Dissertation

Development and application of novel genetic transformation technologies in maize (*Zea mays* L.)

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Abstract

Plant genetic engineering approaches are of pivotal importance to both basic and applied research. However, rapid commercialization of genetically engineered crops, especially maize, raises several ecological and environmental concerns largely related to transgene flow via pollination. In most crops, the plastid genome is inherited uniparentally in a maternal manner. Consequently, a trait introduced into the plastid genome would not be transferred to the sexually compatible relatives of the crops via pollination. Thus, beside its several other advantages, plastid transformation provides transgene containment, and therefore, is an environmentally friendly approach for genetic engineering of crop plants. Reliable *in vitro* regeneration systems allowing repeated rounds of regeneration are of utmost importance to development of plastid transformation technologies in higher plants. While being the world's major food crops, cereals are among the most difficult-to-handle plants in tissue culture which severely limits genetic engineering approaches. In maize, immature zygotic embryos provide the predominantly used material for establishing regeneration-competent cell or callus cultures for genetic transformation experiments. The procedures involved are demanding, laborious and time consuming and depend on greenhouse facilities. In one part of this work, a novel tissue culture and plant regeneration system was developed that uses maize leaf tissue and thus is independent of zygotic embryos and greenhouse facilities. Also, protocols were established for (i) the efficient induction of regeneration-competent callus from maize leaves in the dark, (ii) inducing highly regenerable callus in the light, and (iii) the use of leaf-derived callus for the generation of stably transformed maize plants. Furthermore, several selection methods were tested for developing a plastid transformation system in maize. However, stable plastid transformed maize plants could not be yet recovered. Possible explanations as well as suggestions for future attempts towards developing plastid transformation in maize are discussed. Nevertheless, these results represent a first essential step towards developing chloroplast transformation technology for maize, a method that requires multiple rounds of plant regeneration and selection to obtain genetically stable transgenic plants.

In order to apply the newly developed transformation system towards metabolic engineering of carotenoid biosynthesis, the daffodil phytoene synthase (*PSY*) gene was integrated into the maize genome. The results illustrate that expression of a recombinant *PSY* significantly increases carotenoid levels in leaves. The beta-carotene (pro-vitamin A) amounts in leaves of transgenic plants were increased by ~21% in comparison to the wild-type. These results represent evidence for maize to have significant potential to accumulate higher amounts of carotenoids, especially beta-carotene, through transgenic expression of phytoene synthases.

Finally, progresses were made towards developing transformation technologies in *Peperomia* (*Piperaceae*) by establishing an efficient leaf-based regeneration system. Also, factors determining plastid size and number in *Peperomia*, whose species display great interspecific variation in chloroplast size and number per cell, were investigated. The results suggest that organelle size and number are regulated in a tissue-specific manner rather than in dependency on the plastid type. Investigating plastid morphology in *Peperomia* species with giant chloroplasts, plasmatic connections between chloroplasts (stromules) were observed under the light microscope and in the absence of tissue fixation or GFP overexpression demonstrating the relevance of these structures *in vivo*. Furthermore, bacteria-like microorganisms were discovered within *Peperomia* cells, suggesting that this genus provides an interesting model not only for studying plastid biology but also for investigating plant-microbe interactions.

Index

Abbreviations

Abbreviation	Details
A	Adenine
BAP	Benzylaminopurine
bp	Base pairs
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower Mosaic Virus
Ch/I	Chloroform/isoamyl alcohol
C	Cytosine
°C	Degree Celsius
cDNA	Complementary DNA
cm	Centimeter
CPR	Chlorophenol red
CTAB	Cetyl trimethyl ammonium bromide
2,4-D	2,4-Dichlorophenoxy acetic acid
DNA	Deoxyribonucleic acid
DMAPP	Dimethylallyl pyrophosphate
dsDNA	Double-stranded DNA
EDTA	Diaminoethanetetra acetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
g	Gram; the metric unit of mass
G	Guanine
GA	Gibberellic acid
GE	Genetically engineering
GFP	Green fluorescent protein
GGPP	Geranylgeranyl pyrophosphate
GM	Genetically modified
GMO	Genetically modified organisms
HPLC	High-Performance Liquid Chromatography
IAA	Indole acetic acid
IBA	Indole butyric acid

IPP	Isopentenyl pyrophosphate
IR	Inverted repeat region
kb	Kilo base pairs
kDa	Kilo Dalton
l	Liter
LCY	Lycopene cyclase
ml	Milliliter
MOPS	3-(N-Morpholino) propane sulfonic acid
NAA	Naphthalene acetic acid
NEP	Nuclear-encoded RNA polymerase
PCR	Polymerase chain reaction
PD ring	Plastid-dividing ring
PDS	Phytoene desaturase
PEG	Polyethylene glycol
PEP	Plastid-encoded RNA polymerase
Ph/Ch/I	Phenol/chloroform/isoamyl alcohol
PMI	Phosphomannose isomerase
Prrn	Ribosomal RNA operon promoter
PSK	Phytosulfokine
PSY	Phytoene synthase
ptDNA	Plastid DNA
PzmZ27	*Zea mays* 27 kDa zein gene promoter
RNA	Ribonucleic acid
rRNA	ribosomal RNA
rpm	Rounds per minute
s	Second; unit of the time
SAM	Shoot apical meristem
SD	Shine-Dalgarno sequence
SDS	Sodium dodecyl sulfate
SSC	Small single copy region
T	Thymine
TAE	Tris-Acetate-EDTA buffer
tRNA	transfer RNA
UTR	Untranslated region

UV	Ultraviolet light
V	volt
v/v	Volume per volume
WHO	World Health Organization
Wt	Wild-type
w/v	Weigh per volume
Zea	Zeatin
ZDS	ζ-carotene desaturase

Publications

A part of this work has been recently published in the journal "Transgenic Research": "Ahmadabadi, M., Ruf, S., and Bock, R. (2006) A leaf-based regeneration and transformation system for maize (*Zea mays* L.). *Transgenic Res.*".

1 Introduction

1.1 Totipotency and *in vitro* regeneration of plants

Totipotency is the ability of a single cell to proceed through all stages of development and thus produce a normal adult. Many plant somatic cells are totipotent, and typically undergo *in vitro* regeneration via one of two pathways: (i) organogenesis (adventitious shoot meristem formation), or (ii) somatic embryogenesis (formation of somatic embryo) (Thorpe, 1994). During organogenesis, totipotent cells or tissues produce a unipolar shoot structure with the vascular system often connected to the parent tissue. In contrast, in somatic embryogenesis, a bipolar embryo containing a root and shoot axis with a closed independent vascular system is produced (Thorpe, 1994). In many plant species, *in vitro* culture studies of isolated somatic cells and protoplasts from different tissues confirm that a single isolated cell is able to develop normally into a whole plant via either organogenesis or somatic embryogenesis (Vasil and Vasil, 1992).

1.2 Factors controlling the *in vitro* regeneration response in plants

1.2.1 The genetic control of somatic embryogenesis and organogenesis

The ability of single cells to proceed through a complete developmental pathway suggests that the complete genetic information necessary to generate the entire plant is contained in cells. Consequently, plant cells have the capability to dedifferentiate, re-enter the cell cycle, proliferate, and finally form new plants via organogenesis or somatic embryogenesis. Recently, there have been many attempts to characterize the genes triggering the *in vitro* development program. Several genes including *LEC* (*LEAFY COTYLEDON*), *FUS* (*FUSCA*), *PT* (*PRIMORDIAL TIMING*) and *CLV* (*CLAVATA*) have been identified to affect somatic embryogenesis in *Arabidopsis* (Mordhorst *et al.*, 1998; von Recklinghausen *et al.*, 2000; Gaj *et al.*, 2005). *clv* and *pt* mutants display opposite characteristics to *lec* and *fus* mutants in terms of their morphology and capability for somatic embryogenesis. In *lec* mutants, cotyledons similar to true leaves (''leafy cotyledons'') are formed, while in *pt* mutants leaves show cotyledon-like structure (Conway and Poethig, 1997). As a consequence, in *pt* and *clv* mutants, embryogenic competence is prolonged until the seedling stage and therefore post-embryonic cells in shoot apical meristems (SAMs) can respond *in vitro* and form somatic embryos (Mordhorst *et al.*, 1998; von Recklinghausen *et al.*, 2000). Studies on *Arabidopsis lec* mutants suggest the existence of independent mechanisms that trigger two developmental pathways for organogenesis and somatic embryogenesis (Gaj *et al.*, 2005): While *lec* mutants were strongly impaired in their embryogenic response, they were found to exhibit efficient

shoot regenerability via organogenesis from root explants. In maize, expression of ZmLEC1, a homolog of *Arabidopsis* LEC1, was strongest in very young embryogenic callus cells and decreased in later stages of somatic embryo development (Zhang *et al.*, 2002b) suggesting a role of *LEC* genes in somatic embryogenesis. Recent evidences indicate the direct role of LEC2 and FUS3 transcription factors in down-regulation of gibberellin biosynthesis during plant embryogenesis in *Arabidopsis* (Curaba *et al.*, 2004). Previously, it was also shown that addition of gibberellic acid (GA) to maize culture media results in either no effect or a slight inhibitory effect on callus induction (Sheridan, 1975), however some other data have shown that GAs are required for plant embryogenesis (Singh *et al.*, 2002). Zhang *et al.* (1998) showed that expression of maize CDC2Zm, a protein involved in cell division, generally correlated with *in vitro* cell proliferation. However, there is a complex cross-talk among different plant hormones and a variety of developmental and metabolic signals controlling plant growth and development (Gazzarrini and McCourt, 2003). Thus, complicated molecular relations between different genes and other components of embryogenesis pathway, including plant hormones, are thought to be involved in the *in vitro* regeneration response in plants (Gaj *et al.*, 2005).

1.2.2 Application of growth regulators in culture media

Induction of cell division and subsequent callus formation in *in vitro* cultures usually require the exogenous application of hormones such as auxin and cytokinin (Riou-Khamlichi *et al.*, 1999). The plant hormone auxin [principally indole acetic acid (IAA)] has been well known for its effects on plant embryogenesis (for a review see Jenik and Barton, 2005). In plant tissue culture, exogenously applied auxins, such as 2,4-dichlorophenoxy acetic acid (2,4-D), play an important role in *in vitro* regeneration response. In sugarcane (*Poaceae*), for example, auxin concentration in tissue culture media has been shown to play an important role in determining the embryogenic and organogenic potential of cultured leaf tissues (Lakshmanan *et al.*, 2006). Recent data demonstrate that auxin application causes rapid changes in transcription through the destruction of transcriptional repressors (for review see e.g., Jenik and Barton, 2005). While auxin seems to have a key role in embryogenesis, of course, it does not act alone but rather in combination with other regulators of plant growth and development. It has been shown that auxin enhances the gibberellin-induced destabilization of DELLA proteins (named for the presence of a conserved amino acid sequence) which are thought to be transcriptional repressors of gibberellic acid-inducible genes (Jenik and Barton, 2005). Ethylene, a plant growth regulator produced by cultured tissues, is known to affect *in vitro* morphogenesis and addition of ethylene-inhibitors, such as silver nitrate, in culture

media has been confirmed to promote *in vitro* regeneration (Brar *et al.*, 1999; Turhan, 2004). Recently, the ability of ethylene to inhibit auxin activity has been explained by its capacity to destabilize ARF2 (AUXIN RESPONSE FACTOR) proteins (Li *et al.*, 2004). Finally, auxin has been shown to rapidly repress cytokinin biosynthesis (Nordstrom *et al.*, 2004). Cytokinins which are purine derivatives, play an important role in promotion and maintenance of plant cell division in cultures (reviewed e.g. in Hartig and Beck, 2006). In addition, they are involved in various differentiation processes including shoot initiation and growth, photo-morphogenesis, and senescence. In *Arabidopsis*, it has been demonstrated that activation of cell division by cytokinin occurs through induction of cyclin (Cyc) proteins (Riou-Khamlichi *et al.*, 1999). These data also showed that constitutive CycD3 expression can replace the requirement for exogenous cytokinin in callus induction and proliferation from *Arabidopsis* cells cultures.

In 1996, Matsubayashi and Sakagami identified an active factor in tissue culture systems as phytosulfokine (PSK), a 5-amino acid peptide with sulfated tyrosine (Tyr) residues [Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln]. PSK acts as an extracellular ligand and is involved in the initial steps of cellular dedifferentiation, proliferation and re-differentiation. PSK has been identified in both monocotyledonous and dicotyledonous plants (Matsubayashi and Sakagami, 1996; Matsubayashi *et al.*, 1997; Hanai *et al.*, 2000; Yang *et al.*, 2000) and its five-amino acid sequence has been shown to be uniformly conserved (Lorbiecke and Sauter, 2002). In several studies, application of synthetic PSK in culture media has been reported to stimulate proliferation and somatic embryogenesis (Matsubayashi and Sakagami, 1996; Matsubayashi *et al.*, 1997; Hanai *et al.*, 2000; Igasaki *et al.*, 2003;). PSK is secreted from single cells in response to changes in the auxin and cytokinin levels (Matsubayashi *et al.*, 1999). In these studies, biological activity of PSK was strongly correlated with the signal transduction pathway mediated by auxin and cytokinin. Recently, it has been reported that PSK improves genetic transformation efficiencies by promoting the proliferation of surviving transgenic cells on selective media (Matsubayashi *et al.*, 2004).

The polyamines, such as spermine, spermidine and putrescine, are small basic molecules that play an important role in the promotion of DNA replication, cell division, plant growth and development by activating the nucleic acids biosynthesis pathway (Galston and Kaur-Sawhney, 1995; Tiburcio *et al.*, 1997). Exogenously added polyamines have been reported to induce embryogenesis and plant regeneration in *in vitro* cultures (Kevers *et al.*, 2000; Monteiro *et al.*, 2002; Tanga *et al.*, 2004). Spermidine has been described as the most effective polyamine (Kevers *et al.*, 2000).

1.2.3 Changes in the chromatin structure

In eukaryotic chromatin, there is a strong interaction between DNA and histones restricting access of regulatory protein factors involved in DNA replication and transcription to their target sequences. Histone acetylation is generally correlated with transcriptionally active euchromatin, whereas deacetylation shows correlation with transcriptionally silent heterochromatin (Khochbin *et al.*, 2001). Sodium butyrate has been shown to have inhibitory effect on histone deacetylation *in vitro* and *in vivo* (Boffa *et al.*, 1976). In addition, DNA methylation is known to play a significant role in the regulation of gene expression in eukaryotic cells. It can inhibit transcription directly by modifying target sites of transcription factors, thereby blocking their binding (Ng and Bird, 1999). It has been reported that DNA methylation profiles significantly differ between somatic embryogenesis and organogenesis in rose (*Rosa hybrida* L.) *in vitro* cultures and demethylation occurs at high frequency during somatic embryogenesis (Xu *et al.*, 2004). Similar results were observed in Siberian ginseng (*Eleuterococcus senticosus*) *in vitro* culture where genomic DNA methylation was significantly decreased in embryogenic callus in comparison to non-embryogenic calli (Chakrabaty *et al.*, 2003). Several other data suggest that the ability of tissues to produce somatic embryo in *in vitro* cultures depends on a certain level of DNA methylation, and that addition of DNA methylation inhibitors, such as 5-azacytidine, can alter somatic embryogenesis response (Santos and Fevereiro, 2002; Leljak-Levanic *et al.*, 2004; Yamamoto *et al.*, 2005).

1.3 *In vitro* regeneration and transformation systems in maize

One of the largest monocotyledonous families is the grass family (*Gramineae*). It arose 65 million years ago and includes more than 10,000 species adapted to one-third of the arable land on earth (Kellogg, 2001). As cereals, such as rice, maize (corn) and wheat (examples of *Gramineae* species), provide a major source of food and feed for humans, workable and reproducible techniques facilitating the efficient genetic engineering of these monocotyledonous plants are of particular importance. In maize tissue culture, the first somatic embryos were produced by Green and Phillips (1975). However, fertile maize plants could be regenerated almost fifteen years later from protoplasts isolated from embryogenic cell suspension cultures (Prioli and Sondahl, 1989; Shillito *et al.*, 1989). Maize embryogenic calli can be classified in two groups, type I and type II (Armstrong and Green, 1985). In type I callus, the cells are compact and generate somatic embryos showing complex and organized structures. Type II embryogenic callus is friable and maintains its ability to regenerate plants

over time. Cell suspension and protoplast cultures can be established from type II callus due to its friable nature. However, type II callus tends to be initiated at a lower efficiency than type I callus and could be obtained from fewer genotypes.

Different transformation methods and explants can be used to generate genetically modified maize. The first fertile transgenic maize plants were produced based on microprojectile bombardment (Gordon-Kamm *et al.*, 1990). This method has been used in several other reports of corn genetic transformation (Kemper *et al.*, 1996; Zhong *et al.*, 1996; Wright *et al.*, 2001). However, often also the *Agrobacterium tumefaciens*-mediated transformation method has been used to generate transgenic corn plants (Ishida *et al.*, 1996; Negrotto *et al.*, 2000; Frame *et al.*, 2002; Miller *et al.*, 2002; Frame *et al.*, 2006; Sidorov *et al.*, 2006; Zhao and Ranch, 2006). Successful maize transformation has also been described using tissue electroporation (D'Halluin *et al.*, 1999). At present, immature zygotic embryos are the most reliable explant source to establish regeneration-competent callus cultures, cell suspensions or protoplasts for corn genetic transformation (for review see e.g., Armstrong, 1999; Bilang *et al.*, 1999; Hansen and Wright, 1999). Regeneration of fertile maize plants has also been reported from calli initiated from anthers (Ting *et al.*, 1981), glumes (Suprasanna *et al.*, 1986) and immature tassels (Rhodes *et al.*, 1986; Songstad *et al.*, 1992). However, these techniques are laborious, time consuming, restricted to a limited number of maize genotypes and often require plant growth to maturity and continued crosses between low-yield inbred lines to provide a continuous source of immature zygotic embryos (Bilang *et al.*, 1999).

As an alternative, a system for the induction of transformation-competent callus from maize shoot apical meristems (SAMs) has been established using either shoot apices or immature inflorescences (Zhong *et al.*, 1992a, 1992b; Zhong *et al.*, 1996; Zhang *et al.*, 2002a). However, the SAM is only a small mount of cells at the plant apex and provides very little cell material for culturing on synthetic medium.

