesis has been reported by several groups (Negrutiu *et al.*, 1975; Huang and Yeoman, 1984). However, high regeneration efficiency was not achieved by those groups. Thus, we also tried to improve this type of regeneration protocol. To allow the selection of transformed plastomes in the presence of selective agents, we have chosen a regeneration method with a long period of callus induction. For this reason, seeds were first incubated in a callus induction media for four weeks. With a combination of the callus induction medium PG20KN and the shoot induction medium SRAKN, we have reached efficiencies of up to 97 %. If each shoot per explant is counted independently, we could reach an efficiency of 500 to 700 % (Figure 6.4).

Atta *et al.*, (2009), presented some evidence that the origins of shoot regeneration are pericycle cells. They demonstrated that xylem pericycle is much more pluripotent than previously thought. In this study, when seed derived calli were transferred to regeneration medium shoots were formed either from roots or from hypocotyls. This data supports previous findings by Atta *et al.*, (2009) because xylem pericycle cells can be found in hypocotyls and roots.

Protoplast culture

Plastid transformation efficiencies using PEG-mediated DNA transfer or the particle gun method have almost the same transformation efficiencies in tobacco (Kofer *et al.*, 1998). This makes protoplasts a good alternative for plastid transformation. Successful protoplast cultivation depends on the following factors: the correct environmental conditions, source of protoplasts, protoplast density, media composition, osmotic pressure, light, pH and temperature conditions (Dovzhenko *et al.*, 1998).

In this work, an efficient regeneration protocol for protoplast culture in *Arabidopsis thaliana* was established. Embedding protoplasts in alginate has the advantage that it allows better the cell division and thus the regeneration of fertile plants out of leaf protoplasts (Damm and Willmitzer, 1988). Plating efficiencies (number of protoplast divisions per embedded protoplasts) of up to 16 % could be reached after embedding of *Arabidopsis thaliana* leaf protoplasts in thin alginate layers (Luo and Koop, 1997).

Previously, it was shown that fertile plants could also be regenerated from different sources like protoplasts from suspension cultures (Ford, 1990), roots (Mathur *et al.*, 1995), leaves (Luo and Koop, 1997) and cotyledons (Dovzhenko *et al.*, 2003).

As a base for our experiments, we used a protocol proposed by Dovzhenko *et al.*, (2003) with some modifications that provided plating efficiencies between 40 to 60 %. An important modification of the method by Dovzhenko *et al.* was the replacement of the Dicamba (auxin) phytohormone with a combination of the auxin NAA and the cytokinin 2ip in the protoplast culture medium (PCA). Another important change was the addition of the antibiotic carbenicillin to avoid contamination of protoplast cultures with bacteria. Once protoplast derived colonies were obtained, grids were transferred to a shoot regeneration medium with Kinetin and NAA as phytohormones (SRAKN).

Regeneration in SRAKN medium was highly efficient and more than 150 shoots per grid were obtained. Shoots appeared on SRAKN medium after just 7 days of culture (Figure 6.7).

To allow the segregation of transformed chloroplasts, a regeneration medium with a callus phase was established. Therefore, SRA2N medium was supplemented with 2ip and NAA as phytohormones which allow a long callus phase before shoot formation.

Protoplast-derived calli contain a lower number of plastids compared to leaves (Thomas and Rose, 1983). This could facilitate to a faster selection of transformed

chloroplasts because the starting ratio of transformed plastids to untransformed plastids is higher from the beginning on. Thus, the transformed plastomes will have a better opportunity of being transmitted to daughter cells (Huang *et al.*, 2002).

7.1.2 Species specific vectors in *Arabidopsis thaliana*

Plastid transformation not only requires a reliable regeneration method, it also needs species specific vectors with appropriate regulatory elements, selective markers and specific recombination sites.

Construction of species specific vectors

Plastid transformation follows the principles of homologous recombination (Carrer and Maliga, 1995). The transgene has to be flanked at each site by plastome sequences ensuring the integration of the transgene into a specific region of the plastome (Figure 3.4) (Svab *et al.*, 1990).

Plastid transformation has been achieved using heterologous sequences to allow the integration of transgene in plastomes. Nevertheless, a lack of complete homology may reduce the transformation efficiency, for example, when petunia flanking sequences are used to transform tobacco plants (DeGray *et al.*, 2001). Thus, the use of species-specific vectors for each crop is suggested (Table 7.1).

Selection of integration sites and size of homologous flanks could be an important factor to achieve transplastomic plants. In this work we have used three homologous flanks to the *Arabidopsis thaliana* plastome for the insertion of transgenes. These three regions are between *rps12/7-trnV* (Zoubenko *et al.*, 1994); *trnfM-trnG* (Ruf *et al.*, 2001) and *rbcL-accD* genes (Svab and Maliga, 1993).

In summary, insertion sites used in this study for plastid transformation vectors in *Arabidopsis thaliana* have been previously reported in several plants as shown in Table 7.1.

Regulatory elements in plastid transformation

Regulation of plastid expression in higher plants is similar to that in eubacteria. The plastome encodes the RNA polymerase (PEP) and RNA polymerases (NEP) as RPOTp and RPOTpm are imported into plastids from the nucleus (Allison, 2000).

In plastid transformation the most common promoter used is the rrn promoter (Prrn) of the 16S plastid rRNA operon. This Prrn promoter is regulated during development and its operon can be transcribed by both, PEP and NEP polymerases. Regulation of the rrn operon is complicated as this operon has three promoters: P1, PC and P2. Tobacco chloroplasts lack the factor required for PC recognition, however other species like spinach and *Arabodopsis thaliana* are able to initiate transcription from PC sites. In *Arabidopsis thaliana* the RPOTmp transcribes the rrn operon using the PC promoter only in early developmental stages (Courtois *et al.*, 2007).

In contrast to this evidence, one of the initial findings in *Arabidopsis thaliana* transplastomic plants was that Prrn from tobacco is functional in *Arabidopsis thaliana* plants even if it lacks the PC region (Sriraman *et al.*, 1998). This fact can be explained by the presence of the P1 promoter in tobacco. Figure 7.1 shows the structure of the rrn operon promoters.

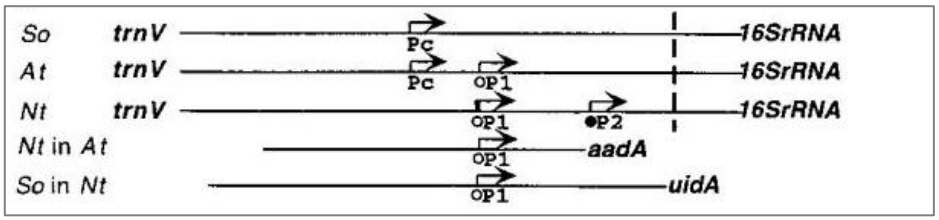


Figure 7.1 Schematic representation of transcription-initiation sites in the promoters of the rrn operon in spinach (So), *Arabidopsis* (At), and tobacco (Nt). Transcription-initiation sites are marked with horizontal arrows PEP and nuclear-encoded plastid RNA polymerase promoters are marked with open and filled circles, respectively. Figure from Sriraman *et al.*, 1998.

Due to the fact that the tobacco Prrn promoter has been proven to function in *Arabidopsis thaliana,* we decided to use this promoter in our plastid transformation vectors.

The psbA promoter was another promoter we chose. The *psbA* gene encodes the D1 protein of photosystem II and its promoter is often used in plastid transformation (Staub and Maliga, 1993; Tangphatsornruang *et al.*, 2010).

Post-transcriptional RNA levels are also tightly regulated in chloroplasts (Stern *et al.*, 2010). Stem-loop secondary structures in the 5´ UTR of the mRNA are important for the translation and the stability of the mRNA (Eibl *et al.*, 1999; Monde *et al.*, 2000b). For these reasons, a suitable 5 ´UTR region which includes a ribosomal binding site (RBS) is important for successful plastid transformation. Usually, a 5´UTR of a highly expressed plastid gene like *atpB*, *psbA* or *rbcL* is used (Staub and Maliga, 1993).

It has been shown that a 5´UTR of the gene 10 from the bacteriophage T7 (T7g10) increases the translation rate and thus the accumulation of proteins (Kuroda and Maliga, 2001). Several groups have shown that extensions or modifications of the N-terminus of the transgene protein also increase the expression level of proteins (Kuroda and Maliga, 2001; Ye *et al.*, 2001; Apel *et al.*, 2010).

Another important element for transcript stability is the 3´UTR. Several 3´UTRs have been tested. Eibl *et al.,* (1999) found that the 3´UTR from the *rpl32* gene could increase the amount of *uidA* transcripts up to threefold compared to the *psbA* 3´UTR. However, GUS activity was not significantly influenced in transplastomic tobacco plants. Stability of transcripts has also been confirmed using different 3´UTR sequences from *rbcL*, *psbA*, *petD*, *rpoA* and *E. coli rrnB* genes and *gfp* as reporter gene (Tangphatsornruang *et al.*, 2010). In summary, regulatory elements are important for plastid transformation and should be considered carefully. Table 7.1 gives an overview of the regulatory elements used in this study, their references and a comparison with other plants.

Table 7.1 Plastid transformation in higher plants*

Plants	Insertion sites	Genes/ regulatory elements	Results	References
Arabidopsis thaliana	*rbcL/accD*	Prrn/RBS::*aadA*::TrbcL Prrn/g10ASGFP::*nptII*::TrbcL Prrn/g10::*gfpbar*::TrbcL	126 putative lines. All lines bleached after strict selection conditions.	This study
	trnV/rps12/7	PpsbA/g10::*gfpbar*::TrbcL Prrn/RBS::*aadA*::TrbcL		
	trnfM/trnG	Prrn/RBS::*aadA*::TrbcL Prrn/g10MASIS::*AmCyan*::Trpl3-PpsbA::*aadA*::TrbcL Prrn/g10MASIS::*DsRed*::Trpl32-PpsbA::*aphA6*::TrbcL		
Arabidopsis thaliana	*trnV/rsp12/7*	Prrn::*aadA*::TrbcL	2 homoplasmic T_0 lines from 200 shots. Sterile plants	(Sikdar *et al.*, 1998)
Lesquerella fendleri	*trnV/rsp12/7*	Prrn/rbcL-RBS::*aadA-gfp*::psbA	2 homoplasmic T_0 lines from 51 shots. T_1 progeny from a grafted shoot.	(Skarjinskaia *et al.*, 2003)
Cauliflower	*rbcL/accD*	Prrn/rbcLRBS::aadA::TpsbA	1 homoplasmic T_0 line from $3X10^6$ treated protoplasts.	(Nugent *et al.*, 2006)
Oil seed rape	*trnI/trnA*	Prrn/g10::*aadA*::TpsbA	1 heteroplasmic T_0 line from 4 shots.	(Cheng *et al.*, 2010)
Petunia	*rbcL/accD*	Prrn/rbcLRBS::aadA::TpsbA	3 homoplasmic T_0 lines from 31 shots. Homoplasmic in T_1 progeny.	(Zubko *et al.*, 2004a)
Potato	*rbcL/accD* *trnV/rsp12/7*	Prrn::*gfp*::Trps16 PpsbA::*aadA*::TpsbA	6 homoplasmic T_0 lines from 150 shots. No seeds.	(Sidorov *et al.*, 1999)
Tomato	*trnfM/trnG*	Prrn/rbcL-RBS::*aadA*::TpsbA	6 homoplasmic T_0 lines from 60 shots. Homoplasmic in T_1 progeny.	(Ruf *et al.*, 2001)
Soybean	*trnV/rps12/7*	Prrn/rbcL-RBS::*aadA*::TpsbA	18 homoplasmic T_0 lines from 8 shots.	(Dufourmantel *et al.*, 2004)
Cotton	*trnI/trnA*	Prrn/g10::*aphA6*::Trps16 PpsbA::*nptII*::TpsbA	30 homoplasmic T_0 lines from 199 shots.	(Kumar *et al.*, 2004)
Rice	*rrn16/rps12/7*	Prrn/g10::*aadA16gfp*::psbA	12 heteroplasmic T_0 lines from 25 shots. No seeds	(Khan and Maliga, 1999)
	trnI/trnA	Prrn/RBS::*aadA*-RBS-*gfp*::psbA	6 heteroplasmic T_0 lines from 10 shots. Heteroplasmic in T_1 progeny.	(Lee *et al.*, 2006)
Sugar beet	*rrn16/rps12/7*	Prrn::*aadA*-PpsbA-*gfp*::TpsbA	1 homoplasmic T_0 lines from 36 shots.	(DeMarchis *et al.*, 2009)
Cabbage	*rrn16S/rrn23S*	Prrn/rbc-LRBS::*aadA*::TpsbA Prrn/rbcL-RBS::*cry1Ab*::TpsbA	3 homoplasmic lines from 150 shots.	(Liu *et al.*, 2007)
Lettuce	*rbcL/accD*	Prrn/RBS::*aadA*::TpsbA PpsbA::*gfp*::rps16	6 heteroplasmic from 10 shots. Homoplasmic in T_1 progeny	(Kanamoto *et al.*, 2006)
	trnI/trnA	Prrn/rbcL-RBS::*aadA*::TpsbA Prrn/rbcL::*gfp*::TrrnB	9 homoplasmic lines from $5.6X10^6$ treated protoplasts.	(Lelivelt *et al.*, 2005)
Carrot	*trnI/trnA*	Prrn/RBS::*aadA*::TpsbA Pg10::*badh*::Trps16	9 homoplasmic T_0 lines from 284 shots	(Kumar *et al.*, 2004b)
Poplar	*rbcL/accD*	Prrn/RBS::*aadA*::TpsbA PpsbA::*gfp*::Trps16	10 homoplasmic T_0 lines from 30 shots	(Okumura *et al.*, 2006)
Wheat	*atpB/rbcL*	Prrn::*gfp*::TpsbA Prrn::*nptII*::TpsbA	1 homoplasmic T_0 line and 2 heteroplasmic T_1 from 57 shots	(Cui *et al.*, 2011)
Eggplant	*trnV/rsp12/7*	PpsbA::*aadA*::TpsbA	2 homoplasmic T_0 lines from 21 shots.	(Singh *et al.*, 2010)

*Only representative papers are given. Genes: *aadA* (aminoglycoside 3´-adenyltrannsferase), *nptII* (neomycin phosphotransferase II), *aphA6* (aminoglycoside phosphotransferase VI), *cry1Ab* (*Bacillus thuringiensis* crystal proteins), *gfp* (green fluorescent protein), *gus* (β-glucuronidase), *badh* (betaine aldehyde dehydrogenase). Regulatory elements:P (promoter), T (terminator), RBS (ribosome binding site) g10 (ribosome binding site from bacteriophage T7 gene 10L).

Combination of visual and selection markers

The combination of a visual with a selection marker allows a simpler selection of transformed cells. In this study, vectors containing selection markers such as *aadA* and *aphA6* outside the homologous flanks and visual markers inside the flanks were constructed.

This strategy is based on the generation of marker free transplastomic plants proposed by Klaus *et al.*, in our group (Klaus *et al.*, 2004). Klaus *et al.*, observed that recombination via a single flank often occurs; this event leads to the formation of vector co-integrates in the plastome. This co-integrate is resolved through a secondary recombination event. Once selection pressure is removed, a secondary

recombination either leads to plastomes identical to those of the target lines or to marker free plastomes containing the gene of interest (Figure 7.2). Klaus *et al.* used a system in which pigment-deficient tobacco knockout plants (Δ*petA* or Δ*rpoA*) were used as a target. The transformation vector carried a gene which encodes a protein involved in photosynthesis; this restored the green pigmentation and introduced the gene of interest with the removal of the selective marker. Homoplastomic sectors in plants were identified by the green color; this strategy reduces significantly the time required to obtain homoplastomic plants and avoids problems with contamination of transgenes (Klaus *et al.*, 2003; 2004).

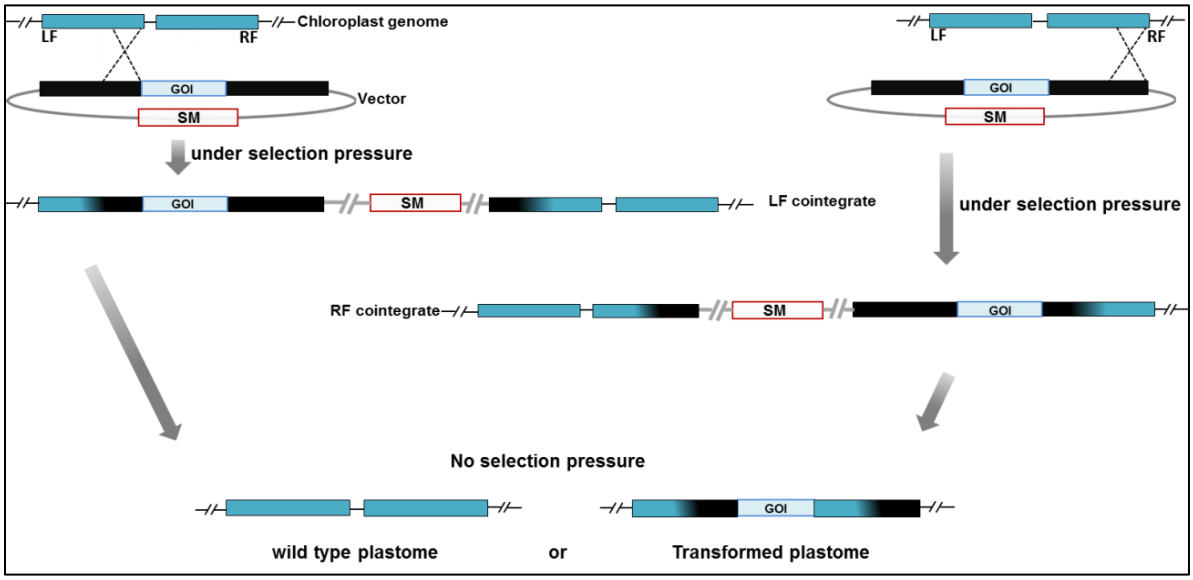


Figure 7.2 Marker free plastid transformation. Plastid transformation vector with a selection marker (SM) outside of the homologous flanks (dark boxes). Recombination can occur via either left of right flanks resulting in co-integration of the vector in the plastome. After several recombination events and without selection pressure the marker gene is lost. Finally only the gen of interest (GOI) is stable integrated or the wild type plastome could be obtained. Uses of a visual marker as GOI can favor the selection of transplastomic lines.