1.4 Plastids

1.4.1 Plastid origin

Plastids are semiautonomous plant organelles responsible not only for photosynthesis but also for many other vital processes such as amino acid, hormone and lipid biosynthesis, as well as nitrate and sulphate assimilation (Galili, 1995; Ohlrogge and Browse, 1995; Aldridge *et al.*, 2005). Plastids have arisen from an endosymbiotic event between a photosynthetic cyanobacterium and a proto-eukaryote in which mitochondria were already established (McFadden, 2001; Bock, 2005). During the gradual integration of the endosymbiont into the

host cell, the organellar genome size was dramatically reduced due to both massive gene loss and gene transfer to the nuclear genome of the eukaryotic host cell (Martin and Herrmann, 1998). As a consequence, in present-day photosynthetic eukaryotes, organellar genome size is rather small. Evidences show that gene transfer from organelles to the nucleus is an ongoing process that takes place at a surprisingly high frequency (reviewed in Bock, 2005). The prochlorophytes (e.g., *Prochlorococcus*) were once suspected to be the closest living relatives of plastids, but more recent findings have shed doubts on that view and still the precise lineage of cyanobacteria that gave rise to plastids remains unknown (Martin *et al.*, 2002).

1.4.2 Plastid types in higher plants

Plastids develop from small colorless undifferentiated proplastids in dividing meristematic cells, which subsequently differentiate into several plastid types during cell differentiation, depending on the developmental stage and cell type. These include chloroplasts (chlorophyll-containing plastids), chromoplasts (colored plastids which are able to store high amounts of carotenoids), amyloplasts (specialized plastids for starch granules storage), elaioplasts (lipid-storing plastids), leucoplasts (colorless plastids which play a role in lipid biosynthesis) and etioplasts (carotenoid-containing plastids) (Kirk and Tiliney-Bassett, 1978; Lopez-Juez and Pyke, 2005). Although different plastid types exhibit a variety of functional roles within the cell, the by far best studied plastid is the green chloroplast due to its presence in photosynthetically active leaf tissues.
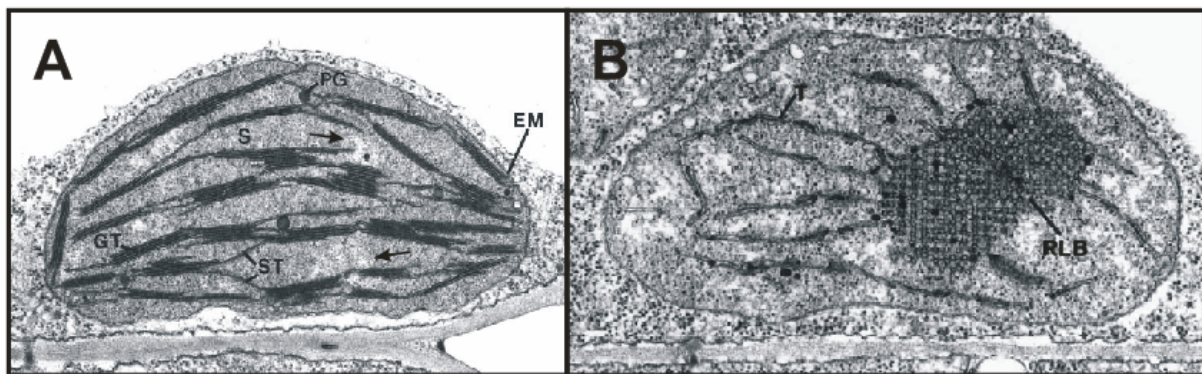
Figure 1.1 Electron microscopy images of plastids in higher plants. (**A**) Electron micrograph of a chloroplast from young tobacco leaves. Two envelope membranes (EM) enclose the chloroplast stroma (S). Stacked grana thylakoids (GT) and unstacked stroma thylakoids (ST) can be seen in stroma. Plastoglobuli (PG) and DNA-containing regions (shown by arrows) are also indicated. (**B**) Electron micrograph of a pea etioplast in a very early stage of light-induced conversion into a chloroplast. Thylakoids (T) and a lattice of tubular membranes known as prolamellar body (PLB) can be recognized (from Staehelin, 2003).

1.4.3 Plastid division

Plastids, like their free-living ancestors, divide by binary fission. During the past years, the cellular mechanisms of the division process have begun to emerge. A number of protein components of the division machinery have been identified through forward and reverse genetics approaches (Pyke and Leech, 1992; Pyke and Leech, 1994; Osteryoung and Pyke, 1998; Colletti *et al.*, 2000; Gao *et al.*, 2003;) and their functions during the division process are becoming increasingly clear (for review see e.g., Pyke, 1999; Osteryoung and Nunnari, 2003; Aldridge *et al.*, 2005; Miyagishima, 2005). Because plastids are surrounded by inner and outer membranes with a different composition, their division machinery must be capable of the coordinated constriction of both membranes and the final separation of the two daughter organelles. Ultrastructurally, the division apparatus is visible as a ring-like structure constricting the organelle [termed the plastid-dividing ring (PD ring)]. The PD ring consists of two distinct ring structures: (i) an outer ring on the cytosolic side of the outer plastid envelope, and (ii) an inner ring on the stromal side of the inner envelope. In addition to the PD ring, several other proteins associated with the inner envelope membrane are also required for plastid division, but how all these components of the division apparatus interact to coordinate the division process in a concerted manner is not yet fully understood (reviewed e.g. in Aldridge *et al.*, 2005).

1.4.4 Plastid genome

The plastid genome of higher plants consists of a circular molecule of double-stranded DNA which is 120-160 kb in size. Identical copies of the plastid genome are present in all plastid types. Generally it forms up to 10-20% of total cellular DNA content (Bock, 2001; Maliga, 2004). Several eubacterial features, such as prokaryotic mechanisms of gene expression and homologous recombination system, have been conserved in plastids from their ancestors (Bock, 2001).

The maize plastid genome consists of 140,384 bp and contains a pair of inverted repeat regions (IR_A and IR_B) (22,748 bp each) separated by a small (12,536 bp) and a large (82,355 bp) single copy region (SSC and LSC; Fig. 1.2). It contains 104 genes (70 protein-encoding genes, 30 tRNA genes and 4 rRNA genes; Fig. 1.2) which are identical with the plastid DNA of the closely related monocotyledonous species rice (*Oryza sativa*) (Maier *et al.*, 1995).
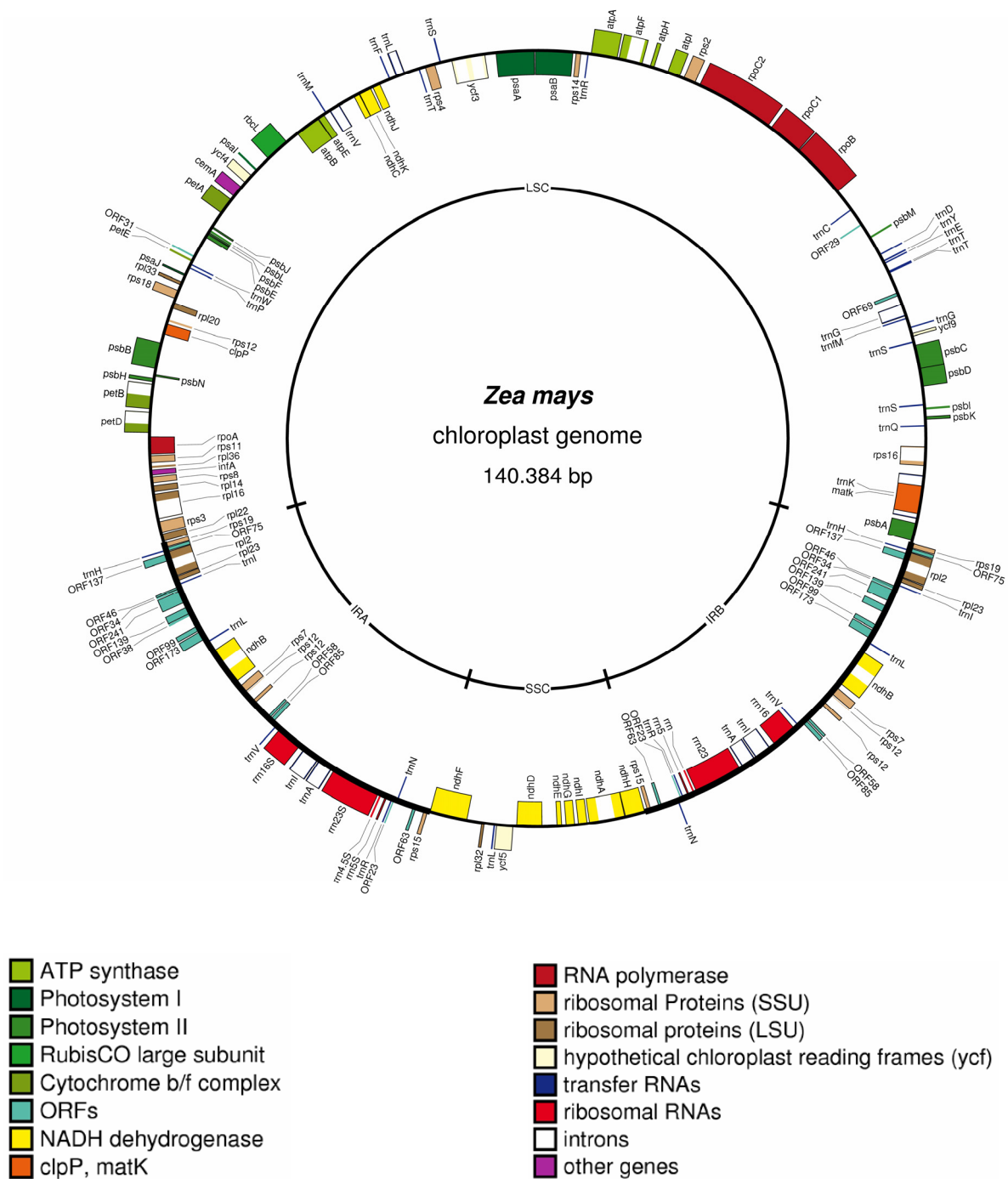
Figure 1.2 Gene organization of the maize (*Zea mays* L.) plastid genome. Two inverted repeat regions IR$_A$ and IR$_B$, divide the rest of the circular chromosome into large (LSC) and small (SSC) single copy regions. Genes drawn inside the circle are transcribed clockwise. The map was created using "*OrganellarGenomeDRAW*" software which has recently been developed by M. Lohse and O. Drechsel in the Bock laboratory.

1.4.5 Gene expression in plastids

Plastids have retained from their ancestors the eubacterial feature of gene organization in operons and principally prokaryotic mechanisms of polycistronic gene expression. However, in contrast to eubacteria, transcripts of plastid-encoded operons are extensively processed into

complex sets of overlapping RNA species and finally often into monocistronic RNAs (Westhoff and Herrmann, 1988). In most cases, both RNA splicing and RNA editing are involved in transcript maturation processes (for review see Maier *et al.*, 1996; Sugita and Sugiura, 1996). In addition to the transcriptional and posttranscriptional stages, the expression and accumulation of the plastid-encoded genes is also significantly controlled at the translational and posttranslational levels (for review see e.g., Manuell *et al.*, 2004; Zerges, 2000).

1.4.5.1 Transcription

Generally, transcription in higher plant plastids is performed by two different RNA polymerases of different phylogenetic origin: (i) a eubacterial (*E. coli*)-like plastid-encoded RNA polymerase (PEP), and (ii) a nuclear-encoded T7 phage-type RNA polymerase (NEP) (for review see e.g., Lysenko and Kuznetsov, 2005).

The plastid genes *rpoA*, *rpoB*, *rpoC1* and *rpoC2* encode subunits of the PEP (reviewed e.g. in Igloi and Kössel, 1992). These subunits are assembled with sigma (δ), a nuclear-encoded plastid promoter-specificity factor, essential for transcription initiation by PEP (Allison, 2000). Based on their function, δ factors can be classified in two groups: (i) general factors involved in the transcription of standard PEP genes, and (ii) specialized factors responsible for recognition of unique promoters in response to developmental and environmental signals. Recent data suggest that the accumulation of specific δ-like factors is controlled by light, plastid type and/or chloroplast developmental stage (reviewed in Allison, 2000). In *Arabidopsis*, for example, mutation in *SIG*5 gene resulted in loss of primary transcripts from the photosynthetic gene *psbD* which is exclusively transcribed by PEP. In contrast, overexpression of SIG5 in dark-adapted protoplasts specifically elevated *psbD* transcripts suggesting that the expression of this δ-like factor is induced by light (Tsunoyama *et al.*, 2004).

NEP is a single-subunit enzyme and in *Arabidopsis*, it consists of three members including plastid-targeted *RpoTp* (originally called *RpoT;3*), mitochondria-targeted *RpoTm* (*RpoT;1*), and *RpoTmp* (*RpoT;2*) which is targeted to both plastids and mitochondria (reviewed e.g. in Khan, 2005). Analysis of the iojap maize mutants lacking the PEP has revealed sequence homology between maize and tobacco NEP promoters suggesting that the NEP transcription machinery is conserved between monocots and dicots (Silhavy and Maliga, 1998).

It has been demonstrated that PEP and NEP polymerases transcribe distinct sets of plastid genes. For example, photosynthetic genes are mainly transcribed by the PEP. Most of the housekeeping genes are transcribed by both RNA polymerases, while a few genes are

transcribed only by the NEP (Hajdukiewicz *et al.*, 1997). Interestingly, the NEP appears to drive the PEP expression. This may have been an important step to bring plastid gene expression under control of the nucleus (Liere and Maliga, 1999).

1.4.5.2 Post-transcriptional RNA processing

Plastid-encoded RNAs are generally subjected to a set of posttranscriptional processing steps including cleavage of the polycistronic RNA chains, intron removal by RNA splicing and complex maturation of the transcript ends (for review see e.g., Monde *et al.*, 2000). The most common step of RNA processing is carried out by ribonucleases. There are two types of ribonucleases: (i) exoribonucleases, which remove nucleotides from either the 5' or 3' end, and (ii) endoribonucleases, which cleave internally. The enzymes involved in generation of monocistronic transcripts from primary polycistronic ones, are thought to include both endo- and exo-ribonucleases (reviewed in Monde *et al.*, 2000). Many plastid genes contain introns which are mainly *cis*-spliced. However, a few of them, such as *rps12* in higher plants, are spliced in *trans* (Sugita and Sugiura, 1996). In plastids of higher plants, the majority of introns are of group II, requiring ribosomes and/or translation for splicing (reviewed in Monde *et al.*, 2000).

RNA editing is another common RNA processing step in organelles (for a review see Bock, 2000). This RNA processing mechanism changes the identity of individual nucleotides, often, altering the information content of the mRNA (messenger RNA). Editing sites in plastid genome are generally found in reading frames and, in some cases, in translation start and stop codons. Interestingly, editing in plastids displays significant similarities to editing in plant mitochondria (Maier *et al.*, 1996). In both cases, editing leads to the conversion of individual cytidine residues to uridine (C-to-U) and, in some cases, the reverse event, U-to-C transitions. In comparison to mitochondria, the number of editing sites is much lower in plastids. However, evidences suggest that the editing process in both plant organelles is evolutionarily linked (Bock, 2000).

In addition, RNA stabilisation/destabilisation has been discovered as a further regulatory point in plastid gene expression (for review see Monde *et al.*, 2000). It has been shown that RNA stability varies significantly between individual transcripts and that relative transcription rates and transcript accumulation of plastid genes often do not correlate. Sequence elements located in the 5' and 3' UTR (untranslated region) as well as in coding sequences have been shown to play an important role in the stability of individual transcripts (reviewed e.g. in Schuster *et al.*, 1999). It has been shown that the stability of individual plastid mRNAs is regulated differentially in a development-specific manner. In spinach, for example, light-

grown plants displayed a significantly increased stability of plastid *psbA* mRNA compared to dark-grown plants (Deng and Gruissem, 1987).

1.4.5.3 Translation

In addition to regulatory steps at the level of transcription and transcript processing, the accumulation and assembly of plastid-encoded proteins is also considerably controlled at the translational and post-translational levels (for review see e.g., Manuell *et al.*, 2004). Translation machinery in plastids exhibits several eubacterial features, such as 70S ribosomes and formylated initiator tRNAs (fMet-tRNA). However, only a few plastid genes contain Shine-Dalgarno (SD)-like sequences that are necessary for eubacterial translation (reviewed e.g. in Sugiura *et al.*, 1998). Also, in some cases, sequence elements not found in prokaryotes seem to replace the function of SD sequence (Hirose and Sugiura, 1996).

Plastid translation has been shown to be regulated in response to light and developmental programs (Zerges, 2000). In several studies, no direct correlation has been observed between mRNA amounts and corresponding proteins. It has been demonstrated that under certain developmental conditions large amounts of thylakoid proteins are translated from little amounts of mRNA.

Genetic studies have been carried out to characterize *cis*-acting RNA elements and *trans*-acting protein factors required for chloroplast translation. *Trans*-acting translational factors are encoded in the nucleus and interact with *cis* elements located in the 5' and 3' UTRs of plastid mRNAs. Also, proteomic and bioinformatic analyses have been utilized to identify the proteins that function in chloroplast translation, including a complete set of plastid ribosomal proteins and homologues of eubacterial initiation, elongation and termination factors. These analyses have shown that the plastid translational apparatus is related to that of bacteria, but has adopted several eukaryotic mechanisms to facilitate and control translation in plastids (Manuell *et al.*, 2004).

1.4.6 The genetic transformation of plastids

Both biolistic bombardment of leaves or suspension cultures (Svab *et al.*, 1990; Ruf *et al.*, 2001; Langbecker *et al.*, 2004; Kanamoto *et al.*, 2006) and polyethylene glycol (PEG) treatment of protoplast (Golds *et al.*, 1993; O'Neill *et al.*, 1993) have been successfully used for DNA delivery into plant plastids. Plastid transformation involves targeted integration of a foreign DNA fragment into the plastid genome via homologous recombination, a feature of plastids inherited from their eubacterial ancestors. Therefore, in plastid transformation systems, the target-DNA fragment is flanked by two DNA sequences homologue to the

chloroplast genome (ptDNA) (for review see Bock, 2001). Plastid genomes are highly polyploid (Bendich, 1987) and each plastid contains between 10 and 100 genome copies. The plastid number per cell is highly variable (Pyke, 1999) and single mesophyll cells specialized for photosynthesis can contain hundreds of these organelles. Therefore, around 10,000 ptDNA copies can be present in a single leaf mesophyll cell. As a consequence, primary plastid transgenic (transplastomic) cell lines contain a mixed population of wild-type and transgenic plastid genomes (heteroplasmic state). Stable plastid transformation requires elimination of wild-type copies of the plastid genome until all plastid genome copies are transformed (homoplasmic state). The homoplasmic state can be achieved by allowing the heteroplasmic lines for a sufficient number of cell divisions under selective condition. In tobacco, for example, homoplasmic plants are obtained after passing the primary chloroplast transformants through several regeneration cycles under high selective pressure. Typically, small leaf samples are excised from regenerating plants and re-exposed to antibiotic-containing regeneration medium. Generally, homoplasmic plants are obtained after two to four rounds of regeneration on selective medium.

There are three types of selectable markers available for chloroplast transformation experiments, including dominant antibiotic-resistance genes, recessive antibiotic-resistance markers, and recessive photoautotrophic growth-restoring markers (for review see Bock, 2001). The latter, which is available only for *Chlamydomonas reinhardtii*, complements non-photosynthetic mutants restoring their capability to grow photoautotrophically on minimal medium. Recessive antibiotic-resistance markers are available for *Chlamydomonas* and tobacco and encode antibiotic-insensitive alleles of ribosomal RNA genes. Dominant antibiotic-resistance genes are available for both *Chlamydomonas* and tobacco, and so far, they have been most frequently used to establish plastid transformation systems in higher plants. The first plastid-specific antibiotic resistance marker gene developed to improve chloroplast transformation efficiency was chimeric *aadA*, an originally bacterial aminoglycoside 3''-adenylyltransferase gene conferring resistance to a number of aminoglycoside-type antibiotics, including spectinomycin and streptomycin (Goldschmidt-Clermont, 1991; Svab and Maliga, 1993). A chimeric *nptII* gene derived from the bacterial transposon Tn5 and encoding a neomycin phosphotransferase conferring resistance to kanamycin, is another dominant selectable marker gene used for plastid transformation. The *aadA* gene is a highly efficient and plastid-specific selectable marker, while the *nptII* gene appears to be less efficient and produces a significant background of nuclear transformants (Carrer *et al.*, 1993). In order to convert the bacterial *aadA* and *nptII* genes into plastid-

specific selectable markers, their coding sequence is fused to chloroplast expression signals including a 5' DNA segment providing plastid-specific promoter (essential for transcription), 5' untranslated region (UTR) and Shine-Dalgarno (SD) sequence (required for efficient translation) as well as a 3' chloroplast DNA segment providing a stable 3' UTR (essential for transcript stability).

1.4.7 Advantages of plastid transformation

In most flowering plant species, the plastid genome is inherited uniparentally in a strictly maternal manner (Zhang *et al.*, 2003). Consequently, the possibility of transgene flow via pollination to related weeds or crops is minimized if the transgene is targeted to the plastid genome. Thus, maternal inheritance offers containment of plastid transgenes. Another advantage of plastid transformation is the possibility of simultaneous expression of multiple transgenes due to the presence of prokaryotic mechanisms of polycistronic gene expression in plastids (for review see e.g., Bock, 2001; Bock and Khan, 2004). Additionally, site-specific transgene integration into intergenic regions of the ptDNA via homologous recombination eliminates concerns over position effects as well as the introduction of vector sequences that are often raised in nuclear transgenic plants. Furthermore, no post-transcriptional gene silencing has been observed in chloroplasts. Finally, plastid transformation provides readily obtainable high protein levels due to the high copy number of the ptDNA, making transplastomic plastids a potential production platform of therapeutic proteins and nutraceuticals (for review see Daniell, 2006). It has also been shown that chloroplasts are able to accumulate some foreign proteins or their biosynthetic products that could be harmful if they were present in the cytoplasm (Bogorad, 2000).

1.4.8 Development of plastid transformation technology in higher plants

For the first time, successful chloroplast transformation was reported by Boynton *et al.* for the unicellular green alga *Chlamydomonas reinhardtii* (Boynton *et al.*, 1988) and the technology was soon applied to the higher plant species tobacco (*Nicotiana tabacum*; *Solanaceae*) (Svab *et al.*, 1990). In many laboratories, tobacco plastid transformation is now routine, and has been the benchmark for developing plastid transformation protocols for other plant species. Plastid transformation has also been achieved in some other Solanaceous species such as potato (Sidorov *et al.*, 1999), tomato (Ruf *et al.*, 2001) and petunia (Zubko *et al.*, 2004), albeit at a significantly lower efficiency than in the tobacco system. In the case of potato, the transformed plants were sterile and transplastomic seeds could not be obtained. In non-Solanaceous species, plastid transformation has been more difficult to obtain. In *Arabidopsis*,

plastid transformation has been reported, however, the efficiency was rather low and the transformed plants were sterile (Sikdar *et al.*, 1998). Recently, fertile transplastomic plants have been regenerated in *Lesquerella fendleri* (*Brassicaceae*) (Skarjinskaia *et al.*, 2003), soybean (*Fabaceae*) (Dufourmantel *et al.*, 2004), cotton (*Malvaceae*) (Kumar *et al.*, 2004b) and lettuce (*Asteraceae*) (Lelivelt *et al.*, 2005), however, the transformation efficiencies are low. Plastid transformation has also been reported in carrot cell cultures but there is no indication if the transformed plants were fertile (Kumar *et al.*, 2004a). In *Brassica napus* (oilseed rape), only heteroplasmic plastid transformed lines were obtained (Hou *et al.*, 2003). Likewise, plastid transformation of embryonic rice suspension culture cells, using a rice-specific chloroplast transformation vector, resulted in heteroplasmic plants and the homoplasmic state could not be achieved (Khan and Maliga, 1999; Lee *et al.*, 2006).

1.4.9 Plastid biotechnology

Organelle genomes offer significant attractions as a target for the expression of foreign genes, especially when environmental concerns and biosafety issues are taken into account. Both insect and herbicide resistance genes have been successfully expressed in plastids (reviewed e.g. in Maliga, 2004). Recently, several methods have been described for selectable marker gene elimination from transplastomic plants (Corneille *et al.*, 2001; Klaus *et al.*, 2004; Lutz *et al.*, 2006). In several cases, plastid transformation technology has been applied to incorporate foreign genes in the plastid genome for contained high-level expression of recombinant proteins for industrial and pharmaceutical purposes. Chloroplast-based gene expression systems are suitable for the production of recombinant proteins in transplastomic plants at the commercial level (for review see Daniell, 2006). At present, there is considerable interest in chloroplast-based recombinant protein expression in non-toxic, edible plant species, such as tomato. This will not only minimize downstream protein purification and processing costs, but also provide combined production and delivery systems for "edible" protein therapeutics. Recently, Wurbs *et al.* (2007) have aimed to engineer the carotenoid biosynthesis pathway towards the production of healthier tomato fruits with elevated levels of *β*-carotene, a precursor of vitamin A. Their results demonstrated the successful engineering of the nutritionally valuable *β*-carotene biosynthesis pathway in non-green plastids by expression of a prokaryotic lycopene cyclase in chromoplasts of transplastomic tomato fruits.

1.5 Carotenoid biosynthesis in higher plants

1.5.1 General pathway for carotenoid biosynthesis in plants

Carotenoids, one of the most abundant pigments in nature, are essential components of the photosynthetic apparatus. Besides their functions in photosynthetic tissues, carotenoids play significant roles in plant reproduction and seed dispersal by attracting pollinators (Burkhardt *et al.*, 1997). Carotenoids accumulate in nearly all plastid types, albeit at different levels (reviewed e.g. in Howitt and Pogson, 2006). For example, in maize and potato, carotenoids are formed in the amyloplasts of the storage tissues. The first committed step in the carotenoid biosynthesis is the head-to-head condensation of two geranylgeranyl pyrophosphate (GGPP) molecules to form the colorless carotenoid phytoene, which does not usually accumulate in tissues. This reaction is catalyzed by the phytoene synthase enzyme (PSY). Then, four desaturation steps follow, oxidizing the phytoene into the red-colored compound lycopene. Afterwards, two lycopene cyclases (*α*-LCY and *β*-LCY) catalyze the formation of *α*- and *β*-carotene from lycopene (Fig. 1.3).

1.5.2 Carotenoid biosynthesis in maize

Carotenoid biosynthesis in seeds has been considerably studied in maize, mainly because of the availability of white-endosperm mutants deficient in carotenoids. Maize contains two phytoene synthase genes (*PSY*): *PSY*1, which was first identified through the characterization of *y*1 mutants (Buckner *et al.*, 1996), and *PSY*2 (Gallagher *et al.*, 2004). Nucleotide sequences of *PSY*1 and *PSY*2 are conserved except for their N- and C-terminal domains, suggesting that these genes have arisen from a gene duplication event (Gallagher *et al.*, 2004). Studies on other maize white-endosperm mutants have led to the characterization of phytoene desaturase (PDS) and *ζ*-carotene desaturase (ZDS) genes in the carotenoid biosynthesis pathway. Maize PDS and ZDS mediate a desaturation pathway, resulting in the production of prolycopene (Matthews *et al.*, 2003). Both maize *PSY* genes are functional when tested in a heterologous bacterial system (Gallagher *et al.*, 2004), however, only *PSY*1 transcript abundance shows correlation with carotenoid content in maize endosperm (Buckner *et al.*, 1996). Also, recent data suggest that PSY1 and PSY2 enzymes are not equivalent *in planta* and that accumulation of carotenoids in seed endosperm requires PSY1 expression (Gallagher *et al.*, 2004). In addition, they propose that the differences in membrane architecture between endosperm amyloplasts and leaf chloroplasts might be responsible for the loss of PSY2 function in endosperm. On the other hand, phytoene synthase gene expression is likely controlled in a

tissue-specific manner to enable the plant to modify the phytoene synthase gene expression in seeds without any harmful effects on photosynthetic tissues.
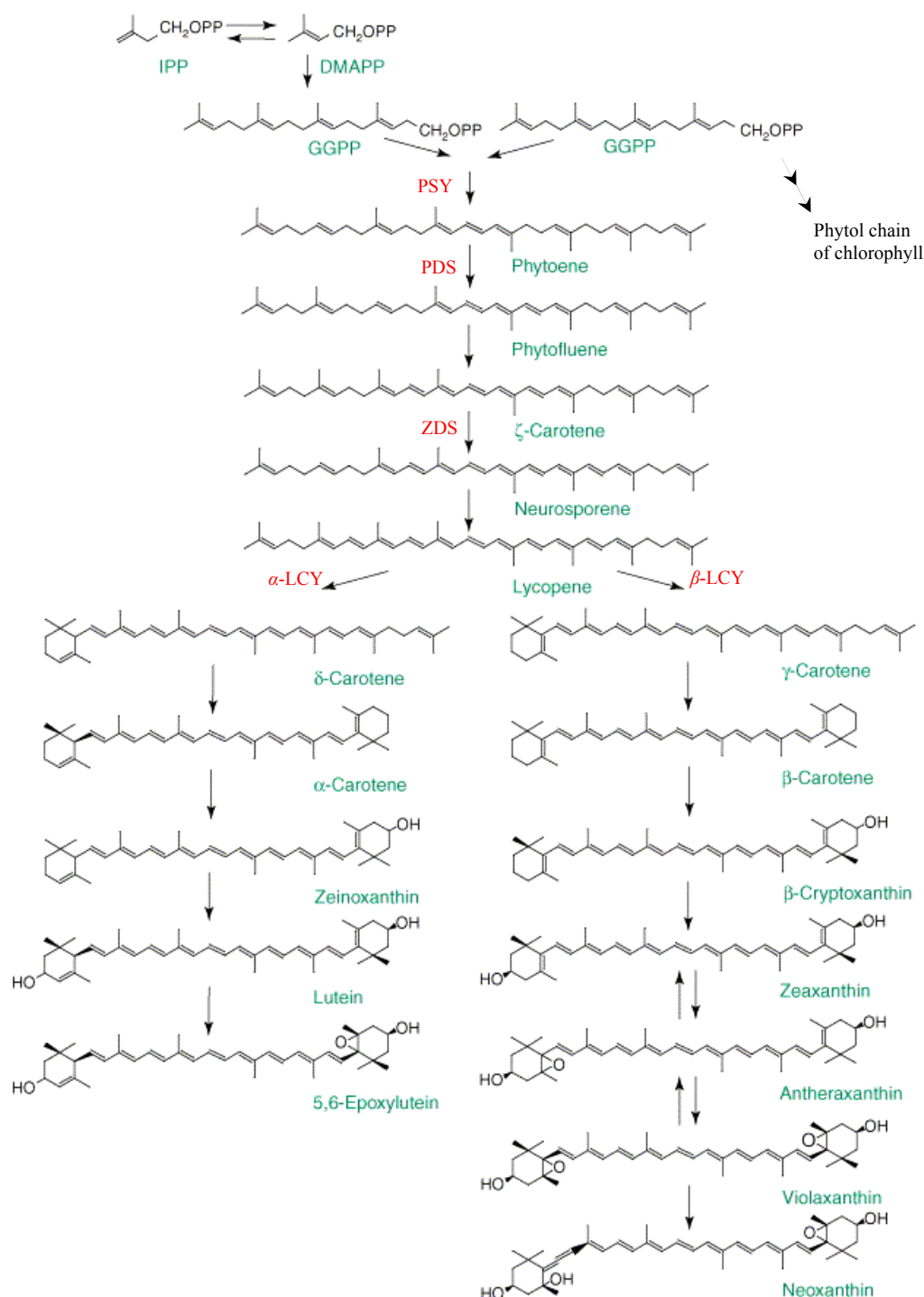
Figure 1.3 Precursor compounds and major carotenes and xanthophylls in the carotenoid biosynthetic pathway in plants. The pathway shows the primary steps found in nearly all plant species. Some of the key enzymes in the pathway are shown in red. Abbreviations: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; α-LCY, lycopene α-cyclase; β-LCY, lycopene β-cyclase (from Kopsell and Kopsell, 2006).

1.5.3 Modification of the carotenoid biosynthesis pathway in higher plants

Vitamin A is an important antioxidant and essential vitamin for human nutrition. β-carotene, one of the most important carotenoids, is a precursor of vitamin A (Yeum and Russell, 2002). Increased levels of several other carotenoids, such as lutein and zeaxanthin, have been implicated in protection against light-induced retinal damage and age-related macular degeneration (Landrum and Bone, 2001). Thus, there is significant potential for providing healthier foods by manipulating the carotenoid content of major crops, such as cereals. As mentioned, PSY is the first key enzyme in the carotenoid biosynthesis pathway. This has been supported experimentally by several studies. For example, in tomato, expression levels of PSY were closely correlated with the level of carotenoids (Giuliano *et al.*, 1993). In marigold, *PSY* transcript abundance in flowers varied among different varieties and correlated positively with carotenoid levels (Moehs *et al.*, 2001).

The carotenoid pathway has been manipulated by transgenic approaches towards elevated levels of carotenoids, especially β-carotene in seeds (reviewed in Howitt and Pogson, 2006). In oilseed canola, overexpression of PSY resulted in a 50-fold increase in carotenoid content in seeds of transgenic plants, which was predominantly in the form of α- and β-carotene (Shewmaker *et al.*, 1999). When the recombinant daffodil PSY was expressed in rice seed endosperm, which does not usually accumulate carotenoids, transgenic seeds accumulated only small mounts of phytoene (Burkhardt *et al.*, 1997). Further studies revealed that overexpression of both PSY and PDS enzymes, is required for carotenoid accumulation in rice endosperm. This became the principle for developing "Golden Rice" (Beyer *et al.*, 2002). Additional studies showed that the source of the *PSY* gene has a significant effect on carotenoid accumulation levels. When the maize instead of the daffodil *PSY* gene was overexpressed in rice endosperm, transgenic lines (called "Golden Rice 2") showed 23-fold more carotenoids than the "Golden Rice" (Paine *et al.*, 2005). In *Arabidopsis*, overexpression of the *PSY* gene has resulted in elevated levels of all carotenoids, with the β-carotene content increasing from 4% to 28% of the total carotenoid pool (Lindgren *et al.*, 2003). These studies provide good evidences for *PSY* to be a key regulator in the carotenoid biosynthesis pathway. They also confirm that seeds have a significant potential to accumulate higher levels of carotenoids, especially β-carotene, through overexpression of the *PSY* gene.

1.6   Objectives of this work

1.6.1   Development of novel transformation technologies in maize

Maize (*Zea mays* L.) is a major crop plant of great agronomical interest and a model plant for genetic studies. Over the past decades, several transgenic strains of this monocotyledonous plant have been produced using available plant genetic engineering approaches. Genetically modified (GM) maize is cultivated in a wide area worldwide and it has been increasing gradually since the mid 1990s and now accounts for 14% of the global area of maize cultivation. In the EU, maize is the only GM crop that is cultivated commercially (Weekes *et al.*, 2006).

In maize, male and female flowers are formed in separate parts of a plant. This leads to a high degree of cross-pollination between plants. It has been reported that field-cultivated corn freely crosses with nearly all members of the genus (Ma, 2005). Individual male flowers (tassel) produce around 4.5×10^6 pollen grains, which are considerably more than required grains for pollination of a single maize plant (Westgate *et al.* 2003). Under natural conditions, pollen can travel from field to field by wind. This factor, together with the naturally high degree of out-crossing in maize (only ∼5% self-pollination occurs), lead to a relatively high degree of gene flow in corn (Hamrick and Godt, 1997).

The genetic diversity maintained in open-pollinated local landraces is recognized as a highly valuable genetic resource. The potential transgene flow via pollination from GM maize into non-transgenic fields, local landraces and wild relatives of maize raises important scientific and political issues (Ortiz-Garcia *et al.*, 2005). In particular, insect-resistant transgenic Bt-maize hybrids cultivated in a wide area worldwide, are the central part of the debates between researchers and recalcitrant organizations to genetically modified organisms (GMOs) (Wisniewski *et al.*, 2002). On the other side, conventional hybrid seeds can be contaminated by pollens originating from transgenic plants cultivated in neighboring fields. This will restrict marketing of the seeds harvested from the contaminated fields (a major issue for corn producers).