7.1.3 Particle gun-mediated plastid transformation

Cotyledons and seed derived calli as target for plastid transformation

In the beginning of this study, cotyledons (6 days old) of *Arabidopsis thaliana* were used for plastid transformation experiments. Petri dishes containing 150 cotyledons were bombarded with appropriate plastid transformation vectors. Due to the fact, that 6 days old cotyledon preparations and their subsequent transfer to

selection medium was a time consuming work and all nine particle gun experiments failed, we decided to simplify our method by the use of seed derived calli.

After the improvement of the regeneration protocol for seed derived calli, the appropriate concentration for the selective reagents as bialaphos and PPT was determined. Therefore, seed derived calli were bombarded with pBA-GFPbar or pC24-GFPbar vectors (Table 7.1). Seed derived calli were further grown in callus induction media containing different concentrations of bialaphos or PPT (data none shown). The *bar* gene encodes for resistance to the herbicides bialaphos and PPT (Lutz *et al.*, 2001). In media containing B5 vitamins (PG1KD) the generation of shoots was inhibited after the addition of 1.5 mg/l bialaphos. Interestingly, in the presence of cocktail 20 almost twice the concentration of bialaphos (3 mg/l) was needed. Cocktail 20 contains a high concentration of nitrogen compounds which may influence herbicide activity. At least 170 Petri dishes containing seed derived calli (one or four weeks old) were bombarded. However, under selective conditions, no resistant line was obtained and no GFP fluorescence could be detected.

To test that the biolistic gun experiment works in principal, transient experiments using a nuclear vector containing the *uidA* gene (data not shown) were performed. The histochemical detection of GUS in cotyledons and seed derived calli demonstrated that the chosen biolistic gun conditions were suitable for DNA delivery into the cells and that those conditions can be used for *Arabidopsis thaliana* plastid transformation.

Protoplast derived colonies as target for transformation

The use of protoplast culture for plastid transformation experiments is not limited to the PEG-mediated plastid transformation method. Protoplast derived colonies can be used as target for particle gun-mediated plastid transformation. In 1998 Dovzhenko *et al.*, described a simplified method for the handling of protoplasts and the embedding of the cells into alginate which enhances cell

divisions and thus the formation of protoplast derived colonies (Dovzhenko *et al.*, 1998). Use of protoplast derived colonies as target for plastid transformation in tobacco reduced the handling effort by 60 % compared to the traditional use of leaves as target material (Huang *et al.*, 2002).

In this study, after the improvement of protoplast culture, more than 350 petri dishes containing protoplast derived colonies were bombarded with DNA coated particles. Several vectors were used (Table 7.1). The first set of experiments was performed with vectors containing DsRed fluorescence marker and the genes *aadA* and *aphA6* to confer resistance to spectinomycin and kanamycin antibiotics 95olyplotively. From this experiment, 12 resistant calli were obtained. However, under more strict selection conditions all calli and shoots bleached. DsRed fluorescence analysis was difficult because the high background due to the auto fluorescence of wounded and highly stressed cells. Using this method no positive lines were detected.

According to a publication by Jach *et al*., (2001) tobacco plants could be successfully transformed with DsRed-Chl via *Agrobacterium tumefaciens*. *DsRed* gene was integrated into the nucleus and the DsRed protein was imported into chloroplasts with the help of a signal peptide. However, DsRed could only be observed in fully developed plant, and not in calli (Jach *et al*., 2001). This could be the reason why we could not detect DsRed in our calli.

In a second set of experiments, the fluorescence marker AmCyan was combined with the *aadA* gene which confers spectinomycin resistance. In these experiments 89 putative lines were obtained. One advantage of AmCyan is that it is highly resistant to photobleaching and it has a relatively high brightness level when compared to CFP (Cyan Fluorescent Protein) from *Aequorea* (Day and Davidson, 2009). Recently, in our group, Torabi (2009) achieved plastid transformation in *Brassica napus* using the AmCyan fluorescence marker. As reported by Torabi (2009), slight blue fluorescence was observed in the cell wall of wild type *Arabidopsis thaliana* (Torabi, 2009). Buschmann *et al*., have shown that the blue green fluorescence (440 nm) derived from ferulic acid (a hydroxycinnamin acid)

bound to the cell wall (Buschmann *et al.*, 2000). Although the low background made the analysis more complicated, we used this marker together with a fluorescence microscope. As a positive control, we took the transgenic *Arabidopsis thaliana* line pt-ck CS16265 (Nelson *et al.*, 2007), expressing AmCyan in chloroplasts (data no shown). This transgenic pt-ck CS16265 was nuclear transformed and AmCyan protein was imported into chloroplasts with the help from a peptide signal. In comparison with this line, all 89 putative lines were negative for AmCyan fluorescence.

One reason for the failure of the selection of transformants is probably the very fast shoot formation. Shoot generation started at the 5th day on SRAKN medium. Once shoots are formed, antibiotics cannot penetrate the inner cells of this shoots, which lead to an insufficient selection. The other hypothesis is that only some plastomes were transformed and that the level was not high enough to detect them by fluorescence microscopy or PCR.

Plastid transformation requires multiple rounds of selection to eliminate wild-type plastomes and to achieve a homoplastomic state. In this study, a regeneration medium with a long callus phase (SRA2N) was used, this allows multiple cycles of selection and regeneration. Additionally, vectors containing *gfpbar* genes or just one selection marker as the *aadA* or the *nptII* genes were used. The *nptII* gene includes the first 14 amino acids of the GFP protein, which improved the selection of transplastomic plants in tobacco (Scharff and Koop, 2007). Despite of all these strategies, all putative lines were negative for transgenes and they bleached after prolonged selection (Table 7.1).

7.1.4 PEG-mediated plastid transformation

An alternative for particle gun experiments is PEG-mediated plastid transformation. One advantage of the PEG method over the particle gun method is that for PEG no expensive equipment is required and in *Nicotiana tabacum* the PEG-mediated plastid transformation method is equally efficient as the biolistic

gun method (Kofer *et al.*, 1998). For PEG-mediated plastid transformation, isolated protoplasts are treated with PEG in the presence of DNA. Successful PEG-mediated plastid transformation needs a regeneration protocol which allows the regeneration of fertile plants from protoplasts. Our group could stably transform chloroplasts in *Nicotiana tabacum* using PEG, for the first time in 1993 (Golds *et al.*, 1993) and shortly afterwards O´Neill *et al.*, were able to repeat the same in *Nicotiana plumbaginifolia* (O'Neill *et al.*, 1993). PEG-mediated plastid transformation was also applied in other plants as *Solanum lycopersicum* (tomato) (Nugent *et al.*, 2005b), *Brassica oleracea* var. botrytis (cauliflower) (Nugent *et al.*, 2005a) and *Lactuca sativa* (lettuce) (Lelivelt *et al.*, 2005). However, more than 15 years later, the precise mechanism of how DNA is transported through the double membrane of chloroplasts is still unknown (Spörlein *et al.*, 1991).

We performed at least 20 PEG-mediated plastid transformation experiments and in each experiment 1 x 10^6 protoplasts were used. Although several different plastid transformation vectors were constructed and used, no transplastomic plant was obtained.

To show that PEG-mediated transformation works in principle and that plants can be regenerated after the PEG treatment, GFP was expressed transiently in the cytoplasm of nuclear transformed protoplasts (Figure 6.23) and plants were regenerated. With this method we also tested different PEG concentrations (20 % and 40 %). We could also regenerate plants out the treated protoplasts confirming that the method allows the transformation and regeneration of plants out of treated protoplasts.

7.2 Towards plastid transformation in Rapid-Cycling *Brassica rapa*

In parallel to *Arabidopsis thaliana* tissue culture, we have worked on the establishment of a regeneration protocol for Rapid-Cycling *Brassica rapa* (Figure 6.25). Although those Rapid-Cycling *Brassica rapa* (RCBr) plants have been recently proposed as a new model organism (Williams and Hill, 1986), this model lacks a

reproducible tissue culture regeneration protocol. Up to the date, just two protocols using cotyledons as explants have been described (Teo *et al.*, 1997; Cogbill *et al.*, 2010). We intended to reproduce their findings, but we could not obtain shoots although using both protocols in RCBr C1 and B3 plants. Then, we modified the explant preparation protocol by Jonoubi *et al.*, for *Brassica napus* (Jonoubi *et al.*, 2005). Hypocotyls were cut at their sagittal axis to expose the vascular tissues. This modification allowed regeneration efficiencies of 50 to 80 % in C1 and 20 to 50 % in B3 lines. However, the high regeneration efficiencies are very sensitive to environmental changes (personal communication with Peter Wehmann). Thus, factors as explant sources and origin of seeds need to be considered for tissue culture.