As mentioned (see 1.4.7), plastids are generally transmitted only by the maternal parent. This means that plastid genes are not present in pollen. Consequently, a trait introduced into the plastid genome would not be unintentionally transferred to the sexually compatible relatives of the crops that might be growing nearby. Thus, beside its other advantages (see 1.4.7), plastid genetic engineering can overcome the major concern of transgene containment in transgenic maize. Generally, development of a plastid transformation system in higher plants requires: (i) a method to deliver foreign DNA into plastids; (ii) plastid-specific vectors

containing selectable marker genes under control of plastid-specific promoters, flanked by two DNA sequences from the plastid genome (required for homologous recombination); and (iii) an effective *in vitro* regeneration system allowing repeated rounds of regeneration (Bock, 2001). Reliable *in vitro* regeneration techniques permitting the production of whole plants from single cells are of central importance to both clonal propagation and successful genetic engineering of plants. Whereas many dicotyledonous plants can readily be regenerated from leaf material, monocotyledonous, and especially gramineous, plant species (such as cereals) are much more difficult to propagate and regenerate in tissue culture (reviewed for e.g. in Bilang *et al.*, 1999; Hansen and Wright, 1999).

For several reasons, leaf material is the preferred source material for transgenic experiments. It is rapidly produced from germinating seedlings, can readily be obtained in large amounts and allows multiple successive rounds of selection and regeneration. The latter is of utmost importance to the development of chloroplast transformation technologies where primary transformants are heteroplasmic and must be purified to homoplasmy by repeated cycles of plant regeneration under selective pressure (Bock, 2001; Maliga, 2004). The impossibility to conduct such successive regeneration rounds in cereal species due to the lack (or extremely low efficiency) of leaf-based regeneration systems is the main reason for the failure to generate genetically stable chloroplast transformants in cereals. While rice chloroplast transformants could be readily obtained, all lines remained heteroplasmic and eventually lost the plastid transgenes in the absence of a protocol for carrying out repeated regeneration cycles (Khan and Maliga, 1999).

In principle, cereals, including maize, have been demonstrated to be capable of undergoing callus induction and plant regeneration via somatic embryogenesis from leaf explants (Conger *et al.*, 1987; Ray and Ghosh, 1990), although the efficiency is often very low. Recently some progress has been made with using immature leaf material for establishing tissue culture systems and protocols for nuclear transformation in oat, barley and sugarcane (Gless *et al.*, 1998a, 1998b; Pasternak *et al.*, 1999; Lakshmanan *et al.*, 2006; Snyman *et al.*, 2006). However, workable leaf-based regeneration and transformation systems for corn are still lacking (Kemper *et al.*, 1996).

In this project we aimed to (i) develop tissue culture systems for maize that can be established from leaf material at high efficiency, (ii) develop a nuclear genetic transformation system for maize that is based on leaf-derived material, (iii) develop plastid-specific transformation vectors for maize, and (iv) test various parameters to optimize selection systems for maize plastid transformation experiments.

1.6.2 Modification of the carotenoid biosynthesis pathway in maize by expression of a recombinant daffodil *PSY* cDNA in the nuclear genome

Carotenoids are essential components of the human diet, primarily as antioxidants and precursors of vitamin A, which plays an essential role in the normal development of humans. The World Health Organization (WHO) has estimated that more than 100 million children are vitamin A-deficient, most of them in developing countries. Around half a million of these children become blind annually and most of them die within 12 month after blindness. Thus, it would be desirable to meet the daily vitamin A requirements by elevating the carotenoid levels in staple foods. Recently, researchers have worked to develop a genetically modified strain of rice, "Golden Rice", with high *β*-carotene level that provides a source of vitamin A (Beyer *et al.*, 2002; Paine *et al.*, 2005). Researchers hope that the "Golden Rice" will provide some improvement in nutrition for the millions of people for whom rice is a primary source of food. However, rice is grown predominantly in Asia, in tropical and subtropical regions. In contrast, maize is cultivated worldwide. It is Africa's second most important food crop, behind cassava, and is grown in a wide range of environments, giving maize a similar position in Africa in terms of dietary importance as rice in Asia. Thus, there is significant potential for providing healthier foods, for example, by manipulating the carotenoid content in major crops, without significantly changing people's diets. In addition, studying regulation of carotenoid accumulation in major cereal crops, such as maize, should facilitate improvement of their nutritional value with important health benefits.

As mentioned (see 1.5.2; 1.5.3), PSY is the first key enzyme in the carotenoid biosynthesis pathway, catalyzing phytoene production. Overexpression of the PSY has resulted in accumulation of carotenoids, especially *β*-carotene, in seed endosperm of several higher plants. In this part of the project, we aimed to engineer the carotenoid biosynthesis pathway in maize towards the production of transgenic maize plants with elevated levels of carotenoids. Using the newly developed leaf-based tissue culture and transformation system, we aimed to manipulate the carotenoid biosynthesis pathway by expression of the recombinant daffodil *PSY* cDNA in the maize genome under the control of two different promoters: (i) the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter, and (ii) the maize seed endosperm-specific 27 kDa zein gene promoter (Schernthaner *et al.*, 1988; Russell and Fromm, 1997). This was also the first biotechnological application of the newly established *in vitro* regeneration and transformation system in maize based on leaf explants as starting material. Thus, the goal of this subproject was to demonstrate the applicability of this method

to engineer nutritionally valuable metabolic pathways and agronomically important traits in maize.

1.6.3   Towards development of transformation technologies in *Peperomia*

The genus *Peperomia* belongs to the *Piperaceae* family and comprises approximately 1500-1700 mostly tropical species making it one of the largest genera of basal angiosperms (Wanke *et al.*, 2006). A well-known species in the genus is *Peperomia metallica* which was reported to contain giant chloroplasts in its palisade parenchyma cells (Schürhoff, 1908). The palisade parenchyma of *P. metallica* consists of a single cell layer with chloroplasts as big as 20-25 µm in diameter (Neumann, 1973). The large size of the chloroplasts is compensated for by a much smaller chloroplast number, which is in the range of only 2-6 per palisade cell (Bartels, 1965). Because of this unique feature, *P. metallica* chloroplasts have been a preferred model object of electrophysiological studies (Bulychev *et al.*, 1972). In addition, the large size of the chloroplasts in palisade cells might facilitate transgene targeting into the chloroplasts, hence facilitating chloroplast transformation in this genus.

On the other hand, while the giant chloroplasts in the palisade parenchyma of *P. metallica* have been intensively investigated, little is known about plastids in other *Peperomia* species. Furthermore, while different types of plastids exhibit a variety of functional roles within the cell, by far the most studied plastid is the green chloroplast. Considering the importance of non-green plastids, their biology has been remarkably little studied. The main reason is that non-green plastids are small and carry little discernible pigment. Thus, development of genetic transformation systems in *Peperomia* will provide a highly useful tool to study plastid biology in this genus.

In this subproject, we aimed to (i) develop a transformation system for *Peperomia*, and (ii) use a set of different *Peperomia* species to analyze plastid morphology, the relationship between cell size and chloroplast size and the regulation of plastid size and number in different tissues.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetone	Roth, Karlsruhe, Germany
Acetonitrile	Roth, Karlsruhe, Germany
Agarose (Seakem® LE agarose)	Cambrex, Rockland, ME
p-Aminobenzoic acid (TC)	Duchefa, Haarlem, Netherlands
Ammonium sulphate (TC)	Duchefa, Haarlem, Netherlands
Ampicillin, sodium salt	Roth, Karlsruhe, Germany
Azacytidine	Sigma-Aldrich, Munich, Germany
Benzylaminopurine (BAP) (TC)	Duchefa, Haarlem, Netherlands
D(+)-Biotin (vitamin H) (TC)	Duchefa, Haarlem, Netherlands
Boric acid (TC)	Duchefa, Haarlem, Netherlands
Bovine serum albumin (BSA)	Roth, Karlsruhe, Germany
Bromophenol blue	Merck, Darmstadt, Germany
Calcium chloride dehydrate (TC)	Duchefa, Haarlem, Netherlands
Casein hydrolysate (TC)	Duchefa, Haarlem, Netherlands
Cetyl trimethyl ammonium bromide (CTAB)	Roth, Karlsruhe, Germany
Cholin chloride (TC)	Duchefa, Haarlem, Netherlands
Cyanocobalamin (vitamin B_{12}) (TC)	Duchefa, Haarlem, Netherlands
Diaminoethanetetra acetic acid (EDTA)	Roth, Karlsruhe, Germany
EDTA, disodium salt (TC)	Duchefa, Haarlem, Netherlands
Dicamba (TC)	Sigma-Aldrich, Munich, Germany
2,4-dichlorophenoxy acetic acid (2,4-D) (TC)	Duchefa, Haarlem, Netherlands
Ethidium bromide	Roth, Karlsruhe, Germany
Ethyl acetate	Merck, Darmstadt, Germany
Folic acid (TC)	Duchefa, Haarlem, Netherlands
Formaldehyde (37%)	Roth, Karlsruhe, Germany
Formamide (deionized)	Roth, Karlsruhe, Germany
Gelrite (TC)	Duchefa, Haarlem, Netherlands
Geneticin (G418)	Sigma-Aldrich, Munich, Germany
Gibberellic acid (GA_3) (TC)	Duchefa, Haarlem, Netherlands
Glucose	Roth, Karlsruhe, Germany

Glycerol	Roth, Karlsruhe, Germany
Glycine (TC)	Duchefa, Haarlem, Netherlands
Hydrochloric acid	Sigma-Aldrich, Munich, Germany
3-Indole acetic acid (IAA) (TC)	Sigma-Aldrich, Munich, Germany
Indole butyric acid (IBA) (TC)	Sigma-Aldrich, Munich, Germany
Iron sodium EDTA	Roth, Karlsruhe, Germany
Isopropanol	Fluka, Buchs, Switzerland
Kanamycin	Duchefa, Haarlem, Netherlands
L-proline (TC)	Duchefa, Haarlem, Netherlands
Lysozyme	Roth, Karlsruhe, Germany
Magnesium chloride hexahydrate (TC)	Sigma-Aldrich, Munich, Germany
Magnesium sulphate heptahydrate	Roth, Karlsruhe, Germany
Manganese (II) chloride, tetrahydrate	Roth, Karlsruhe, Germany
Manganese sulphate monohydrate (TC)	Sigma-Aldrich, Munich, Germany
D-Mannose (TC)	Duchefa, Haarlem, Netherlands
ß-Mercaptoethanol	AppliChem, Darmstadt, Germany
Methanol (HPLC grade)	Merck, Darmstadt, Germany
Micro agar (TC)	Duchefa, Haarlem, Netherlands
3-(N-Morpholino) propane sulfonic acid (MOPS)	Roth, Karlsruhe, Germany
Myo-inositol (TC)	Duchefa, Haarlem, Netherlands
Naphthalene acetic acid (NAA) (TC)	Duchefa, Haarlem, Netherlands
Nicotinamide (TC)	Duchefa, Haarlem, Netherlands
Pantothenate, hemicalcium salt (TC)	Sigma-Aldrich, Munich, Germany
Phenol	Roth, Karlsruhe, Germany
Phenol/chloroform/isoamyl alcohol (Ph/Ch/I) (25/24/1)	Roth, Karlsruhe, Germany
Phytosulfokine alpha (α-PSK)	Neo MPS, Strasbourg, France
Potassium acetate	Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate (TC)	Duchefa, Haarlem, Netherlands
Potassium hydroxide	Roth, Karlsruhe, Germany
Potassium iodide (TC)	Duchefa, Haarlem, Netherlands
Potassium nitrate (TC)	Duchefa, Haarlem, Netherlands
Pro-Q Diamond dye	Mo Bi Tec, Goettingen, Germany
Pyridoxine hydrochloride (vitamin B_6) (TC)	Duchefa, Haarlem, Netherlands
Riboflavin (vitamin B_2) (TC)	Duchefa, Haarlem, Netherlands

Rubidium chloride	Fluka, Buchs, Switzerland
Salicylic acid, sodium salt	Sigma-Aldrich, Munich, Germany
Silver nitrate (TC)	Sigma-Aldrich, Munich, Germany
Sodium acetate	Roth, Karlsruhe, Germany
Sodium butyrate	Sigma-Aldrich, Munich, Germany
Sodium chloride	Roth, Karlsruhe, Germany
Sodium citrate	Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, Germany
Sodium EDTA	Roth, Karlsruhe, Germany
Sodium hydroxide	Fluka, Buchs, Switzerland
Spectinomycin	Duchefa, Haarlem, Netherlands
Spermidine (free base)	Duchefa, Haarlem, Netherlands
Spermidine-3HCl (TC)	Serva, Heidelberg, Germany
Spermine	Sigma-Aldrich, Munich, Germany
Streptomycin	Duchefa, Haarlem, Netherlands
Sucrose (TC)	Duchefa, Haarlem, Netherlands
Thiamine hydrochloride (vitamin B_1) (TC)	Sigma-Aldrich, Munich, Germany
Tris(hydroxymethyl)-aminomethan (Tris)	Roth, Karlsruhe, Germany
Yeast extract	Duchefa, Haarlem, Netherlands
Zinc sulphate heptahydrate (TC)	Duchefa, Haarlem, Netherlands

All stock solutions and media were prepared using chemicals listed above (unless otherwise stated). For plant *in vitro*-culture media, applied chemicals were tested for tissue culture by manufacturer (labeled with "TC" for "Tissue Culture" in the list). Highly pure deionized distilled water was used to prepare all media and stock solutions.

2.1.2   Enzymes and kits

Restriction enzymes

The restriction enzymes used in this study were mainly purchased from New England Biolabs (NEB, Frankfurt a. M., Germany), MBI Fermentas (St. Leon-Rot, Germany), Promega (Mannheim, Germany) and Roche (Mannheim, Germany).

Other enzymes

Enzyme	Produced by
T4-DNA ligase	Promega (Mannheim, Germany)

Klenow fragment, DNA polymerase I	MBI Fermentas (St. Leon-Rot, Germany)
RNase A (DNase-free)	Roche (Mannheim, Germany)
DNase I (RNase-free)	AppliChem, Darmstadt, Germany
Taq DNA polymerase	Promega (Mannheim, Germany)
Deep Vent® DNA polymerase	NEB (Frankfurt a. M., Germany)

Kits

Kit	Manufacturer
NucleoBond® AX plasmid DNA purification kit	Macherey-Nagel (Dueren, Germany)
Qiagen Plasmid Midi, Maxi kits	Qiagen (Hilden, Germany)
GFX™ PCR DNA and Gel Band Purification kit	GE Healthcare (Buckinghamshire UK)
Nucleospin Extract II kit	Macherey-Nagel (Dueren, Germany)
Megaprime™ DNA labeling system	Amersham (Buckinghamshire, UK)
PeqGold TriFast™	PeqLab (Erlangen, Germany)

2.1.3 Molecular weight markers

DNA markers

1. Gene Ruler™, 1 kb DNA Ladder (MBI Fermentas)

Fragments in bp: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250

2. Gene Ruler™, 100 bp DNA Ladder (MBI Fermentas)

Fragments in bp: 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80

RNA markers

1. 0.24-9.5 kb RNA Ladder (Invitrogen, Karlsruhe, Germany)

Fragments in bp: 9490, 7460, 4400, 2370, 1350, 240

2. 0.5-10 kb RNA Ladder (Invitrogen, Karlsruhe, Germany)

Fragments in bp: 10000, 8000, 6000, 4000, 3000, 2000, 1500, 1000, 500

2.1.4 Oligodeoxynucleotides (primers)

The following primers were used to amplify DNA fragments of interest (i) to confirm the physical presence of transgenes in the selected transgenic lines; (ii) for sequencing; (iii) to amplify a 1.1 kb DNA fragment from maize genomic DNA containing the full length seed endosperm-specific 27 kDa zein gene (zmZ27; NCBI accession no. X53514) promoter (PzmZ27) (Russell and Fromm, 1997) for cloning purposes. The primers used for amplifying PzmZ27 were designed to produce restriction sites (underlined) specific for the enzymes indicated at the end of the primer name.

Primer name	Nucleotide sequence 5'→ 3'
manA-P1	ACAGCCACTCTCCATTCA
manA-P2	GTTTGCCATCACTTCCAG
PMCS5 For	GTTGTAAAACGACGGCCAG
Ppsy Rev	GGGATCTCTATAGCTGAAAC
Pzm27kD Rev	ACCCTTGACGTGCAATTTTG
Pzm cpt1	CTCAGGTTATTCGACGCTC
P10	AACCTCCTATAGACTAGGC
PZ27kD For (*Avr* II)	CAT<u>CCTAGG</u>AAAGCACCAGTTCAACGAT
PZ27kD Rev (*Pvu* II)	ATTT<u>CAGCTG</u>GGTTCTTCTGCGCTC

2.1.5 Bacterial strain and media

The Top 10 F' *Escherichia coli* strain (Invitrogen, genotype F' *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) was used for cloning.

The bacteria were cultured in LB medium containing appropriate antibiotics at suitable concentrations and incubated overnight at 37°C. The liquid cultures were shaken at 180 rpm.

LB liquid medium		LB solid plates		Antibiotic	End concentration
Bacto-Trypton	10 g	Bacto-Trypton	10 g	Ampicillin	100 µg/ml
NaCl	10 g	NaCl	10 g	Kanamycin	50 µg/ml
Yeast extract	5 g	Yeast extract	5 g	Spectinomycin	100 µg/ml
H$_2$O ad 1 l		Bacto Agar	12.5 g		
		H$_2$O ad 1 l			

2.1.6 Selectable marker genes for plant transformation

Marker gene	Function
aadA	Encodes aminoglycoside 3"-adenylyltransferase protein which transfers adenylyl group to spectinomycin and streptomycin converting them into inactive antibiotics (Goldschmidt-Clermont, 1991).
nptII	Encodes neomycin phosphotransferase protein which transfers a phosphate group to kanamycin leading to its inactivation (Wright and Thompson, 1999).
pmi (*manA*)	Encodes phosphomannose-isomerase enzyme which catalyses reversible conversion of mannose-6-phosphate into fructose-6-phosphate (Privalle, 2002).