In this study, at least 10 particle gun-mediated plastid transformation experiments with a species specific vector for RCBr (Fig 6.25) were performed. Although one putative callus was obtained, it bleached after strict selection conditions.

In general, plastid transformation in crops is not as efficient as in tobacco (Table 7.1). In tobacco at least two or more transplastomic plants are obtained per bombardment. However, in crop plants as *Oriza sativa* or *Glycine max* more than 20 particle gun experiments are needed to acquire transplastomic plants (Koop *et al.*, 2007). For this reason, more experiments in RCBr may be necessary to achieve plastid transformation.

7.3 *Nicotiana tabacum,* a positive control in plastid transformation

In this study *Nicotiana tabacum* was used as a positive control for plastid transformation. One the one hand, *Nicotiana tabacum* was taken to demonstrate that the *AmCyan* gene can be used as a new visual marker and on the other hand, it allowed the test of regulatory elements used in *Arabidopsis thaliana* plastid transformation vectors. *Nicotiana tabacum* is the first higher plant in which plastids could be transformed by the particle gun method (Svab *et al.*, 1990). One advantage of tobacco is its high regeneration capacity. Tobacco is still a model

plant for cell and tissue culture research. Protoplast regeneration from *Nicotiana tabacum* was described for the first time in 1971 (Nagata and Takebe, 1971). Here, following a method of Dovzhenko *et al.*, high platting efficiencies could be achieved allowing shoot formation in less than two weeks (Dovzhenko *et al.*, 1998).

Leaves and protoplast derived colonies were bombarded with the pKAA vector containing aadA and AmCyan expression cassettes (Figure 6.28). The pKAA vector contains flanking regions from the pKCZ vector for the insertion of the transgene between the *trnN-trnR* genes from *Nicotiana tabacum* (Zou *et al.*, 2003).

First putative lines were obtained after four weeks under selection conditions. Although AmCyan fluorescence could not be detected using a fluorescence stereoscope, blue fluorescence could clearly been observed using a laser scanning microscope (Figure 6.29). T_0 and T_1 lines expressing AmCyan in chloroplasts of *Nicotiana tabacum* were obtained. Transformation efficiencies were comparable to those obtained by using other selection markers in *Nicotiana tabacum* (Huang *et al.*, 2002).

Further analysis of these lines by southern blotting have shown a very faint band suggesting that wild-type plastome sequences could still be present in these lines, even after seven cycles of regeneration. One explanation for this faint signal could be the presence of plastid DNA sequences in the nuclear genome (Ayliffe *et al.*, 1989; Huang *et al.*, 2002). This hypothesis is supported by the fact that reciprocal crosses and screening of the progeny allowed the confirmation of the homoplasmic status of the analyzed lines (Figure 6.31).

In summary, these results show that the reef coral fluorescent protein AmCyan can be used as visual marker in plastid transformation of *Nicotiana tabacum*. The results also clearly show that all the expression cassettes used were fully functional.

7.3.1 Plastid transformation in *Arabidopsis thaliana* and *Nicotiana tabacum*

For plastid transformation experiments in *Arabidopsis thaliana* several experimental approaches could be established: 1. A highly reliable regeneration protocol was developed. 2. Plant specific vectors for plastid transformation were constructed. 3. Vectors with different fluorescence and selection markers were tested and 4. Particle gun- and PEG-mediated plastid transformation methods were performed. Notwithstanding, after all this attempts, no plastid transformant could be obtained in *Arabidopsis thaliana*. More than 10 years have passed since the transformation of *Arabidopsis thaliana* plastids was reported (Sikdar *et al.*, 1998) and several groups have tried stable transformation of plastids in *Arabidopsis thaliana*, but no success has been reported so far. It is still not clear where the bottleneck is.
Until now, *Nicotiana tabacum* is still the best plant model organism where plastid transformation has been achieved (Koop *et al.*, 2007; Maliga and Bock, 2011).
Recently, evidence for microhomology and nonhomologous end joining (NHEJ) repair mechanisms of double strand breaks (DSB) was described in chloroplasts of *Arabidopsis thaliana* (Kwon *et al.*, 2010). Interestingly, in *Nicotiana tabacum* chloroplasts lack the capacity to repair DSB in their plastomes by the NHEJ mechanism (Kohl and Bock, 2009). Chloroplasts are constantly exposed to reactive oxygen species generated during photosynthesis which may cause permanent DNA damage (Maréchal and Brisson, 2010).
The most common form of DNA damage is a double strand break (DSB). In plants mechanisms of DSB repair includes homologous recombination (HR), microhomology and NHEJ. According to Kohl and Bock, (Kohl and Bock, 2009) it has been shown with the help of an insertion sequences (IS) of *Escherichia coli* that this IS element is mobilized inside the chloroplast. These results suggest that plastids of *Nicotiana tabacum* cannot repair DSB through NHEJ which is important for their implications in genome stability. Understanding of the mechanisms of DSB repair may help to understand the underlying mechanisms of plastid transformation in *Arabidopsis thaliana.*

7.4 Future directions in plastid transformation of *Arabidopsis thaliana*

Several groups attribute failures of plastid transformation methods in *Arabidopsis thaliana* to the homologous recombination machinery (Sikdar *et al.*, 1998). Although this argument cannot completely be discarded; recent studies have shown that *Arabidopsis thaliana* has an efficient homologous recombination system (Rowan *et al.*, 2010).

Despite the highly reactive forms of oxygen in plastids, plastomes are still as conservative genomes as nuclear or mitochondrial genomes (Kwon *et al.*, 2010), meaning that efficient DNA repair mechanisms must exist in plastids. Despite the need of a better understanding of homologous recombination in plastids, there is little knowledge on the mechanisms and enzymes involved in this process. In *Arabidopsis thaliana* some proteins involved in plastid DNA replication, recombination and repair (RRR) have been identified. These proteins include RecA-like recombinases and two families of plant specific single-stranded DNA (ssDNA)-binding proteins: the organelle ssDNA-binding proteins (OSBs) and the Whirly proteins (Marechal *et al.*, 2009; Maréchal and Brisson, 2010).

In *E. coli* the rec-A protein is well characterized. It is involved in the reduction of mutations and in the repair of DNA by homologous recombination. Homologues of *recA* have been identified in algae and in the nuclear genomes of several plants, including *Arabidopsis thaliana* (Cerutti *et al.*, 1992). It has also been shown that the absence of *recA* gene induces aberrant plastomes in *Arabidopsis thaliana.* Interestingly, the variegated phenotype of *RecA* mutants could be observed only after four generations (Rowan *et al.*, 2010).

Transgenic *Arabidopsis thaliana* plants overexpressing a *RecA* gene could increase the success of homologous recombination and thus the stable transformation of plastids after bombardment with DNA covered particles. This strategy has already been tested in *Nicotiana tabacum* with positive results (Liu, 2005).

In *Arabidopsis thaliana* it has been shown that plastid DNA copy number remains constant during leaf development (Li *et al.*, 2006; Zoschke *et al.*, 2007). This means,

that each daughter cell receives in theory an equal number of plastids and that the plastid genomes are inherited strictly randomly. Plastid transformation requires several regeneration cycles under selective conditions to allow the segregation of transformed plastomes. As suggested by Bogorad (2000), the reduction of the number of chloroplasts per cell may also favour the selection of transformed plastomes.

Reduced numbers of chloroplasts are observed in mutants or transgenic plants with macrochloroplasts in *Arabidopsis thaliana*. Through antisense mechanisms that inhibit the expression of either the *AtFtsZ1-1* or the *AfFtsZ2-1* genes in *Arabidopsis thaliana,* the number of 100 chloroplasts has been reduced to one macrochloroplast (MCP) per leaf cell (Osteryoung *et al.*, 1998). Plants containing MCP were obtained by the overexpression of the *AtMinE1* or the *AtMinD1* in the nucleus (Colletti *et al.*, 2000; Maple *et al.*, 2002; Aldridge *et al.*, 2005; Glynn *et al.*, 2007). The use of transgenic plants containing macrochloroplasts in plastid transformation has been further explored in *Nicotiana tabacum* plants (Chikkala, 2009). The plastid division gene *MinD* from cauliflower was overexpressed in tobacco plants and afterwards transgenic plants containing macrochloroplasts were used for plastid transformation experiments. Interestingly transformation efficiencies were not increased. However, only one regeneration cycle was necessary to obtain homoplasmy (Chikkala, 2009). This significant reduction of selection cycles would make transgenic plants containing MCP a good alternative for plastid transformation in *Arabidopsis thaliana*.

Another possibility to improve plastid transformation could be the use of tetraploid *Arabidopsis thaliana* plants for particle gun experiments. In this regard, it is interesting to note that most plants which could successfully plastid transformed are polyploidy. Most prominent is *Nicotiana tabacum* with four copies of the genome. In contrast, *Arabidopsis thaliana* has just two copies of its genome. Thus, it would be an interesting experiment to try plastid transformation of polyploidy *Arabidopsis thaliana*.