2.1.7   Plasmid list

Plasmid name	Cloning strategy

pRB104
: ZmPrrnPEP+NEP-G10L-14aaGFP-nptII-petD3' cassette (for a physical map, see Fig. 6.3) containing the *nptII* selectable marker gene under control of the maize 16S ribosomal RNA operon promoter (Prrn) and *petD* gene terminator (from pMON49295, kindly provided by Monsanto, St. Louis, MO) was cloned into pBSII KS (BlueScript basic vector, NCBI accession no. X52327) as *EcoR* I/*Not* I. The Prrn promoter which has recognition sites for both the plastid-encoded RNA polymerase (PEP) and the nuclear-encoded RNA polymerase (NEP) (Hajdukiewicz *et al.*, 1997) was fused with the short leader sequence (g10L) of the gene 10 from bacteriophage T7. The g10L sequence (GAATTGTAGA AATAATTTTG TTTAACTTTA AG<u>AAGGAG</u>AT ATACC) contains a perfect plastid ribosome binding site (RBS) (nucleotides underlined) and has been documented as an excellent leader sequence for the expression of various heterologous proteins (Olins *et al.*, 1988). In addition the neomycin phosphotransferase enzyme (NPTII) was translationally fused with 14 N-terminal amino acids of the green fluorescent protein (GFP), which has been demonstrated to enhance translation rate in plastids (Maliga, 2003b).

pRB108
: DNA fragment containing the *aadA* selectable marker gene under control of a modified tobacco plastid ribosomal RNA operon promoter (Prrn) and the 3' region of the plastid *psbA* gene (*psbA*3') from pZS179 (Svab and Maliga, 1993) as *Kpn* I/*Dra* I was cloned into the plasmid pRB104 (*Kpn* I/*EcoR* V).

pZmC303PstI/SacI
: A 9.55 kb fragment of *Zea mays* chloroplast DNA (NCBI accession no. X86563) from *ndhB* gene to *rpl22* gene as *Pst* I/*Sac* I (nucleotides position: 81931…91488; for a physical map, see Fig. 3.11A) was cloned into pUC19 (NCBI accession no. X02514) (*Pst* I/*Sac* I).

pGPTVII.kan.gfp
: Kindly provided by the group of Jörg Kudla, University of Muenster, Germany. The plasmid is a derivative of pGPTV.kan (Becker *et al.*, 1992) in which the *uidA* gene is replaced by the *GFP* reporter gene.

pNOV2820   A vector kindly provided by Syngenta (Greensboro, NC) carrying the *pmi* (*manA*) gene from *E. coli* under the control of CMPS (Cestrium Yellow Leaf Curling Virus) promoter.

pPCVpsy   This vector was kindly provided by P. Beyer, University of Freiburg, in which the daffodil phytoene synthase (*PSY*) cDNA (NCBI accession no. X78814) under control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (*BamH* I/*EcoR* V) was cloned into vector pPCV812 (Koncz *et al.*, 1994) (*BamH* I/*Ecl136* II). The *PSY*-cDNA contains a 5' sequence coding for a functional plastid transit peptide (Bonk *et al.*, 1997).

pMA1   The pZmC303PstI/SacI "vector" was opened using *Nhe* I restriction site. The "insert" DNA fragment containing the *aadA* gene and *nptII* gene cassettes was excised from pRB108 (as *Acc65* I/*Ecl136* II). Both vector and insert DNA fragments were then blunted by Klenow polymerase treatment and ligated to each other (for a physical map, see Fig. 3.11A).

pMA2   The TP-gfp [transit peptide from Rubisco small subunit (RbcS) + *GFP* reporter gene] fragment from plasmid p35S-TP-GFP (from AG Bock plasmids) as *BamH* I/*Sac* I was cloned into vector pGPTVII.kan.gfp (*BamH* I/*Sac* I) (Fig. 3.25).

pMA3   An *EcoR* I/*Hind* III DNA fragment from pMA2 containing TP-gfp (transit peptide from RbcS + *GFP* reporter gene) was cloned into vector pGreen0029 (Hellens *et al.*, 2000) (*EcoR* I/*Hind* III) (Fig. 3.25).

pMA4   The daffodil phytoene synthase gene cDNA under control of CaMV 35S promoter was excised from pPCVpsy (*Hind* III/*Sac* II) and cloned into vector pMCS5 (Mo Bi Tec, Goettingen, Germany) (*Hind* III/*Sac* II).

pMA5   The CMPS+pmi+Tnos (including CMPS promoter, *pmi* gene sequence and nopaline synthase gene terminator) fragment from pNOV2820 (*Sac* II/*Sfo* I) was cloned into pMA4 (*Sac* II/*Hpa* I) (see Fig. 3.17).

pMA6   The 35S promoter of phytoene synthase (*PSY*) gene was removed (as *Avr* II/*Sma* I) from pMA5 and PCR-amplified 1.1 kb DNA fragment containing the full-length maize seed endosperm-specific 27 kDa zein gene (zmZ27; NCBI accession no. X53514) promoter (Russell and Fromm, 1997) was inserted upstream of the *PSY* gene (*Avr* II/*Pvu* II). The PCR was performed using PZ27kD-forward (*Avr* II) and PZ27kD-reverse (*Pvu* II) primer pair (see Fig. 3.17).

2.1.8 Plant material

Maize (*Zea mays* L.) F1 hybrid of Pa91 × H99 (unless otherwise stated) was used for optimizing tissue culture and transformation protocols as well as for modifying the carotenoid biosynthesis pathway. Four *Peperomia* species, *P. metallica*, *P. pedunculosa*, *P. argyreia* and *P. serpens*, were used to study plastid morphology. Plants were obtained from the Botanical Gardens Freiburg and Berlin. For comparison, wild-type tomato plants (*Solanum lycopersicum* cv. IPA-6) were analyzed.

2.1.9 Plant tissue culture stock solutions

10× RM Macro salts (Linsmaier and Skoog, 1965)

KNO_3	19 g
$MgSO_4 \times 7\ H_2O$	3.7 g
$CaCl_2 \times 2\ H_2O$	4.4 g
KH_2PO_4	1.7 g
$(NH_4)NO_3$	16.5 g
H_2O ad 1 l	

100× RM Micro salts (Linsmaier and Skoog, 1965)

$MnSO_4 \times 1\ H_2O$	1.69 g
H_3BO_3	620 mg
$ZnSO_4 \times 7\ H_2O$	860 mg
KJ	83 mg
$Na_2MoO_4 \times 2\ H_2O$	25 mg
$CuSO_4 \times 5\ H_2O$	2.5 mg
$CoCl_2 \times 6\ H_2O$	2.5 mg
H_2O ad 1 l	

10× ML1(N6) Macro salts (Chu *et al*., 1975)

$CaCl_2 \times 2\ H_2O$	1.66 g
KH_2PO_4	4 g
KNO_3	28.3 g
$MgSO_4 \times 7\ H_2O$	1.85 g
$(NH_4)_2SO_4$	4.63 g
H_2O ad 1 l	

100× ML1(N6) Micro salts (Chu *et al*., 1975)

H_3BO_3	160 mg
KJ	80 mg
$MnSO_4 \times 1\ H_2O$	333 mg
$ZnSO_4 \times 7\ H_2O$	185 mg
H_2O ad 1 l	

KT vitamins (Horn *et al*., 1983)

p-Aminobenzoic acid	5 mg
D(+)-Biotin	10 mg
Cholin chloride	10 mg
Folic acid	5 mg
Nicotinamide	20 mg
Pantothenate	10 mg
Pyridoxine hydrochloride	20 mg
Riboflavin	5 mg
Thiamine hydrochloride	10 mg
Cyanocobalamin	15 µg
H_2O ad 100 ml	

Hormone stock solutions

Hormone	Concentration	Dissolved in
IAA	1 mg/ml	0.1 M NaOH
NAA	1 mg/ml	0.1 M NaOH
BAP	1 mg/ml	0.1 M HCl
2,4-D	2.2 mg/ml	EtOH, ad H_2O
GA_3	0.1 mg/ml	H_2O
α-PSK	100 µM	H_2O

Vitamin stock solution was filter sterilized and stored at -70°C. To prepare 2,4-D stock solution, 220 mg powder was dissolved in 1 ml EtOH and the volume of solution was adjusted to 100 ml with pure water. Hormone stock solutions were filter sterilized and stored at 4°C, except α–PSK which was stored at -20°C.

2.1.10 Plant propagation and tissue culture media

Following media were used in most of the tissue culture and regeneration experiments (unless otherwise stated). For selection of transgenic cell lines following transformation, proper antibiotics (filter sterilized) were added into media after autoclaving under aseptic conditions.

<u>0.5× RM medium for maize seeds germination</u>

10× RM Macro salts	50 ml
100× RM Micro salts	5 ml
1% FeNaEDTA	2.5 ml
Sucrose	5 g
H_2O ad 1 l	
Adjust pH 5.8 with 2 M KOH	
Gelrite	2 g

<u>RM medium for *Peperomia* propagation</u>

10× RM Macro	100 ml
100× RM Micro	10 ml
1% FeNaEDTA	5 ml
Sucrose	30 g
H_2O ad 1 l	
Adjust pH 5.8 with 2 M KOH	
Micro Agar	5.6 g

These media were prepared in 1 liter bottles and heated by microwave (~10 min) to dissolve gelling agent. The medium was shaken carefully (to homogenize gelling agent), divided into Magenta tissue culture boxes (~50 ml per box) and autoclaved for 20 min at 121°C. For *Peperomia* medium, 1 ml of sterile (autoclaved) activated carbon (15% stock solution; 0.3% end concentration) was added into every box under sterile condition.

<u>ML1C2 maize callus culture medium</u>

10× ML1 macro salts	100 ml
100× ML1 micro salts	10 ml
1% FeNaEDTA	5 ml
KT vitamin stock	1 ml
Glycine (1 mg/ml stock)	2 ml
Myo-inositol	100 mg
L-proline	2880 mg
$MgCl_2 \times 6H_2O$	650 mg
Casein hydrolysate	100 mg
Sucrose	20 g
2,4-D (2.2 mg/ml stock)	1 ml

<u>ML1G1 maize callus induction medium</u>

10× ML1 macro salts	100 ml
100× ML1 micro salts	10 ml
1% FeNaEDTA	5 ml
KT vitamin stock	1 ml
Glycine (1 mg/ml stock)	2 ml
Myo-inositol	100 mg
L-proline	2880 mg
$MgCl_2 \times 6H_2O$	650 mg
Casein hydrolysate	100 mg
Sucrose	20 g
2,4-D (2.2 mg/ml stock)	1 ml

H$_2$O ad 1 l		H$_2$O ad 1 l	
Adjust pH 5.8, 2 M KOH		Adjust pH 5.8, 2 M KOH	
Gelrite	2.7 g	Gelrite	2.7 g
AgNO$_3$ (10 mg/ml stock)	1 ml	AgNO$_3$ (400 mg/ml stock)	2 ml
		Spermidine (1 M stock)	0.5 ml

Silver nitrate and spermidine were filter-sterilized and added to the medium after autoclaving. A highly concentrated silver nitrate stock solution (400 mg/ml) was used to prepare ML1G1 medium to avoid significant changes in the end volume and concentration of the medium. The medium was then poured into 100 mm × 15 mm Petri dishes (SARSTEDT, Nuembrecht, Germany) (~30 ml per plate) under aseptic conditions. Note that ML1C1 medium, which was used as basic medium to optimize the callus induction media in preliminary steps (see 3.1.1), was similar to ML1C2 medium except containing lower level of L-proline (690 mg/l).

ML1R3 maize regeneration medium		PIBG *Peperomia* regeneration medium	
10× ML1 macro salts	100 ml	10× RM macro salts	100 ml
100× ML1 micro salts	10 ml	100× RM micro salts	10 ml
1% FeNaEDTA	5 ml	1% FeNaEDTA	5 ml
KT vitamin stock	1 ml	Myo-inositol	100 mg
Glycine (1 mg/ml stock)	2 ml	Sucrose	20 g
Myo-inositol	100 mg	Thiamine (1 mg/ml stock)	1 ml
L-proline	690 mg	IAA (1 mg/ml)	150 μl
MgCl$_2$ × 6H$_2$O	650 mg	BAP (1 mg/ml)	3 ml
Casein hydrolysate	100 mg	H$_2$O ad 1 l	
Sucrose	20 g	Adjust pH 5.8, 2 M KOH	
BAP (1 mg/ml stock)	0.5 ml	Agar	5.8 g
H$_2$O ad 1 l		GA$_3$ (0.1 mg/ml stock)	70 μl
Adjust pH 5.8, 2 M KOH			
Gelrite	2.7 g		

Gibberellic acid (GA$_3$) was filter sterilized and added to the medium after autoclaving. The regeneration media were poured into 100 × 20 mm Petri Dishes (OPTILUX^{TM}, Becton Dickinson Labware, Bedford, MA) (~40 ml per plate) under aseptic conditions.

2.1.11 Growth condition

For callus induction in the dark, plates were incubated in Percival chambers (CLF Plant Climatics, Emersacker, Germany) with daily temperature cycles of 16 h at 25°C followed by 8 h at 20°C. For maize seed germination and plant regeneration from callus tissue, the Percival chamber was adjusted to a diurnal cycle of 16 h light (60 μE m^{-2} s^{-1}) at 25°C followed by 8 h dark at 20°C. Regenerated plantlets were cultured in soil and placed in growth chambers for two weeks (diurnal cycle: 16 h light of 120 μE m^{-2} s^{-1} at 25°C; 8 h dark at 20°C). Subsequently, they were transferred to the greenhouse (diurnal cycle: 16 h light of 250 μE m^{-2} s^{-1} at 22°C; 8 h dark at 20°C) for seed production. *Peperomia* and tomato plants were grown under a growth regime of 16 h light of 25 μE m^{-2} s^{-1} at 25°C followed by 8 h dark at 20°C.

2.2 Methods

2.2.1 Methods for plant tissue culture and transformation

2.2.1.1 Surface sterilization of maize seeds

For surface sterilization, maize seeds were incubated in 70% technical EtOH. After 10 min, seeds were washed once with sterile water and soaked for 30 min in 2.6% sodium hypochloride containing 0.1% tween® 20 (Roth, Karlsruhe, Germany). Seeds were then washed several times with sterile water and cultured in Magenta boxes containing 50 ml of 0.5× RM medium.

2.2.1.2 Induction of callus cultures from maize leaves in the dark and in the light

Surface-sterilized maize seeds were germinated on 0.5× RM medium (Murashige and Skoog, 1962) under aseptic conditions. When the seedlings were 5 to 10 cm high, shoot fragments of 1 to 2 cm were excised from the bottom part of the shoot (Fig. 3.1A). Following coleoptile removal, individual leaf segments were longitudinally sectioned with a sharp scalpel in 2 to 4 thin strips. The strips were then sliced in thin cross sections generating leaf pieces of approximately 1-2 mm × 1 mm. The leaf segments were subsequently transferred onto callus induction medium (ML1G1) (Fig. 3.1B). Induced embryogenic calli were then propagated on ML1C2 medium in the dark.

For callus induction in the light, leaf segments were cultured on ML1C2 medium complemented with 30 to 75 nM phytosulfokine-alpha (α-PSK; Matsubayashi, 1997). Silver nitrate was not added to the media in these experiments. The cultures were incubated in

Percival chamber with diurnal cycle of 16 h light (60 μE m^{-2} s^{-1}) at 25°C followed by 8 h dark at 20°C.

2.2.1.3   Callus induction from leaves of greenhouse-grown maize plants

For callus induction from leaves of greenhouse-grown plants, six-week old maize plants were harvested and the three to four outermost (oldest) leaves were removed. Subsequently, a ~10-20 cm section from bottom of the plant was excised and two additional old leaves were removed under sterile conditions. Then, leaves number seven, eight and nine were separated from the innermost (youngest) leaves and briefly surface sterilized with 70% ethanol (20-30 s) followed by washing with sterile water. 2 mm leaf strips were cut from the basal 2-3 cm of each leaf sample and sliced in pieces of 1-2 mm, thus producing leaf segments of roughly 2 mm × 2 mm. The leaf segments were then placed on ML1G1 medium and incubated in the dark.

2.2.1.4   Embryogenic callus induction from immature tassel in maize

To induce embryogenic calli from tassel tissue, seven-week old greenhouse-grown maize plants were harvested and surface sterilized as described under 2.2.1.3. Immature tassels (around 1 cm in size) were then excised, cut in 1 mm segments and cultured on ML1G1 medium in the dark.

2.2.1.5   Plant regeneration from embryogenic maize calli

Plant regeneration from callus tissue was induced in the light (diurnal cycle as described in 2.1.11) on regeneration medium ML1R3. Alternatively, ML1R4 medium (a modified ML1R3 medium in which BAP was replaced by 2 mg/l NAA) was used, which stimulates root and shoot formation simultaneously.

2.2.1.6   Biolistic transformation of maize plants

2.2.1.6.1   Gold particle preparation for biolistic transformation

For every single hepta-shot bombardment using a biolistic gun (BioRad, Salt Lake City, UT), 1.4-1.5 mg gold particles of 0.6 μm in diameter (BioRad) were washed with 140 μl pure (water-free) EtOH for 1 min (by vortexing vigorously) followed by centrifugation at 5000 rpm for 1 s. After removing the supernatant, the particles were washed once more with 1 ml H$_2$O. Note that all solutions used for gold particle preparation were cooled down (4°C) and all steps were carried out on ice.

<u>Precipitation of DNA on gold particles</u>

After gold particle cleaning, the plasmid DNA was precipitated onto the particles according to the following protocol:

- the washed gold particles were resuspended carefully in 172 µl H_2O
- the following substances were added into the test tube (after addition of each substance, the sample was briefly vortexed to keep the particles in suspension):

<u>Added amount</u>	<u>Concentration of the substance</u>
7-10 µl of plasmid DNA (~20 µg)	2-3 µg/µl
175 µl CaCl2	2.5 M stock
35 µl Spermidine (free base)	0.1 M stock

- particles were then incubated for 10 min on ice, and vortexed 1× every minute
- samples were centrifuged very briefly at 3500 rpm and the supernatant was discarded
- the pellet was resuspended in 600 µl EtOH followed by centrifugation at 5000 rpm for 1 s and removal of the supernatant. This step was repeated two times.
- the pellet was then resuspended in 50 µl of pure EtOH (sufficient for one hepta-shot)
- the particles were then mixed and 6.5 µl aliquots were spread carefully (by dropping from close distance using a micropipette) on macrocarriers (BioRad) which were placed in the holes of the hepta-adaptor after surface sterilization with EtOH.

2.2.1.6.2 Maize biolistic transformation

A filter paper (ashless; Schleicher&Schuell, Dassel, Germany) was placed on high osmotic medium, ML1TC1 (modified ML1C2 medium supplemented with 120 g/l sucrose). Propagated yellow embryogenic calli (3 weeks old) were collected, cut in small pieces with a sharp scalpel and spread on the filter paper. After half an hour incubation, the plates were bombarded with gold particles (0.6 µm in diameter) coated with the appropriate plasmid DNA using a helium-driven biolistic gun (PDS-1000/He; BioRad; for details on design, operation and performance, see Kikkert, 1993) and 1.100 psi (pounds-force per square inch) rupture disks. After transformation, the calli remained on high osmotic medium for one to three days and were then transferred onto selection medium containing the proper selection agent.