8 Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
aadA	3´Aminoglycoside-adenyltransferase
aphA6	3´Aminoglycoside phosphotransferase type VI
ATP	Adenosin 5´-triphosphat
B5	Gamborg´s medium
BAP	Benzylaminopurine
BD	Binding domain
cm	Centimeter
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EM	Electron microscopy
EtBr	Ethidium bromide
EtOH	Ethanol
GFP	Green fluorescent protein
h	Hour
IAA	3-Indole acetic acid
IBA	Indole butyric acid
IR	Inverted repeat region
KoAc	Potassium acetate
mRNA	messenger Ribonucleic acid
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NEP	Nuclear-encoded RNA polymerase
nptII	Neomycin phosphotransferase II
^{o}C	Degree Celsius
p.a	Analytical reagent quality
pat	Phosphinothricin acetyl transferase
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEP	Plastid-encoded RNA polymerase
pp	Protoplasts
PPT	Phosphinothricin
Prrn	Plastid 16 S r RNA promoter
RCBr	Rapid-Cycling *Brassica rapa*
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Seconds
SD	Shine-Dalgarno sequence
SDS	Sodium dodecyl sulfate
Spec	spectinomycin
UTR	Untranslated region
UV	Ultraviolet light
V	volt
wt	wild-type

9 References

Acedo, N. (1986). Regeneration of *Arabidopsis* callus in vitro. *Plant Cell, Tissue and Organ Culture* 6, 109-114.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell, fourth edition, (New York: *Garland Science*).

Aldridge, C., Maple, J., and Møller, S. G. (2005). The molecular biology of plastid division in higher plants. *Journal of experimental botany* 56, 1061-1077.

Allison, L. A. (2000). The role of sigma factors in plastid transcription. *Biochimie* 82, 537-548.

Apel, W., Schulze, W. X., and Bock, R. (2010). Identification of protein stability determinants in chloroplasts. *Plant J* 63, 636-650.

Arlen, P., Falconer, R., Cherukumilli, S., Cole, A., Cole, A., Oishi, K., and Daniell, H. (2007). Field production and functional evaluation of chloroplast-derived interferon-alpha2b. *Plant Biotechnol J* 5, 511 - 525.

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarc'h, A., Carnero, E., Giraudat-Pautot, V., Rech, P., and Chriqui, D. (2009). Pluripotency of *Arabidopsis* xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. *Plant J* 57, 626-644.

Ausubel, F. M. (2000). *Arabidopsis* Genome. A Milestone in Plant Biology. *Plant Physiol* 124, 1451-1454.

Ayliffe, M., Scott, N., and Timmis, J. (1989). Analysis of Plastid DNA-like Sequences Within the Nuclear Genomes of Higher Plants. *Mol Biol Evol* 15, 738-745.

Azhagiri, A. K., and Maliga, P. (2007). Exceptional paternal inheritance of plastids in *Arabidopsis* suggests that low-frequency leakage of plastids via pollen may be universal in plants. *Plant J* 52, 817-823.

Barghchi, M., Turgut, K., Scott, R., and Draper, J. (1994). High-frequency transformation from cultured cotyledons of *Arabidopsis thaliana* ecotypes "C24" and "Landsberg erecta". *Plant Growth Regulation* 14, 61-67.

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.

Baur, E. (1909). Das Wesen und die Erblichkeitsverhältnisse der "Varietates albomarginatae hort."von *Pelargonium zonale*. *Zeits ind Abst Vererb* 1, 330-351.

Bendich, A., and Smith, S. (1990). Moving pictures and pulsed-field electrophoresis show linear DNA molecules from chloroplasts and mitochondria. *Curr Genet* 17, 421-425.

Bendich, A. J. (1987). Why do chloroplasts and mitochondria contain so many copies of their genome? *Bioessays* 6, 279-282.

Bendich, A. J. (2004). Circular Chloroplast Chromosomes: The Grand Illusion. *The Plant cell* 16, 1661-1666.

Birky, C. W. (2001). THE INHERITANCE OF GENES IN MITOCHONDRIA AND CHLOROPLASTS: Laws, Mechanisms, and Models. *Annu Rev Genet* 35, 125.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research* 7, 1513-1523.

Blowers, A. D., Bogorad, L., Shark, K. B., and Sanford, J. C. (1989). Studies on Chlamydomonas chloroplast transformation: foreign DNA can be stably maintained in the chromosome. *The Plant cell* 1, 123-132.

Bock, R. (1998). Analysis of RNA editing in plastids. *Methods.* 15, 75-83.

Bock, R. (2001). Transgenic plastids in basic research and plant biotechnology. *J Mol Biol* 312, 425-438.

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

Bock, R., Hagemann, R., Kossel, H., and Kudla, J. (1993). Tissue- and stage-specific modulation of RNA editing of the psbF and psbL transcript from spinach plastids--a new regulatory mechanism? *Mol Gen Genet* 240, 238-244.

Bock, R., and Khan, M. S. (2004). Taming plastids for a green future. *Trends Biotechnol* 22, 311-318.

Bock, R., and Maliga, P. (1995). Correct splicing of a group II intron from a chimeric reporter gene transcript in tobacco plastids. *Nucleic acids research* 23, 2544-2547.

Bogorad, L. (2000). Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions and products. *Trends Biotechnol* 18, 257-263.

Boynton, J., Gillham, N., Harris, E., Hosler, J., Johnson, A., Jones, A., Randolph-Anderson, B., Robertson, D., Klein, T., Shark, K., and et, a. (1988). Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. *Science* 240, 1534-1538.

Bressan, R. A., Zhang, C., Zhang, H., Hasegawa, P. M., Bohnert, H. J., and Zhu, J.-K. (2001). Learning from the *Arabidopsis* Experience. The Next Gene Search Paradigm. *Plant Physiol* 127, 1354-1360.

Briggs, W. H., and Goldman, I. L. (2006). Genetic variation and selection response in model breeding populations of *Brassica rapa* following a diversity bottleneck. *Genetics* 172, 457-465.

Buhot, L., Horvath, E., Medgyesy, P., and Lerbs-Mache, S. (2006). Hybrid transcription system for controlled plastid transgene expression. *Plant J* 46, 700-707.

Buschmann, C., Langsdorf, G., and Lichtenthaler, H. K. (2000). Imaging of the Blue, Green, and Red Fluorescence Emission of Plants: An Overview. *Photosynthetica* 38, 483-491.

Candela, M., Velazquez, I., De la Cruz, B., Sendnino, A. M., and De la Pena, A. (2001). Differences in in vitro plant regeneration ability among four *Arabidopsis thaliana* ecotypes. *In Vitro Cellular and Development Biology -Plant* 37.

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.

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.

Cerutti, H., Osman, M., Grandoni, P., and Jagendorf, A. T. (1992). A homolog of *Escherichia coli* RecA protein in plastids of higher plants. *Proc Natl Acad Sci U S A* 89, 8068-8072.

Chang, S. S., Park, S. K., Kim, B. C., Kang, B. J., Kim, D. U., and Nam, H. G. (1994). Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation in planta. *The Plant Journal* 5, 551-558.

Chaudhury, A. M., and Signer, E. R. (1989). Relative regeneration proficiency of *Arabidopsis thaliana* ecotypes. *Plant Cell Reports* 8, 368-369.

Chebola, S., and Daniell, H. (2007). Stable expression of Gal/GalNAc lectin of *Entamoeba histolytica* in transgenic chloroplasts and immunogenicity in mice towards vaccine development for amoebiasis. *Plant Biotechnol J* 5, 230 - 239.

Chen, Z., and Celio, J. (2000). Particle bombardment transformation of *Brassica*. United States Patent 6051756.

Cheng, L., Li, H.-P., Qu, B., Huang, T., Tu, J.-X., Fu, T.-D., and Liao, Y.-C. (2010). Chloroplast transformation of rapeseed (*Brassica napus*) by particle bombardment of cotyledons. *Plant Cell Reports* 29, 371-381.

Chikkala, V. R. N. (2009) Production and Transformation of tobacco and *Brassica* containing Macrochloroplasts. *RMIT University,* phD.

Cogbill, S., Faulcon, T., Jones, G., McDaniel, M., Harmon, G., Blackmon, R., and Young, M. (2010). Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. *Plant Cell, Tissue and Organ Culture* 101, 127-133.

Colletti, K. S., Tattersall, E. A., Pyke, K. A., Froelich, J. E., Stokes, K. D., and Osteryoung, K. W. (2000). A homologue of the bacterial cell division site-determining factor MinD mediates placement of the chloroplast division apparatus. *Curr Biol* 10, 507-516.

Correns, C. (1909). Vererbungsversuche mit blass (gelb) grünen und buntblättrigen Sippen bei Mirabilis, Urtica und Lunaria. *Zeits ind Abst Vererb* 1, 281-329.

Courtois, F., Merendino, L., Demarsy, E., Mache, R., and Lerbs-Mache, S. (2007). Phage-type RNA polymerase RPOTmp transcribes the rrn operon from the PC promoter at early developmental stages in *Arabidopsis. Plant physiology* 145, 712-721.

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*.

Damm, B., and Willmitzer, L. (1988). Regeneration of fertile plants from protoplasts of different *Arabidopsis thaliana* genotypes. *Molecular and General Genetics MGG* 213, 15-20.

Daniell, H. (2002). Molecular strategies for gene containment in transgenic crops. *Nat Biotechnol* 20, 581-586.

Daniell, H., Chebolu, S., Kumar, S., Singleton, M., and Falconer, R. (2005). Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine* 23, 1779-1783.

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

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

Daniell, H., Ruiz, G., Denes, B., Sandberg, L., and Langridge, W. (2009). Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. *BMC Biotechnol* 9, 33.

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 U S A* 87, 88-92.

Day, R. N., and Davidson, M. W. (2009). The fluorescent protein palette: tools for cellular imaging. *Chem Soc Rev* 38, 2887-2921.

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. *Nature biotechnology* 19, 71-74.

De Marchis, F., Wang, Y., Stevanato, P., Arcioni, S., and Bellucci, M. (2008). Genetic transformation of the sugar beet plastome. *Transgenic research*.

DeGray, G., Rajasekaran, K., Smith, F., Sanford, J., and Daniell, H. (2001). Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant physiology* 127, 852-862.

Dhingra, A., and Daniell, H. (2006). Chloroplast genetic engineering via organogenesis or somatic embryogenesis. *Methods Mol Biol* 323, 245-262.

Dovzhenko, A. (2001) Towards plastid transformation in rapedseed (*Brasica napus L.*) und sugarbeet (*Beta vulgaris L.*). *Ludwig-Maximilians-Universität München*, Dissertation.

Dovzhenko, A., Bergen, U., and Koop, H. U. (1998). Thin-alginate-layer technique for protoplast culture of tobacco leaf protoplasts: Shoot formation in less than two weeks. *Protoplasma* 204, 114-118.

Dovzhenko, A., Dal Bosco, C., Meurer, J., and Koop, H. U. (2003). Efficient regeneration from cotyledon protoplasts in *Arabidopsis thaliana*. *Protoplasma* 222, 107-111.

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

Eibl, C. (1999) In vivo-Analyse plastidärer UTR-Elemente von Tabak (*Nicotiana tabacum*) durch Chloroplastentransformation. *Ludwig-Maximilians-Universität München*, Dissertation.

Eibl, C., Zou, Z., Kim, M., Mullet, J., and Koop, H. (1999). In vivo analysis of plastid psbA, rbcL, and rpl32 UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. *Plant J* 19, 333 - 345.

Feldmann, K., and Marks, M. (1986). Rapid and efficient regeneration of plants from explants of *Arabidopsis thaliana*. *Plant Science* 47, 63-69.

Fernandez-San Millan, A., Mingo-Castel, A., Miller, M., and Daniell, H. (2003). A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnol J* 1, 71-79.

Ford, K. G. (1990). Plant regeneration from *Arabidopsis thaliana* protoplasts. *Plant Cell Reports* 8, 534-537.

Fulgosi, H., Gerdes, L., Westphal, S., Glockmann, C., and Soll, J. (2002). Cell and chloroplast division requires ARTEMIS. *Proc Natl Acad Sci U S A* 99, 11501-11506.

Gamborg, O. L., Miller, R. A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50, 151-158.

Gamborg, O.L., and Eveleigh, D.E. (1967). Culture methods and detection of glucanases in suspension cultures of wheat and barley. *Can J Biochem* 46, 417-421.

Gleba, Y., Klimyuk, V., and Marillonnet, S. (2007). Viral vectors for the expression of proteins in plants. *Curr Opin Biotechnol* 18, 134-141.

Glenz, K., Bouchon, B., Stehle, T., Wallich, R., Simon, M. M., and Warzecha, H. (2006). Production of a recombinant bacterial lipoprotein in higher plant chloroplasts. *Nature biotechnology* 24, 76-77.

Glimelius, K., Djupsjöbacka, M., and Fellner-Feldegg, H. (1986). Selection and enrichment of plant protoplast heterokaryons of Brassicaceae by flow sorting. *Plant Science* 45, 133-141.

Glynn, J. M., Miyagishima, S.-y., Yoder, D. W., Osteryoung, K. W., and Vitha, S. (2007). Chloroplast Division. *Traffic* 8, 451-461.

Golds, T., Maliga, P., and Koop, H. (1993). Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. *Bio/Technology* 11, 95-97.

Gresshoff, P. M., and Doy, C. H. (1972). Development and differentiation of haploid *Lycopersicon esculentum* (tomato). *Planta* 107, 161-170.

Griffiths, A. J., Gelbart, W. M., Miller, M. H., and Lewontin, R. (1999). Modern Genetic Analysis, (New York: *W.H. Freeman*).

Guda, C., Lee, S., and Daniell, H. (2000). Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. *Plant Cell Rep* 19, 257-262.

Hagemann, R. (2000). Erwin Baur or Carl Correns: who really created the theory of plastid inheritance? *Journal of Heredity* 91, 435-440.

Hagemann, R. (2010). The foundation of extranuclear inheritance: plastid and mitochondrial genetics. *Molecular genetics and genomics : MGG* 283, 199-209.

Hagemann, R., and Schröder, M. (1989). The cytological basis of the plastid inheritance in angiosperms. *Protoplasma* 152, 57-64.

Hager, M., and Bock, R. (2000). Enslaved bacteria as new hope for plant biotechnologists. *Applied Microbiology Biotechnology* 54, 302-310.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* 166, 557-580.

Herz, S., Fussl, M., Steiger, S., and Koop, H. U. (2005). Development of novel types of plastid transformation vectors and evaluation of factors controlling expression. *Transgenic research* 14, 969-982.

Hibberd, J., Linley, P., Khan, M., and Gray, J. (1998). Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. *Plant J* 16, 627-632.

Hong, C. P., Kwon, S. J., Kim, J. S., Yang, T. J., Park, B. S., and Lim, Y. P. (2008). Progress in understanding and sequencing the genome of *Brassica rapa*. *International journal of plant genomics* 2008, 582837.

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 research* 12, 111-114.

Huang, B., and Yeoman, M. (1984). Callus proliferation and morphogenesis in tissue cultures of *Arabidopsis thaliana* L. *Plant Science Letters* 33, 853-863.

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.

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.

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

Jach, G., Binot, E., Frings, S., Luxa, K., and Schell, J. (2001). Use of red fluorescent protein from *Discosoma* sp. (dsRED) as a reporter for plant gene expression. *Plant J* 28, 483-491.

Jonoubi, P., Mousavi, A., Majd, A., Salmanian, A. H., Jalali Javaran, M., and Daneshian, J. (2005). Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*. *Biologia Plantarum* 49, 175-180.

Kanamoto, H., Yamashita, A., Asao, H., Okumura, S., Takase, H., Hattori, M., Yokota, A., and Tomizawa, K.-I. (2006). Efficient and Stable Transformation of *Lactuca sativa* L. cv. Cisco (lettuce) Plastids. *Transgenic Res* 15, 205-217.

Kang, T. J., Loc, N. H., Jang, M. O., Jang, Y. S., Kim, Y. S., Seo, J. E., and Yang, M. S. (2003). Expression of the B subunit of E. coli heat-labile enterotoxin in the chloroplasts of plants and its characterization. *Transgenic research* 12, 683-691.

Keskitalo, J., Bergquist, G., Gardestrom, P., and Jansson, S. (2005). A Cellular Timetable of Autumn Senescence. *Plant Physiol* 139, 1635-1648.

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

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.

Kofer, W., Eibl, C., Steinmüller, K., and Koop, H.-U. (1998). PEG-mediated plastid transformation in higher plants. *In Vitro Cell Dev Biol Plant* 34, 303-309.

Kohl, S., and Bock, R. (2009). Transposition of a bacterial insertion sequence in chloroplasts. *The Plant Journal* 58, 423-436.

Koop, H.-U., Herz, S., Golds, T., and Nickelsen, J. (2007). The genetic transformation of plastids. In Cell and Molecular Biology of Plastids, R. Bock, ed. (*Springer Berlin / Heidelberg*), pp. 457-510.

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.

Koornneef, M., and Meinke, D. (2010). The development of *Arabidopsis* as a model plant. *The Plant Journal* 61, 909-921.

Krause, K., Maier, R. M., Kofer, W., Krupinska, K., and Herrmann, R. G. (2000). Disruption of plastid-encoded RNA polymerase genes in tobacco: expression of only a distinct set of genes is not based on selective transcription of the plastid chromosome. *Mol Gen Genet* 263, 1022-1030.

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

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 physiology* 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. (2001). Sequences downstream of the translation initiation codon are important determinants of translation efficiency in chloroplasts. *Plant physiology* 125, 430-436.

Kwon, T., Huq, E., and Herrin, D. L. (2010). Microhomology-mediated and nonhomologous repair of a double-strand break in the chloroplast genome of *Arabidopsis*. *Proc Natl Acad Sci U S A* 107, 13954-13959.