2.2.1.7 Selection of maize nuclear transgenic lines based on the PMI/mannose system

Phosphomannose isomerase (PMI), an enzyme not present in many plants, catalyzes the reversible inter-conversion of mannose-6-phosphate and fructose-6-phosphate. Plant cells

lacking this enzyme are incapable of surviving on synthetic medium containing mannose as the only carbon source. Thus PMI/mannose selection can be utilized in the identification of transformed plant cells (Privalle, 2002). To select maize nuclear transgenic lines expressing *pmi* following biolistic transformation using a plasmid carrying the *pmi* selectable marker gene, the calli remained on high osmotic medium for one to three days and were then transferred onto ML1TC2 medium, a modified ML1C2 medium in which sucrose is replaced with 10 g/l mannose and 20 mg/l glucose as described by Wright *et al*. (2001). After 4 weeks incubation on ML1TC2 medium without subculturing, surviving cell lines were transferred onto ML1TC3 medium which contains 15 g/l mannose as the only carbon source, but otherwise is identical with ML1TC2. Surviving colonies were transferred onto fresh ML1TC3 medium every two weeks. After two to four rounds of selection on ML1TC3, transgenic maize lines were isolated, propagated and callus samples were transferred onto ML1R3 regeneration medium. Multiple shoots could be regenerated from a single small callus piece on medium containing either mannose or sucrose. Regenerated plantlets were transferred into boxes containing 0.5× RM medium in which sucrose was replaced by mannose. After root formation, plants were transferred to the greenhouse for continued growth and seed production.

2.2.1.8 Chlorophenol red assay

An assay using the pH indicator chlorophenol red (CPR; Kramer *et al.*, 1993; Wright *et al.*, 2001) was used to test for *pmi* gene expression in candidate transgenic lines. Small callus pieces from lines growing on mannose-containing medium were placed in wells of microtiter plates that contained ML1C2 callus culture medium with 50 mg/l CPR dye and supplemented with either 5 g/l mannose or 10 g/l sucrose (as a control). The pH of the medium was adjusted to 6.0 at which the color of the medium is deep red. Cells capable of metabolizing the respective sugar will acidify the medium resulting in a color change from red to yellow. Plates were incubated in the dark for 2-3 days and then visually inspected for color change of the medium.

2.2.1.9 Growth test to confirm stable inheritance of the *pmi* gene into next generation

To confirm stable *pmi* transgene inheritance into the T1 generation, intact embryos were isolated from mature seeds of selfed T0 plants using a sharp scalpel. The endosperm-free embryos were then cultured in Magenta boxes containing 0.5× RM salts supplemented with 10 g/l mannose and solidified with 2 g/l gelrite. Whereas mannose-metabolizing transgenic

embryos can grow under these conditions, the wild type embryos cease to grow on this medium.

2.2.2 Molecular biology work with nucleic acids

2.2.2.1 Preparation of bacterial competent cells

2.2.2.1.1 *E. coli* heat-shock-competent cells

Heat-shock competent cells of *E. coli* were prepared based on the method described by Hanahan (1985).

Required solutions

RF1		RF2	
100 mM	RbCl	10 mM	RbCl
50 mM	$MnCl_2 \times 4\ H_2O$	10 mM	MOPS (pH 6.5 with KOH)
30 mM	KAc	75 mM	$CaCl_2$
10 mM	$CaCl_2$	15%	Glycerol (v/v)
15% Glycerol (v/v)		pH 6.5 with KOH	
pH 5.8 with acetic acid			

Procedure

- 5 ml LB medium was inoculated with a single *E. coli* Top10 F' colony followed by shaking at 180 rpm, 37°C for 8 h
- 30 µl bacteria culture was added into 30 ml LB medium and incubated overnight at 37°C with shaking at 180 rpm
- 2 ml of overnight culture was poured into a 1 liter flask containing 200 ml LB medium and bacteria were grown until OD_{578}=0.3-0.4
- after 10 min incubation on ice, the cells were collected by centrifugation for 10 min at $2500 \times g$, 4°C
- the bacterial pellet was resuspended in 18 ml cold RF1 solution followed by incubation on ice for 30 min
- the cells were centrifuged for 10 min at $2500 \times g$, 4°C
- the pellet was resuspended in 4 ml solution RF2; aliquots of 100 µl were frozen in liquid nitrogen and stored at -70°C

2.2.2.1.2 *E. coli* electroporation-competent cells

The procedure is similar to the production of heat-shock competent cells with a few modifications: all centrifugation steps were performed at 4000-4500 × g. In addition, the wash steps were performed using 30 ml H_2O and 6 ml of 10% glycerin instead of the RF1 and RF2 solutions, respectively. Finally, the cells were resuspended in 300 µl of 10% glycerin; aliquots of 50 µl were frozen and stored at -70°C.

2.2.2.2 Transformation of bacterial cells

2.2.2.2.1 Heat-shock transformation

For heat-shock transformation, 10 to 50 ng of plasmid DNA (for retransformation) or half of the ligation reaction (7.5 µl) was added to *E. coli* competent cells followed by incubation on ice for 30-60 min. The sample was then subjected to 42°C for 42 s and incubated on ice for 2 min. After addition of 1 ml LB medium, the sample was shaken at 180 rpm at 37°C for 1 h. Then, 20 to 200 µl of transformed bacterial cells were plated on solid LB medium containing appropriate antibiotics and incubated at 37°C overnight.

2.2.2.2.2 Transformation of *E. coli* cells via electroporation

In this method, salt removal from DNA solution is essential because high salt concentrations present in ligation reactions are known to reduce electroporation efficiency. For this purpose a MF-Millipore membrane filter (Millipore, Eschborn, Germany) (0.025 µm in diameter pores) was placed on pure water filled in a well of a microtiter plate. The ligation reaction was placed on the membrane filter for 30 min and then used for electroporation. Transformation was performed in sterile 2 mm electrocuvettes (Peqlab, Erlangen, Germany) using a Multiporator (Eppendorf, Hamburg, Germany) with 2500 mV for 5 ms.

2.2.2.3 Isolation of nucleic acids

2.2.2.3.1 Plasmid DNA isolation

This method, which is also called "plasmid DNA minipreparation", is based on a protocol developed by Birnboim and Doly (1979). The bacteria were lysed by treatment with a solution containing SDS and NaOH. SDS denatures proteins while NaOH denatures chromosomal and plasmid DNA. This mixture is neutralized by addition of potassium acetate, leading to a fast renaturation of the plasmid DNA but not of bacteria chromosomal DNA.

The extracted plasmid DNA can be used for restriction with endonucleases and further cloning steps such as ligation of DNA fragments into vectors and transformation of bacterial

cells with plasmid DNA. For experiments (e.g. biolistic transformation) requiring purer DNA, the plasmid DNA isolation was performed using Qiagen Plasmid Purification kit or Nucleobond® AX kit (anion exchange columns) following the manufacturer's instructions.

2.2.2.3.2 Isolation of nucleic acids from maize plant tissues

The method optimized by Doyle and Doyle (1990) was used to extract total genomic DNA from maize tissues. This method is based on the lysis of the plant cell by CTAB, an ionic detergent, and cleaning of the nucleic acids using chloroform/isoamyl alcohol (Ch/I) extraction followed by isopropanol precipitation. The extraction buffer consists of 2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8, 100 mM Tris/HCl pH 8 and 100 mM β-mercaptoethanol. Usually, nucleic acids isolated based on the CTAB method contain both DNA and RNA. To remove RNA, RNase A (DNase-free) was added to the samples and incubated at 37°C for 15-30 min followed by phenol/chloroform (Ph/Ch) extraction and EtOH precipitation.

This method provides nucleic acids that can be used for PCR, Southern blotting and further DNA techniques.

2.2.2.3.3 RNA isolation from maize tissues

Total cellular RNA was extracted from leaves of transgenic lines and wild-type plants with the peqGOLD TriFast™ reagent (Peqlab, Erlangen, Germany) (Chomczynski and Sacchi, 1987; Chomczynski, 1993) following the manufacturer's instructions.

2.2.2.4 Determination of nucleic acids concentration

The concentration of nucleic acids was determined by optical density measurement. A solution of double-stranded DNA (dsDNA) in a 10 mm path-length cell with an optical density of 1.0 has a concentration of 50 µg/ml (40 µg/ml for RNA). Nucleic acids can be quantified at 260 nm. In addition, optical densities for three further wavelengths (230, 280 and 320 nm) are measured as reference points for the purity of the nucleic acids. The extraction of nucleic acids from cells is usually accompanied by low-level contamination with protein. The 260 nm/280 nm ratio gives an indication of the purity of the sample. Pure DNA and RNA preparations have ratios of 1.8-2.0 and 1.9-2.1, respectively. Deviations from these ratios indicate the presence of impurities in the sample. An elevated absorbance at 230 nm can indicate the presence of impurities as well. 230 nm is near the absorbance maximum of peptide bonds and also indicates buffer contamination since Tris, EDTA and other buffer salts absorb at the same wavelength. When measuring RNA samples, the 260 nm/230 nm ratio

should be > 2.0. The optical density at 320 nm shows the effects of turbidity in the measuring solution and in pure samples, it should be close to zero.

2.2.2.5 Precipitation and purification of nucleic acids

Ethanol (EtOH) or isopropanol precipitation methods were performed to concentrate nucleic acids and/or remove salts that could disturb subsequent steps. In the EtOH precipitation procedure, 0.1 volume of 3M sodium acetate (pH 4.8) (unless the solution contains salts) and 2.5 volumes of 100% EtOH were added. After each step, the samples were mixed carefully by inverting. In the isopropanol procedure, 0.7-1 volume of isopropanol was added followed by mixing. In both methods, nucleic acids were precipitated by a subsequent centrifugation step at 12000 rpm for 20-30 min followed by a wash step using 70% EtOH. The pellet was then air-dried and dissolved in pure water.

2.2.2.6 Phenol/chloroform/isoamyl alcohol (Ph/Ch/I) extraction of nucleic acids

The standard method to remove proteins from nucleic acid solutions was the extraction with Ph/Ch/I (25/24/1). Generally, 1 volume of Ph/Ch/I was added to the samples followed by mixing thoroughly in order to reach the emulsion consistency. After centrifugation, the aqueous phase (containing the nucleic acids) was transferred into a fresh test tube followed by EtOH precipitation.

2.2.2.7 Digestion of the nucleic acids

Digestion with restriction enzymes

Digestion of plasmid DNA or plant genomic DNA was performed by appropriate endonucleases in the suitable buffer under conditions recommended by the manufacturer.

Nonspecific cleavage of DNA

DNase I (RNase-free), an endonuclease that nonspecifically cleaves DNA, was used to remove DNA molecules from extracted RNA samples. The RNA pellet was dissolved in $1\times$ DNase I buffer (40 mM Tris/HCl pH 7.5, 6 mM $MgCl_2$, 1 mM DTT) followed by addition of 1-2 units of DNase I enzyme. The samples were then incubated at 37°C for 10-20 min followed by Ph/Ch/I extraction and EtOH precipitation.

Nonspecific cleavage of RNA

To remove RNA from DNA samples, RNase A (DNase-free) was added to the DNA samples. The reaction was then incubated at 37°C for 10-30 min followed by Ph/Ch/I extraction and EtOH precipitation. Note that RNase A is active in most restriction enzyme buffers.

2.2.2.8 Ligation of DNA fragments

T4 DNA ligase catalyzes the formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA. Since base pairing is only possible between complementary sequences, "sticky ends" produced by one restriction enzyme cannot pair with ends produced by another enzyme, unless the other enzyme produces the same sticky end. Two arbitrary "blunt-end" fragments can be ligated independently of their origin with each other. To ligate two DNA fragments, the following reaction mixture was prepared in a test tube and incubated at 16°C overnight

Linear vector DNA	100-300 ng
Insert DNA	1:1 to 3:1 molar ratio of insert DNA termini to vector DNA
10× ligation buffer for T4 DNA Ligase	1.5 µl
T4 DNA Ligase	0.2-0.4 µl (1-2 U) for sticky ends, 1 µl (5 U) for blunt ends
H_2O	ad 15 µl

2.2.2.9 Fill-in reaction with Klenow DNA polymerase

The Klenow fragment (the large fragment of DNA polymerase I from *E. coli*) (MBI Fermentas) was used to fill single stranded termini of double-stranded DNA fragments to produce blunt ends. The Klenow polymerase I exhibits 5'→3' polymerase activity and 3'→5' exonuclease (proofreading) activity, but lacks the 5'→3' exonuclease activity of DNA polymerase I. After digestion of DNA, the restriction endonuclease was inactivated by heating (according to the manufacturer's advice). Then, 1 µl of a dNTP mix (2.5 mM each) and 2 units of Klenow fragment were added to the sample and incubated at 25°C for 15 min. The enzyme was then inactivated by adding 10 mM EDTA followed by heating at 75°C for 20 min.

2.2.2.10 Electrophoresis of nucleic acids

2.2.2.10.1 DNA gel electrophoresis

Depending on DNA fragment sizes, 0.8 to 1.5% agarose gels were used to separate DNA fragments after digestion with restriction enzymes or to check the quality of extracted genomic DNA. 1 µl of 10 mg/ml ethidium bromide (EtBr) stock solution was added to 100 ml of melted agarose in 1× TAE buffer (40 mM Tris, 0.11% acetic acid (v/v), 1 mM Na_2EDTA) to stain DNA molecules. EtBr is a fluorescent dye that intercalates between bases of DNA and RNA and can be visualized by exposure to UV light. The DNA samples were mixed with loading buffer (0.2% bromophenol blue, 48% glycerin, 0.2 M Na_2EDTA, pH 8.0) and

separated at 70-90 V in 1× TAE buffer followed by visualization of DNA bands by exposure to UV light.

2.2.2.10.2 RNA gel electrophoresis

10× MOPS buffer		Loading buffer	
0.2 M	MOPS	50%	Formamide, deionized
0.05 M	NaAc	6.5%	Formaldehyde
0.01 M	EDTA	20%	Glycerin
pH 5.5-7.0		1.25 mM	EDTA, pH 8.0
		0.005%	Ethidium bromide
		0.2%	Xylencyanol
		0.2%	Bromophenol blue
		in 1× MOPS buffer	

Procedure:

Generally, 1% formaldehyde gel consists of 10 ml 10× MOPS buffer, 87.5 ml H_2O and 1 g agarose. The agarose was melted and cooled to 50-60°C followed by addition of 2.5 ml formaldehyde (37%). RNA samples were mixed with 3 volumes of loading buffer, denatured by incubation at 95°C for 5-10 min and loaded on the gel in 1× MOPS buffer at 70-90 V.

2.2.2.11 DNA purification from agarose gel

GFX™ PCR DNA and Gel Band Purification kit (Amersham) or Nucleospin Extract II kit (Macherey-Nagel) were used to elute DNA from agarose gels. A chaotropic agent which is the main substance of the "capture buffer" of the kit, dissolves agarose, denatures proteins and promotes the binding of dsDNA to a glass fiber matrix. Afterwards, a wash step follows with which protein and salt impurities are removed. The DNA was purified from agarose gels according to the manufacturer's protocol and eluted in water.

2.2.2.12 Polymerase chain reaction (PCR)

To confirm the physical presence of transgenes or amplify maize genomic DNA fragments, PCR reactions with specific primers were performed. In 50 µl reactions, 50 ng total genomic DNA was amplified in a reaction mixture containing 200 µM of each dNTP, 2.0 mM $MgCl_2$, 80 ng of each primer and 2 U *Taq* DNA polymerase (Promega). The standard PCR program was 30 cycles of 30 s at 95°C, 30 s at 60°C (variable depending on the primer pair) and 45 s at 72°C with a 3 min extension of the first cycle at 95°C and a 6 min final extension at 72°C. PCR products were analyzed by electrophoretic separation in 1% agarose gels. For amplification of the maize endosperm-specific zein (27 kDa) promoter, 0.5 U *Deep Vent*®

DNA polymerase (which performs proofreading activity during DNA polymerization) was added to the reaction to achieve error-free amplification. The PCR program for this experiment was 30 cycles of 60 s at 95°C, 60 s at 58°C and 1 min and 30 s at 72°C with a 3 min extension of the first cycle at 95°C and a 6 min final extension at 72°C.

2.2.2.13 Southern blot analysis

Required solutions

Southern I	Southern II	Southern III	Southern IV
0.25 M HCl	0.5 M NaOH	0. 5 M NaOH	1 M Tris
		1.5 M NaCl	3 M NaCl
			pH 6.5 with HCl

Southern blot procedure was developed by Edward M. Southern (Southern, 1975). It allows determination of the molecular weight of a restriction fragment, to measure relative amounts in different samples and to detect a particular sequence of DNA within a complex mixture. Generally, 5-10 µg DNA was digested with proper restriction enzymes, separated by gel electrophoresis in 0.8-1% agarose gels (depending on the fragment sizes), denatured by incubation in NaOH-containing solutions (Southern I, II, III and IV, for 15, 30, 30 and 15 min, respectively) and transferred onto Hybond nylon membranes (Amersham) by capillary blotting using standard protocols. The membrane was then illuminated with UV light at 0.120 Joules cm^{-2} (using a UV-Crosslinker; Peqlab, Erlangen, Germany) leading to covalent binding of the DNA to the membrane.

2.2.2.14 Northern blot analysis

The procedure for Northern blots is the same as for Southern blots with the difference that the RNA gel blot does not need the denaturation step (RNA molecules are single stranded). 5-10 µg of total cellular RNA samples were separated by electrophoresis in formaldehyde-containing 1% agarose gels and directly blotted onto Hybond nylon membranes (Amersham).

2.2.2.15 Preparation of radioactively labeled probes

A probe is a nucleic acid fragment [purified (as described in part 2.2.2.11) as a restriction fragment or PCR product] which can be used to prove the presence of certain sequences in a mixture of DNA or RNA fragments by complementary interaction. Probes were labeled with the isotope ^{32}P using Megaprime™ DNA labeling system kit (Amersham) according to the manufacturer's protocol.

2.2.2.16 Hybridization of membranes with radioactively labeled DNA probes

Hybridizations were performed at 65°C in Rapid-Hyb buffer (Amersham) following the manufacturer's protocol. Nonspecifically bound probe was then removed by the following wash steps: 20 min with solution I (2× SSC, 0.1% (w/v) SDS) at room temperature and twice 10 min with solution II (0.5× SSC, 0.1% (w/v) SDS) at 65°C. The membranes were then exposed to X-ray films to detect radioactive signals. Alternatively membranes were exposed to phosphoscreens (Amersham) followed by radioactive signal detection using the Typhoon™ TRIO⁺ scanner (Amersham).