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

Lee, S., Kwon, H., Kwon, S., Park, S., Jeong, M., Han, S., Byun, M., and Daniell, H. (2003). Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. . *Nature, Molec Breed* 11, 1-13.

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.

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

Li, W., Ruf, S., and Bock, R. (2006). Constancy of organellar genome copy numbers during leaf development and senescence in higher plants. *Mol Genet Genomics* 275, 185-192.

Li, W., Ruf, S., and Bock, R. (2010). Chloramphenicol acetyltransferase as selectable marker for plastid transformation. *Plant Mol Biol*, 1-9.

Lilly, J. W., Havey, M. J., Jackson, S. A., and Jiang, J. (2001). Cytogenomic Analyses Reveal the Structural Plasticity of the Chloroplast Genome in Higher Plants. *The Plant cell* 13, 245-254.

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.

Liu, J.-r. D., KR), Jeong, Won-joong (Daejeon, KR), Min, Sung-ran (Daejeon, KR), Jbong, Seok-won (Daejeon, KR), Han, Su-kyoung (Daejeon, KR) (2005). Method for recombinating plastid using procaryotic recombinase gene. United States Patent Application 20050204433.

López-Juez, E. (2007). Plastid biogenesis, between light and shadows. *Journal of experimental botany* 58, 11-26.

Lopez-Juez, E., and Pyke, K. (2005). Plastids unleashed: their development and their integration in plant development. *The International journal of developmental biology* 49, 557-577.

Lossl, A., Bohmert, K., Harloff, H., Eibl, C., Muhlbauer, S., and Koop, H. U. (2005). Inducible trans-activation of plastid transgenes: expression of the R. eutropha phb operon in transplastomic tobacco. *Plant & cell physiology* 46, 1462-1471.

Lossl, A., Eibl, C., Harloff, H. J., Jung, C., and Koop, H. U. (2003). Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of polyhydroxybutyrate are associated with growth reduction. *Plant Cell Rep* 21, 891-899.

Luo, Y. (1997) Studies on *Arabidopsis thaliana* cells in vitro: somatic embryogenesis and protoplast culture, *Ludwig-Maximilians-Universität, München* Dissertation.

Luo, Y., and Koop, H. U. (1997). Somatic embryogenesis in cultured immature zygotic embryos and leaf protoplasts of *Arabidopsis thaliana* ecotypes. *Planta* 202, 387-396.

Lutz, K. A., Knapp, J. E., and Maliga, P. (2001). Expression of bar in the Plastid Genome Confers Herbicide Resistance *Plant physiology* 125, 1585-1590.

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

Maliga, P. (2003). Progress towards commercialization of plastid transformation technology. *Trends Biotechnol* 21, 20-28.

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

Maliga, P., and Bock, R. (2011). Plastid biotechnology: food, fuel and medicine for the 21st century. *Plant Physiol*, pp.110.170969.

Maple, J., Chua, N.-H., and Møller, S. G. (2002). The topological specificity factor AtMinE1 is essential for correct plastid division site placement in *Arabidopsis*. *The Plant Journal* 31, 269-277.

Maple, J., and Møller, S. G. (2007). Plastid Division: Evolution, Mechanism and Complexity. *Annals of Botany* 99, 565-579.

Maréchal, A., and Brisson, N. (2010). Recombination and the maintenance of plant organelle genome stability. *New Phytologist* 186, 299-317.

Marechal, A., Parent, J. S., Veronneau-Lafortune, F., Joyeux, A., Lang, B. F., and Brisson, N. (2009). Whirly proteins maintain plastid genome stability in *Arabidopsis. Proc Natl Acad Sci U S A* 106, 14693-14698.

Márton, L., and Browse, J. (1991). Facile transformation of *Arabidopsis. Plant Cell Rep* 10, 235-239.

Mathur, J., Koncz, C., and Szabados, L. (1995). A simple method for isolation, liquid culture, transformation and regeneration of *Arabidopsis thaliana* protoplasts. *Plant Cell Rep* 14, 221-226.

McBride, K. E., Svab, Z., Schaaf, D. J., Hogan, P. S., Stalker, D. M., and Maliga, P. (1995). Amplification of a chimeric Bacillus gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Bio/technology (Nature Publishing Company)* 13, 362-365.

McCabe, M. S., Klaas, M., Gonzalez-Rabade, N., Poage, M., Badillo-Corona, J. A., Zhou, F., Karcher, D., Bock, R., Gray, J. C., and Dix, P. J. (2008). Plastid transformation of high-biomass tobacco variety Maryland Mammoth for production of human immunodeficiency virus type 1 (HIV-1) p24 antigen. *Plant Biotechnol J.*

Meinke, D. W., Cherry, J. M., Dean, C., Rounsley, S. D., and Koornneef, M. (1998). *Arabidopsis thaliana*: A Model Plant for Genome Analysis. *Science* 282, 662-682.

Mere-Villanueva, G., and Vazquez-Alejandro, V. (2003) Bombardeo de callos embriogénicos de zanahoria (*Daucus carota* L). y su regeneración con la proteína G del virus de la rabia., *UNAM,* Tesis de licenciatura.

Meyerowitz, E. M. (2001). Prehistory and History of *Arabidopsis* Research. *Plant Physiol* 125, 15-19.

Meyerowitz, E. M., and Pruitt, R. E. (1985). *Arabidopsis thaliana* and Plant Molecular Genetics. *Science* 229, 1214-1218.

Michaelis, P. (1962a). Über gehäufte Plastiden-Abänderungen. *Planta* 58, 34-49.

Michaelis, P. (1962b). Über Zahlengesetzmäßigkeiten plasmatischer Erbträger, insbesondere der Plastiden. *Protoplasma* 55, 177-231.

Monde, R. A., Greene, J. C., and Stern, D. B. (2000a). The sequence and secondary structure of the 3'-UTR affect 3'-end maturation, RNA accumulation, and translation in tobacco chloroplasts. *Plant molecular biology* 44, 529-542.

Monde, R. A., Schuster, G., and Stern, D. B. (2000b). Processing and degradation of chloroplast mRNA. *Biochimie* 82, 573-582.

Muhlbauer, S. K., Lossl, A., Tzekova, L., Zou, Z., and Koop, H. U. (2002). Functional analysis of plastid DNA replication origins in tobacco by targeted inactivation. *Plant J* 32, 175-184.

Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* 15, 473-497.

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic acids research* 8, 4321-4325.

Musgrave, M. E. (2000). Realizing the Potential of Rapid-Cycling *Brassica* as a Model System for Use in Plant Biology Research. *Journal of Plant Growth Regulation* 19, 314-325.

Nagata, N. (2010). Mechanisms for independent cytoplasmic inheritance of mitochondria and plastids in angiosperms. *J Plant Res* 123, 193-199.

Nagata, T., and Takebe, I. (1971). Plating of isolated tobacco mesophyll protoplasts on agar medium. *Planta* 99, 12-20.

Negrutiu, I., Beeftink, F., and Jacobs, M. (1975). *Arabidopsis thaliana* as a model system in somatic cell genetics I. Cell and tissue culture. *Plant Science Letters* 5, 293-304.

Nelson, B. K., Cai, X., and Nebenfuhr, A. (2007). A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J* 51, 1126-1136.

Neuhaus, H. E., and Emes, M. J. (2000). Nonphotosynthetic metabolism in plastids. *Annual Review of Plant Physiology and Plant Molecular Biology* 51, 111-140.

Nugent, G. D., Coyne, S., Nguyen, T. H., Kavanagh, T. A., and Dix, P. J. (2005a). Nuclear and plastid transformation of *Brassica oleracea* var. botrytis (cauliflower) using PEG-mediated uptake of DNA into protoplasts *Plant Sci* 170, 135-142

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

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.

O'Neill, C. M., and Mathias, R. J. (1993). Regeneration of Plants from Protoplasts of *Arabidopsis thaliana* L. cv. Columbia (C24), Via Direct Embryogenesis. *Journal of experimental botany* 44, 1579-1585.

Oey, M., Lohse, M., Kreikemeyer, B., and Bock, R. (2008). Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J* 3, 436-445.

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 research* 15, 637-646.

Osteryoung, K. W., Stokes, K. D., Rutherford, S. M., Percival, A. L., and Lee, W. Y. (1998). Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial ftsZ. *The Plant Cell Online* 10, 1991-2004.

Patton, D. A., and Meinke, D. W. (1988). High-frequency plant regeneration from cultured cotyledons of *Arabidopsis thaliana*. *Plant Cell Reports* 7, 233-237.

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

Raghavan, V. (2004). Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. *Am J Bot* 91, 1743-1756.

Reed, M. L., Wilson, S. K., Sutton, C. A., and Hanson, M. R. (2001). High-level expression of a synthetic red-shifted GFP coding region incorporated into transgenic chloroplasts. *Plant J* 27, 257-265.

Richly, E., and Leister, D. (2004). An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of *Arabidopsis* and rice. *Gene* 329, 11-16.

Rochaix, J. (1997). Chloroplast reverse genetics: new insights into the function of plastid genes. *Trends in plant science* 2, 419-425.

Rosales-Mendoza, S. (2007) Obtención y caracterización de plantas productoras de antígenos relevantes de *Escherichia coli* enterotoxigénica., *IPICYT*, phD.

Rowan, B. A., Oldenburg, D. J., and Bendich, A. J. (2010). RecA maintains the integrity of chloroplast DNA molecules in *Arabidopsis*. *Journal of experimental botany* 61, 2575-2588.

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.

Sambrook, J., Russell, D. W., and MacCallum, P. (2001). Molecular cloning : a laboratory manual 3rd. ed. edn (Cold Spring Harbor, N.Y. :: *Cold Spring Harbor Laboratory*).

Sato, N., Misumi, O., Shinada, Y., Sasaki, M., and Yoine, M. (1997). Dynamics of localization and protein composition of plastid nucleoids in light-grown pea seedlings. *Protoplasma* 200, 163-173.

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., and Tabata, S. (1999). Complete Structure of the Chloroplast Genome of *Arabidopsis thaliana*. *DNA Res* 6, 283-290.

Scharff, L., and Koop, H.-U. (2006). Linear molecules of tobacco ptDNA end at known replication origins and additional loci. *Plant Mol Biol* 62, 611-621.

Scharff, L. B., and Koop, H. U. (2007). Targeted inactivation of the tobacco plastome origins of replication A and B. *Plant J* 50, 782-794.

Schmidt, R., and Willmitzer, L. (1988). High efficiency *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. *Plant Cell Rep* 7, 583-586.

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., Serino, G., Chaudhuri, S., and Maliga, P. (1998). Plastid transformation in *Arabidopsis thaliana*. *Plant Cell Rep* 10, 20-24.

Singh, A., Verma, S., and Bansal, K. (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 research* 12, 115-122.

Somerville, C., and Koornneef, M. (2002). A fortunate choice: the history of *Arabidopsis* as a model plant. *Nat Rev Genet* 3, 883-889.

Spörlein, B., Streubel, M., Dahlfeld, G., Westhoff, P., and Koop, H. (1991). PEG-mediated plastid transformation: a new system for transient gene expression assays in chloroplasts. *Theor Appl Genet* 82.

Sriraman, P., Silhavy, D., and Maliga, P. (1998). Transcription from heterologous rRNA Operon promoters in chloroplasts reveals requirement for specific activating factors. *Plant Physiol* 117, 1495-1499.

Staub, J. M., Garcia, B., Graves, J., Hajdukiewicz, P. T., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J. A., Spatola, L., *et al.* (2000). High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat Biotechnol* 18, 333-338.

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

Staub, J. M., and Maliga, P. (1993). Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the psbA mRNA. *The EMBO journal* 12, 601-606.

Stern, D. B., Goldschmidt-Clermont, M., and Hanson, M. R. (2010). Chloroplast RNA metabolism. *Annu Rev Plant Biol* 61, 125-155.

Sugiura, M. (1992). The chloroplast genome. *Plant Mol Biol* 19, 149-168.

Sugiura, M. (2003). History of chloroplast genomics. *Photosynthesis Research* 76, 371-377.

Sugiura, M., Hirose, T., and Sugita, M. (1998). Evolution and mechanism of translation in chloroplasts. *Annu Rev Genet* 32, 437-459.

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

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

Szmidt, A. E., Alden, T., and Hallgren, J. E. (1987). Paternal inheritance of chloroplast DNA in Larix. *Plant Mol Biol* 9, 59-64.

Tangphatsornruang, S., Birch-Machin, I., Newell, C., and Gray, J. (2010). The effect of different 3′ untranslated regions on the accumulation and stability of transcripts of a *gfp* transgene in chloroplasts of transplastomic tobacco. *Plant Mol Biol*, 1-12.

Teo, W., Lakshmanan, P., Kumar, P., Goh, C., and Swarup, S. (1997). Direct shoot formation and plant regeneration from cotyledon explants of rapid-cycling *Brassica rapa*. *In Vitro Cellular and Developmental Biology - Plant* 33, 288-292.

Thomas, M., and Rose, R. (1983). Plastid number and plastid structural changes associated with tobacco mesophyll protoplast culture and plant regeneration. . *Planta* 158, 329-338.

Torabi, S. (2009) Plastidentransformation in *Brassica napus. Ludwig-Maximilians-Universität München* Diplom.

Tregoning, J. S., Nixon, P., Kuroda, H., Svab, Z., Clare, S., Bowe, F., Fairweather, N., Ytterberg, J., van Wijk, K. J., Dougan, G., and Maliga, P. (2003). Expression of tetanus toxin Fragment C in tobacco chloroplasts. *Nucleic acids research* 31, 1174-1179.

Valkov, V., Gargano, D., Manna, C., Formisano, G., Dix, P., Gray, J., Scotti, N., and Cardi, T. (2010). High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences. *Transgenic research*, 1-15.

Valvekens, D., Montagu, M. V., and Lijsebettens, M. V. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci U S A* 85, 5536-5540.

Vasil, I., and Vasil, V. (1972). Totipotency and embryogenesis in plant cell and tissue cultures. *In Vitro Cell Dev Biol Plant* 8, 117-125.

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

Wakasugi, T., Tsudzuki, T., and Sugiura, M. (2001). The genomics of land plant chloroplasts: Gene content and alteration of genomic information by RNA editing. *Photosynth Res* 70, 107-118.

Waters, M. T., and Langdale, J. A. (2009). The making of a chloroplast. *The EMBO journal* 28, 2861-2873.

Watson, J., Koya, V., Leppla, S. H., and Daniell, H. (2004). Expression of *Bacillus anthracis* protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. *Vaccine* 22, 4374-4384.

Wenck, A., Pugieux, C., Turner, M., Dunn, M., Stacy, C., Tiozzo, A., Dunder, E., van Grinsven, E., Khan, R., Sigareva, M., *et al.* (2003). Reef-coral proteins as visual, non-destructive reporters for plant transformation. *Plant Cell Rep* 22, 244-251.

Wendell, D. L., and Pickard, D. (2007). Teaching human genetics with mustard: Rapid Cycling *Brassica rapa* (Fast Plants Type) as a model for human genetics in the classroom laboratory. *CBE Life Sci Educ* 6, 179-185.

Whatley, J. M. (1978). A suggested cycle of plastid developmental interrealationships. *New Phytologist* 80, 489-502.

Williams, P. H., and Hill, C. B. (1986). Rapid-Cycling Populations of *Brassica*. *Science* 232, 1385-1389.

Wise, R. R. (2006). The Diversity of Plastid Form and Function. In The Structure and Function of Plastids, R.R. Wise, and J.K. Hoober, eds. (*Springer Netherlands*), pp. 3-26.

Yang, M., Zhang, X., Liu, G., Yin, Y., Chen, K., Yun, Q., Zhao, D., Al-Mssallem, I. S., and Yu, J. (2010). The Complete Chloroplast Genome Sequence of Date Palm (*Phoenix dactylifera* L.). *PLoS ONE* 5, e12762.

Ye, G.-N., Colburn, S. M., Xu, C. W., Hajdukiewicz, P. T. J., 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.

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.

Yoo, S.-D., Cho, Y.-H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protocols* 2, 1565-1572.

Zerges, W. (2000). Translation in chloroplasts. *Biochimie* 82, 583-601.

Zhao, Q.-h., Fisher, R., and Auer, C. (2002). Developmental phases and STM expression during *Arabidopsis* shoot organogenesis. *Plant Growth Regulation* 37, 223-231.

Zhou, F., Karcher, D., and Bock, R. (2007). Identification of a plastid intercistronic expression element (IEE) facilitating the expression of stable translatable monocistronic mRNAs from operons. *The Plant journal* 52, 961-972.

Zoschke, R., Liere, K., and Börner, T. (2007). From seedling to mature plant: *Arabidopsis* plastidial genome copy number, RNA accumulation and transcription are differentially regulated during leaf development. *The Plant Journal* 50, 710-722.

Zou, Z., Eibl, C., and Koop, H. U. (2003). The stem-loop region of the tobacco psbA 5'UTR is an important determinant of mRNA stability and translation efficiency. *Mol Genet Genomics* 269, 340-349.

Zoubenko, O. V., Allison, L. A., Svab, Z., and Maliga, P. (1994). Efficient targeting of foreign genes into the tobacco plastid genome. *Nucleic acids research* 22, 3819-3824.

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

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Rosales-Mendoza, S., Alpuche-Solís, A., Soria-Guerra, R., Moreno-Fierros, L., Martínez-González, L., **Herrera-Díaz, A**., Korban, S.S. "Expression of an *Escherichia coli* antigenic fusion protein comprising the heat labile toxin B subunit and the heat stable toxin and its assembly as a functional oligomer in transplastomic tobacco plants". *Plant Journal*. 2009; 57: 45-54.

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12 Erklärung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist. Ich habe weder an anderer Stelle versucht eine Dissertation oder Teile einer solchen einzureichen bzw. einer Prüfungskommission vorzulegen, noch eine Doktorprüfung zu absolvieren.

München, den 28.04.2011