2.2.3 Isolation of carotenoids and chlorophyll from maize leaves

Total pigments were isolated from leaves (at the same developmental stage) of 3-week old greenhouse-grown maize plants. Generally, frozen leaf materials were grinded and 50 to 100 mg (fresh weight) was dried by speed vacuum (Eppendorf) for 30 min at 30°C. After determining dry-weight (DW), the samples were suspended in 500 µl of 80% acetone (extraction buffer), mixed vigorously for 15 min, centrifuged for 10 min at high speed (12000 ×g) and the supernatant was transferred into a fresh test tube followed by incubation on ice. The extraction was repeated using 500 µl of 100% acetone. Both extracts were mixed, centrifuged and ~100 µl of the supernatant was filtered (0.2 µm filter, Schleicher&Schuell, Dassel, Germany; 1 ml syringe, Roth, Germany) into a HPLC column (Agilent, Waldbronn, Germany). The samples were then used for quantification of carotenoids and chlorophylls by HPLC.

2.2.4 Quantification of carotenoids and chlorophyll contents by HPLC analysis

An Agilent 1100 Series HPLC (High-Performance Liquid Chromatography) system (Agilent, Waldbronn, Germany) with a diode array detector (DAD) was used for separation, identification and quantification of the isolated carotenoids and chlorophylls. The separation is based on the method described by Thayer and Björkman (1990) with the following modifications: solvent A consisted of acetonitrile, methanol and 0.1 M Tris HCl pH 8 (72:8:3 respectively). Solvent B contained methanol and ethyl acetate (68:32) and the flow rate was 0.75 ml/min. Pigments were eluted by 100% solvent A for 5 min followed by a shift to 100% solvent B for 20 min. Prior to the next run, the columns were recalibrated with the solvent A for 10 min. All pigments were quantified against known amounts of standards including Neoxanthin, Violaxanthin, Antheraxanthin, Zeaxanthin (CaroteNature, Lupsingen, Switzerland), Lutein, Chlorophyll B, Chlorophyll A and β-carotene (Sigma, Munich,

Germany). The carotenoid and chlorophyll contents were then calculated on the dry-weight bases. For each plant line 3 biological and 3 technical replicas were performed.

2.2.5 Microscopy

In order to study chloroplasts in different cell types, thin cross sections of 50 to 100 μm were prepared from young leaves using a Leica vibrotome (Leica microsystems, Wetzlar, Germany) in water. Samples were analyzed by light microscopy with an Olympus BX41 high resolution light microscope (Olympus America Inc, Melville, NY) connected to an Olympus U-CMAD3 microscopy camera. The cell^P imaging software (Olympus) was used to capture light microscopy images. A Leica TCS SP2 spectral laser-scanning confocal microscope (Leica microsystems, Wetzlar, Germany) was used to analyze chlorophyll autofluorescence (excitation: 543 nm, emission: 580 nm) in leaf cells.

2.2.6 Staining techniques

The starch-staining iodine solution (2% potassium iodide, 1% iodine) was used to label amyloplasts (starch-storing) in root cells. Thin cross sections of root tissues were studied first by light microscopy to visualize amyloplasts without any treatment. To confirm these observations, same samples were stained with iodine solution: small amount of the solution was injected between glass slide and cover slip and washed immediately with water. After removing iodine completely, the samples were used for microscopic studies.

2.2.7 Analysis of plastid morphology

To measure chloroplast and amyloplast size, photographs taken from thin cross sections using high-resolution light microscopy were used. 100-330 chloroplasts and 150-250 amyloplasts were randomly selected and their diameter was measured. Light microscopy of thin leaf cross sections was also employed to determine chloroplast numbers in leaf palisade and spongy parenchyma cells. Chloroplasts were counted in 30 to 90 cells per species. Finally, around 25 palisade cells were randomly selected, their length and width were measured and the cell area was calculated. All microscopic analyses were done in at least three biological replicas.

3 Results

3.1 Development of novel transformation technologies in maize

3.1.1 Development of an efficient leaf-based callus induction and plant regeneration system for maize

All initial experiments towards establishing protocols for callus induction and regeneration were performed using the Pa91 × H99 hybrid. Callus induction was achieved on a modified N6-based tissue culture medium (Chu *et al.*, 1975) containing KT vitamins (Horn *et al.*, 1983) and a number of additional ingredients (see 2.1.10). An approximately 1-2 cm long section at the leaf base of young seedlings (Fig. 3.1A) was identified as best responsive to callus induction (Fig. 3.1D and Fig. 3.2). The leaf segments were longitudinally sectioned in thin strips. These leaf strips are yellowish at the bottom and light green at the top reflecting the developmental gradient in plastid differentiation from etioplasts to chloroplasts. The strips were then sliced in thin cross sections and pieces of approximately 1-2 mm × 1 mm were determined as the optimum size of the segments for callus induction from leaves (Fig. 3.1B-C). A number of different chemicals were tested for their influence on callus induction from leaves, including the growth regulator salicylic acid, known modifiers of chromatin structure (sodium butyrate) and DNA methylation (5-azacytidine) as well as the polyamine spermidine (Table 3.1).

Table 3.1 Chemicals tested at different concentrations for their influence on callus induction from maize leaves

Medium	Chemical	End concentration	Medium	Chemical	End concentration
ML1A1	Salicylic acid	10^{-12} M	ML1A9	Spermidine	0.2 mM
ML1A2	Salicylic acid	10^{-9} M	ML1A10	Spermidine	0.5 mM
ML1A3	Salicylic acid	10^{-6} M	ML1A11	Spermidine	0.7 mM
ML1A4	Salicylic acid	5×10^{-12} M	ML1A12	Spermidine	1.0 mM
ML1A5	Sodium butyrate	1 mM	ML1A13	5-Azacytidine	1 μM
ML1A6	Sodium butyrate	5 mM	ML1A14	5-Azacytidine	10 μM
ML1A7	Sodium butyrate	10 mM	ML1A15	5-Azacytidine	50 μM
ML1A8	Spermidine	0.1 mM	ML1A16	5-Azacytidine	100 μM

The basic medium was ML1C1 which differs from ML1C2 medium (for composition, see 2.1.10) in that it contains 690 mg/l L-proline. All chemicals were filter-sterilized and added to the media after autoclaving.

In these experiments, the addition of 0.5-1 mM spermidine was found to have a strong stimulatory effect on embryogenic callus induction from leaf segments while other chemicals either did not influence or inhibited callus induction (Table 3.2).

Table 3.2 Effects of different chemicals on total (the percentage of induced calli from cultured leaf segments of a single seedling) and embryogenic (the percentage of regenerable calli from total calli induced) callus induction from leaf segments of young maize seedlings. Results are represented as mean ± standard deviation.

Medium	Callus induction (%)	Embryogenic callus (%)	Medium	Callus induction (%)	Embryogenic callus (%)
ML1C1	36.57 ± 6.14	2.78 ± 2.26	ML1A9	49.64 ± 4.08	4.61 ± 1.13
ML1A1	35.28 ± 7.84	3.70 ± 2.21	ML1A10	40.12 ± 5.87	10.83 ± 4.10*
ML1A2	33.37 ± 0.48	0	ML1A11	33.23 ± 2.94	12.49 ± 1.79*
ML1A3	29.33 ± 2.13	0	ML1A12	36.03 ± 5.39	11.60 ± 1.69*
ML1A4	29.45 ± 6.05	0	ML1A13	55.04 ± 3.69	2.94 ± 1.58
ML1A5	34.45 ± 1.62	5.34 ± 2.18	ML1A14	2.56 ± 1.09	0
ML1A6	0	0	ML1A15	0	0
ML1A7	0	0	ML1A16	0	0
ML1A8	48.69 ± 4.62	5.83 ± 2.25			

ML1C1 medium was used as control. Silver nitrate was added to all media at the same concentration of 10 mg/l. Each medium included at least three replicas. The media ML1A1-16 contain ML1C1 components supplemented with various chemicals at different concentrations as described in Table 3.1. The values labeled by "*" show a statistically significant (P value < 0.05) increase in comparison to the control, as revealed by the T-test.

An elevated level of L-proline was found to positively affect callus induction and embryogenic callus formation (Table 3.3) and therefore, a high L-proline concentration was applied in all further callus induction media.

Table 3.3 Effects of L-proline concentration on callus induction from maize leaf segments

	690 mg/l L-proline (control)		2880 mg/l L-proline	
Medium	Callus induction (%)	Embryogenic callus (%)	Callus induction (%)	Embryogenic callus (%)
ML1C1	36.57 ± 6.14	2.78 ± 2.26	42.38 ± 3.41	4.87 ± 1.90
ML1A3	29.33 ± 2.13	0	48.58 ± 3.51*	6.38 ± 1.84*
ML1A10	40.12 ± 5.87	10.83 ± 4.10	43.78 ± 4.19	10.31 ± 1.04

Note that sodium butyrate- and 5-azacytidine-containing media did not induce callus formation from leaf pieces at both L-proline levels. "*": statistically significant (P value < 0.05) increase in comparison to the control.

Furthermore, the effects of a pretreatment of the leaf explants with osmotic medium on callus induction were tested. For osmotic treatment, leaves from young seedlings were cut into small pieces (for description, see 2.2.1.2) in a liquid ML1C2 medium containing 0.7 M mannitol. The leaves were then cultured on solid ML1C2 medium without mannitol. This treatment did not support better callus formation from leaf explants (Table 3.4) and therefore, was not performed in other experiments.

Table 3.4 Effects of pretreatment of leaf explants with mannitol-containing (0.7 M) liquid medium prior to culturing leaf pieces onto callus induction medium

ML1C2 (control)		Mannitol treatment	
Callus induction (%)	Embryogenic callus (%)	Callus induction (%)	Embryogenic callus (%)
42.38 ± 3.41	4.87 ± 1.90	44.59 ± 2.90	3.61 ± 2.61

In addition, the silver nitrate concentration was found to affect callus induction in the dark. Generally, higher concentrations of silver nitrate resulted in a better response to callus induction from leaf segments. Even very high concentrations (1 g/l) were not toxic to the leaf explants (Table 3.5).

Table 3.5 Effects of silver nitrate on callus induction from young leaf segments

Silver nitrate concentration (mg/l)	Callus induction (%)	Embryogenic callus (%)
10 (ML1C2)	49.88 ± 2.69	4.07 ± 1.00
20	43.46 ± 5.37	3.70 ± 2.01
50	69.38 ± 5.85*	2.99 ± 0.23
100	68.05 ± 4.47*	4.14 ± 0.60
250	60.42 ± 5.35*	3.67 ± 0.48
500	61.36 ± 1.61*	5.49 ± 1.17
1000	62.76 ± 1.58*	4.09 ± 0.17

ML1C2 medium containing 10 mg/l silver nitrate was used as control. Note that silver nitrate was filter-sterilized and added to the media after autoclaving. The values labeled by "*" showed statistically significant (P value < 0.05) increase compared to the control, as revealed by the T-test.

The optimum concentrations for silver nitrate and spermidine were then determined as 800 mg/l and 0.5 mM, respectively. The optimized medium, which was called ML1G1 (see 2.1.10), gives rise to callus development and embryogenic callus formation from leaf segments at high frequency (on average 65% of all cultured leaf pieces displayed callus induction and the fraction of embryogenic calli reached up to 16%) (Fig. 3.1D-E and Fig. 3.2)

Phytosulfokine-alpha (α-PSK), a recently discovered peptide hormone (Matsubayashi *et al.*, 1997; Matsubayashi *et al.*, 2002), was also tested for its effects on callus induction in the dark. In these experiments, addition of α-PSK at nanomolar concentrations (10–75 nM) to both the ML1C2 and the optimized ML1G1 media did not influence callus induction significantly (Fig. 3.2). However, callus growth was slightly promoted in PSK-containing media (Fig. 3.2).
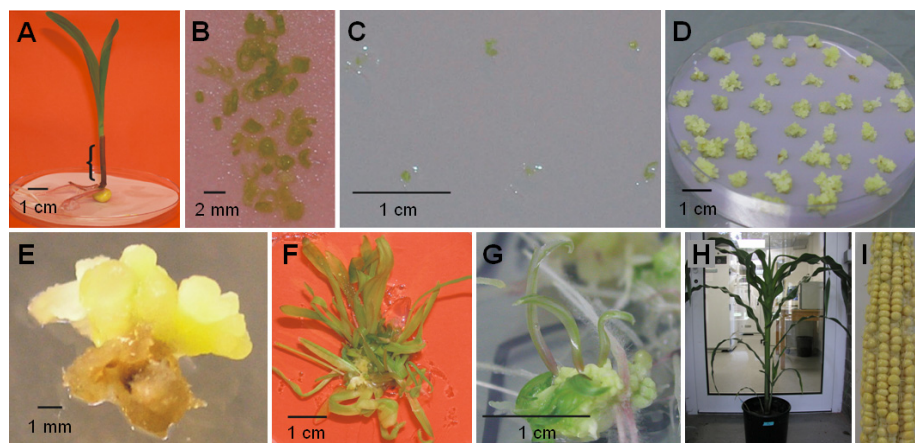
Figure 3.1 Callus induction from young maize leaves and plant regeneration. (**A**) Excision of regenerable leaf tissue from young maize seedlings grown in sterile culture. The segment used to establish callus cultures from leaf pieces is indicated by the brace. (**B**) Cut-up leaf pieces prior to their exposure to callus-inducing medium. (**C**) Leaf pieces placed on callus induction medium ML1G1. (**D**) Callus induction after five weeks incubation of leaf pieces on medium ML1G1 in the dark. A progressive series of leaf pieces from a young leaf of a sterile maize seedling is shown. The series starts with pieces from the leaf base (upper right corner) and proceeds towards the leaf tip (lower left corner). (**E**) Close-up of a type I-like yellow callus induced from leaf segments of young maize plants. The brown tissue at the bottom is the leaf piece from which the callus was induced. (**F**) Shoot induction from leaf-derived maize calli. Callus pieces were placed on medium ML1R3 and incubated for four weeks in the light. (**G**) Shoot and root initiation from leaf-derived calli. Callus samples were incubated for three weeks in the light on medium ML1R4. (**H**) Growth of a regenerated maize plant to maturity in the soil. (**I**) Seed production from regenerated maize plants. Regenerated plants that have passed through the regeneration procedure are fertile and seed production is indistinguishable from plants that were obtained directly from seeds.

Callus induction is most efficient from the basal 1-2 cm segment of the leaf and decreases towards the leaf tip as the tissue becomes more differentiated. Visible callus growth occurred after 4-6 weeks of incubation of the leaf segments on callus-inducing medium in the dark. In general, two distinguishable callus types are induced: (i) light-yellow, friable, non-embryogenic callus with a tendency towards early differentiation into roots and (ii) a dense dark yellow embryogenic type I-like callus which is highly regenerable and produces fertile plants (Fig. 3.1E-I). This callus type maintained its embryogenic potential and regeneration capacity over at least one year, without the appearance of phenotypic abnormalities in regenerated plants. The optimized medium (ML1G1) produced the highest amount of the desirable yellow type I-like callus. These calli can be easily further propagated and large amounts of callus material can be readily generated in a short time. This simple way of rapidly establishing a fresh and highly regenerable maize callus culture at any time (without being dependent on immature embryos and greenhouse facilities) thus provides a cost-effective and time-saving alternative to existing *in vitro* culture systems for maize (Ahmadabadi *et al.*, 2006).
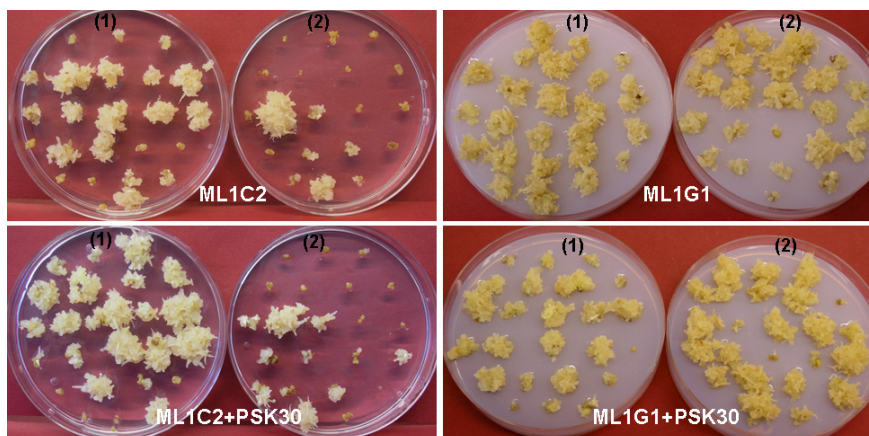
Figure 3.2 Effects of α-PSK on callus induction from maize leaves in the dark. Callus induction after six weeks incubation of leaf pieces on ML1C2 (left side) and ML1G1 (right side) media without (upper row) or with (lower row) application of α-PSK (30 nM is shown as example). Note that the leaf pieces and calli on two plates (labeled with number 1 and 2) are from a single maize seedling and a progressive series of leaf pieces is shown. The series starts with segments from the leaf base (upper right corner of plate 1) and proceeds towards the leaf tip (lower left corner of plate 2).

Various hormones, such as 2,4-D (0.5 mg/l), BAP (0.5 mg/l), NAA (2 mg/l), IBA (0.5 mg/l), zeatin (2 mg/l) and a combination of IBA and BAP (0.5 and 0.3 mg/l, respectively) were tested for their effects on regeneration of leaf-derived calli. Small pieces of embryogenic calli were regenerated to multiple shoots on medium containing 0.5 mg/l BAP (ML1R3; see 2.1.10) at high frequency (100%) (Fig. 3.1F). Regenerated shoots were able to form roots on hormone-free RM medium (see 2.1.10) at high efficiency. When BAP was replaced with 2 mg/l NAA (the medium was called ML1R4), both shoots and roots were initiated simultaneously (Fig. 3.1G). However, the shoot initiation frequency was higher on ML1R3 medium and therefore, it was used in further experiments as the regeneration medium. In addition, several methods including a three-step regeneration system (Armstrong, 1994), were tested to regenerate plants from non-embryogenic calli, and in some cases, greening of the apical callus regions was observed. However, regeneration into plants was not feasible.

In preliminary experiments, the genotype dependency of the leaf-based tissue culture system was assessed. While embryogenic calli could not be obtained from leaf bases of several commercial maize varieties (Fig. 3.3), regeneration-competent callus induction from leaves and subsequent shoot initiation from callus tissue was obtained for several other genotypes, including the two parental lines of the hybrid (Pa91 and H99) and a commercial variety of sweet corn (Fig. 3.4).
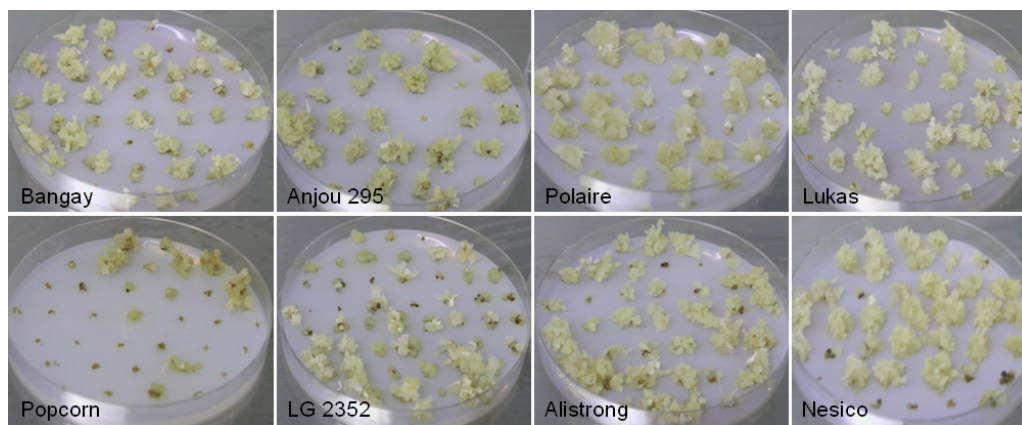
Figure 3.3 Callus induction from leaf bases of several commercial maize varieties. Callus formation was observed in all cultivars after four weeks incubation on ML1G1 medium. However, the calli were exclusively of the non-embryogenic type.

Furthermore, the developed tissue culture and regeneration system was successfully applied for sugarcane (Fig. 3.4), although the callus induction efficiency was lower than in maize. However, whether or not the system is generally applicable for a wide range of maize elite inbred lines or other monocotyledonous species remains to be investigated.
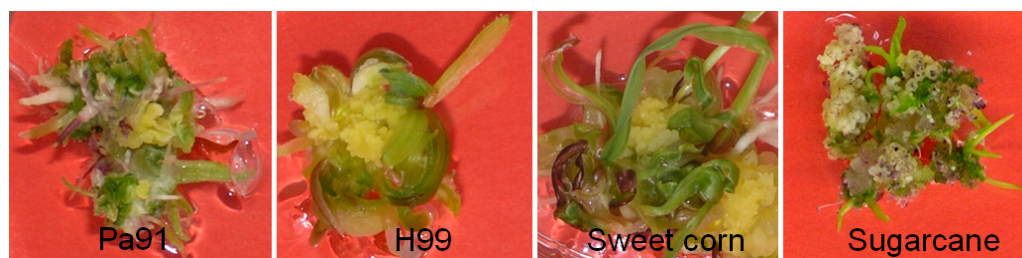
Figure 3.4 Regeneration of multiple shoots from leaf-derived embryogenic calli induced on ML1G1 medium from leaf segments of different maize genotypes (two inbred lines Pa91 and H99, and a commercial variety of sweet corn) as well as a sugarcane variety. The cultures were incubated on ML1R4 regeneration medium for three weeks (for maize) or five weeks (for sugarcane).

3.1.2   Nuclear transformation of leaf-derived calli

3.1.2.1   Biolistic gun-based transformation of embryogenic calli

To test whether the leaf-derived calli provide suitable source material for genetic transformation of maize, stable genetic transformation experiments were conducted using the vector pNOV2820. This vector carries the phosphomannose isomerase (*pmi*; Wright *et al.*, 2001) selectable marker gene under control of the CMPS promoter. *pmi* gene expression confers the ability to grow on mannose, an otherwise non-metabolizable sugar for maize cells, providing a metabolic marker (Weisser *et al.*, 1996; Wright *et al.*, 2001).
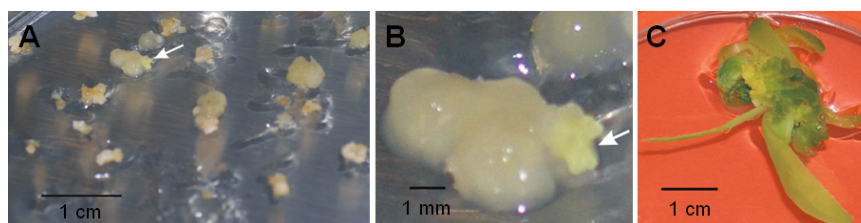
Figure 3.5 Transformation of leaf-derived maize callus tissue. (**A**) Selection of transformed cell lines on mannose-containing media. (**B**) Close-up of a transformed callus growing on selection medium due to its capability of metabolizing mannose. Transgenic callus tissue is indicated by arrows. (**C**) Regeneration of transgenic maize plants from callus tissue on medium ML1R3.

Biolistic transformation with pNOV2820-plasmid-coated 0.6 μm gold particles and selection of transformed cell lines for growth on mannose-containing media produced between one and four transgenic clones per shot (Fig. 3.5; Table 3.6). A prolonged incubation of the calli after biolistic bombardment on high osmotic medium seems to be beneficial and gave a higher transformation frequency (Table 3.6). Calli growing on mannose could be readily regenerated into shoots on medium containing either mannose or sucrose (Fig. 3.5C) and, for seven out of eight transgenic lines produced, yielded fertile plants (Table 3.6) (Ahmadabadi *et al.*, 2006).

Table 3.6 Maize nuclear transformation efficiency using a novel leaf-based tissue culture system

Experiment No.	Post-transformation incubation on osmotic medium (days)	No. of colonies surviving on selection medium	PCR positive	Fertile transgenic lines
1	1	1	1	1
2	1	1	1	1
3	1	2	2	2
4	3	4	4	3

Each experiment represents a single bombardment of a Petri dish with a round filter paper (7 cm diameter) entirely covered with callus material

3.1.2.2 Analysis of putative transformants for *pmi* expression

Different assays were used to confirm presence of the *pmi* transgene and its expression in putative transgenic lines. First, chlorophenol red assays were performed to investigate *pmi* gene expression (Fig. 3.6). *pmi* transgene integration into the nuclear genome was confirmed by PCR (Fig. 3.7) and Southern blot analysis (Fig. 3.8A). In addition, Northern blot analysis was used to detect *pmi* transcripts in transgenic lines (Fig. 3.8B).

To test for *pmi* expression in candidate transgenic lines, chlorophenol red assays were conducted. Metabolization of sugar in the growth medium results in acidification of the medium which can be visually detected by a color change of the indicator substance chlorophenol red (CPR) from red to yellow (Kramer *et al.*, 1993; Wright *et al.*, 2001). All

putative transgenic lines that displayed callus growth on mannose-containing medium were positive in CPR assays (Fig. 3.6) suggesting that they express the *pmi* gene and therefore, are indeed transgenic (Ahmadabadi *et al.*, 2006).
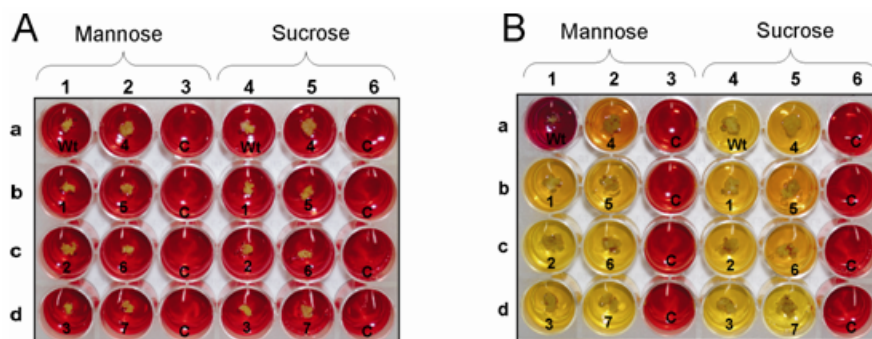
Figure 3.6 Chlorophenol red assay to confirm *pmi* expression in transgenic maize lines. (**A**) Calli from the wild-type (Wt) and seven independently generated transgenic lines (1-7) were incubated on medium with the indicator substance chlorophenol red and either mannose or sucrose as carbon source. (**B**) After two days incubation in the dark, a color shift from red to yellow indicates acidification of the medium as caused by sugar metabolization. While sucrose is metabolized by all calli, mannose can only be utilized by transgenic cell lines expressing the phosphomannose isomerase encoded by the *pmi* transgene. C: control incubation without callus.

To confirm the physical presence of the *pmi* transgene in the selected lines, PCR analyses with *pmi*–specific primers (manA-P1 and manA-P2; see 2.1.4) were performed. A PCR product of the expected size could be amplified from all candidate transgenic lines (Fig. 3.7), ultimately confirming that all lines selected by their growth on mannose are indeed transformed.
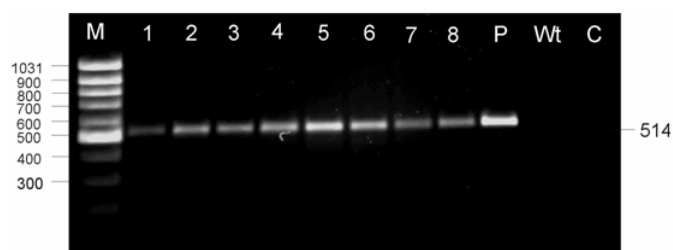
Figure 3.7 PCR assay confirming presence of the *pmi* gene in transgenic maize lines selected on mannose-containing medium. PCR amplification using a *pmi*-specific primer pair yields a specific product of the expected size (514 bp) in all mannose-metabolizing lines (lanes 1-8) but not in the wild-type (Wt). Fragment sizes of the molecular weight marker (M) are given in bp. P: positive control (plasmid pNOV2820); C: buffer control.

To analyze transgene copy numbers in the selected lines, DNA gel blots using a *pmi*-specific probe were conducted. Southern blot hybridization detected multiple bands in all transgenic lines indicating integration of more than one copy of the transgene (Fig. 3.8A). Integration of multiple transgene copies is often observed after biolistic transformation of the plant's nuclear genome (Altpeter *et al.*, 2005; Kohli *et al.*, 1998). If multiple copies are undesirable, the

"minimal-cassette" or "clean-DNA" strategy can be applied in which excision of the transgene(s) from the plasmid vector and transformation with a linearized DNA fragment produces simple integration patterns (Altpeter *et al.*, 2005).
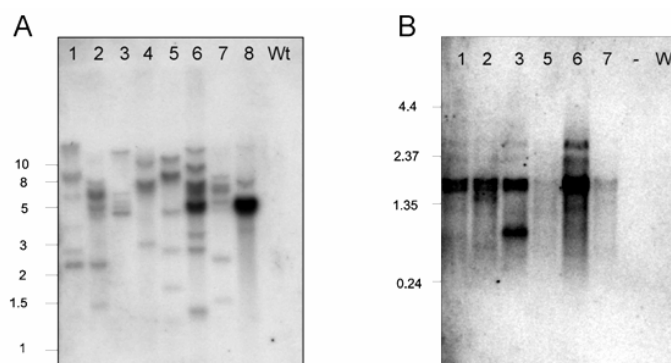
Figure 3.8 Nucleic acid gel blots to confirm integration of the *pmi* transgene into the maize genome and analyze its expression. (**A**) Southern blot analysis using a *pmi*-specific probe. Ten micrograms of total genomic DNA digested with *Hind* III restriction enzyme was used. Multiple bands in all transgenic lines analyzed here indicate integration of more than one copy of the transgene. (**B**) Northern blot to confirm expression of the *pmi* transgene. In most transgenic lines, a single prominent transcript of the expected size (~1.6 kb) is observed. The smaller than expected additional band in lane 3 may be due to expression of a partially deleted transgene copy. Fragment sizes of the molecular weight markers are indicated at the left in kb. Wt: wild type.

Expression of the *pmi* transgene was also confirmed for a number of lines by Northern blot analysis. In most transgenic lines, a single prominent transcript of the expected size was observed (Fig. 3.8B). One of the lines (lane 3 in Fig. 3.8B) showed, in addition to the band corresponding to the full-length *pmi* transcript, a second strongly hybridizing band which was smaller than expected and may be due to expression of a partially deleted transgene copy.

As expected, the *pmi* transgene was stably transmitted into the next generations as revealed by seedling tests and CPR assays in the T1 and T2 generations (Fig. 3.9).
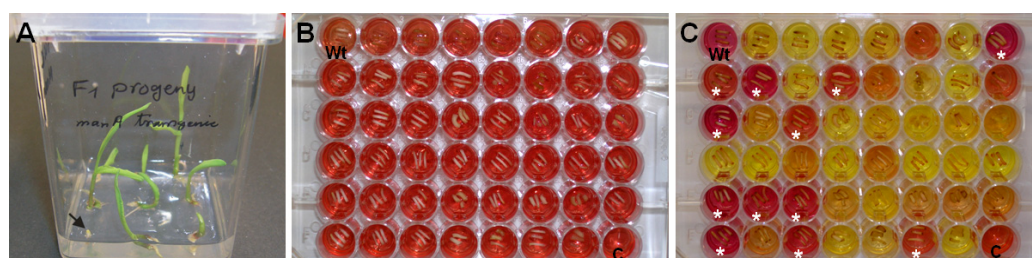
Figure 3.9 Confirmation of stable inheritance of the *pmi* transgene into the T1 (A) and T2 (B) generations. (**A**) Phenotypic segregation in embryos isolated from T1 seeds of a selfed *pmi* transgenic line is shown by assaying growth on mannose-containing medium. While homozygous and heterozygous transgenic embryos can grow on this medium, the embryo indicated by the arrow is wild type and therefore not able to grow on mannose. (**B,C**) Chlorophenol red assay to confirm stable inheritance and expression of *pmi* transgene in the T2 generation. (**B**) Root segments of sterile seedlings from the wild-type (Wt) and T2 progeny (unlabeled wells) of a selfed T1

transgenic line were incubated on medium containing chlorophenol red and mannose as carbon source. (**C**) After two days incubation in the dark, a color change from red to yellow indicates ability of root pieces to utilize mannose due to the expression of the phosphomannose isomerase. Some of the progeny (indicated by asterisks), like Wt, were not able to metabolize mannose indicating that the selfed T1 plant was indeed heterozygote and consequently, the progeny segregated into Wt and transgenic lines. However, the possibility of transgene silencing in some of the progeny cannot be excluded. Variation in the intensity of the color change might reflect different levels of PMI expression in transgenic progeny plants, but could also be due to differences in the physiological status of the excised root segments. C: control incubation without callus.

3.1.3 Regenerable callus induction from leaves and immature tassels of greenhouse-grown maize plants

Next, other explant sources than leaves were tested in the developed culture system. While no regenerable callus could be induced from roots, immature tassels harvested from greenhouse-grown plants produced embryogenic callus at very high frequency (100%: all cultured tassel pieces produced embryogenic calli) (Fig. 3.10A-B). The calli induced from tassels were exclusively of the highly regenerable type I-like kind (Fig. 3.10A-B). Thus, using the ML1G1 medium, tassel tissue provides an optimum source material for the rapid and efficient induction of embryogenic callus and presumably, also for genetic transformation experiments (Ahmadabadi *et al.*, 2006).
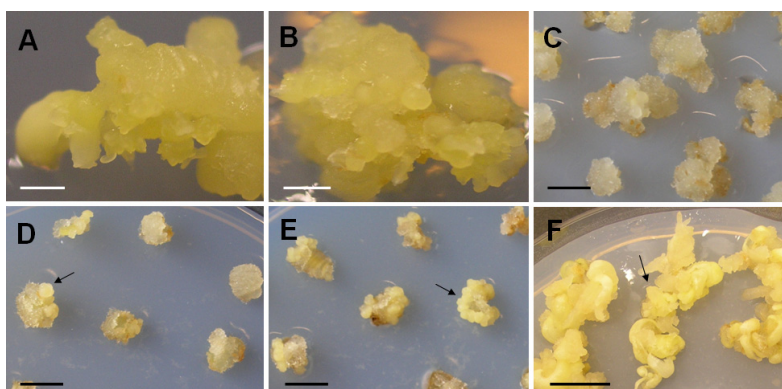
Figure 3.10 Induction of embryogenic callus from maize immature tassel tissue in the dark (A,B) and from six-week old maize leaves harvested from greenhouse-grown plants (C-F). (**A,B**) Embryogenic yellow callus induced from immature tassels after three weeks of incubation on ML1G1 medium in the dark. (**C-F**) Callus induction from leaves of greenhouse-grown plants. Leaves seven to eleven (counted from the bottom of the plant) were surface-sterilized and small pieces were incubated on ML1G1 medium for 5 weeks in the dark. (**C**) Loose white non-embryogenic calli induced from leaf seven. (**D-F**) Yellow embryogenic callus (examples indicated by arrows) induced from leaves eight (**D**), nine (**E**) and the two youngest leaves of greenhouse-grown maize plants (leaves 10 and 11) (**F**). White scale bars: 1 mm, black scale bars: 1 cm.

Finally, it was tested whether leaves from soil-grown maize plants can also serve as explant source for the induction of type I-like embryogenic callus. To this end, leaf samples were

taken from six-week old greenhouse-grown maize plants (see 2.2.1.3) and incubated on ML1G1 medium. Visible callus induction was observed in all samples after 4-6 weeks incubation in the dark. Embryogenic callus induction was not observed in leaf number seven (the oldest leaf tested) which gave only loose non-embryogenic callus (Fig. 3.10C). However, all younger leaves produced yellow type I-like embryogenic callus (Fig. 3.10D-F). As expected, the frequency of the desired embryogenic callus induction increased with decreasing leaf age from young light-green to yellowish leaves with the youngest leaves producing type I-like callus with high efficiency (i.e. on about 20 to 30 % of the leaf pieces) (Ahmadabadi *et al.*, 2006).

3.1.4 Construction of a maize plastid-specific transformation vector

Successful transformation of the leaf-derived embryogenic calli at high frequency (Table 3.6) has motivated us to set up several experiments to develop a plastid transformation system for maize. Stable plastid transformation depends upon the integration of the foreign DNA into the plastid genome by homologous recombination. Thus, in every plastid-specific transformation vector, the DNA fragment to be transferred is flanked by two DNA fragments homologous to sequences in the plastid DNA (ptDNA) (Bock, 2001; Fig. 3.11). Therefore, a vector (pMA1; for details on cloning strategy, see 2.1.7) based on a large non-coding region in the maize plastid genome (Maier *et al.*, 1995; Fig. 3.11), which is not conserved among different plant species, was constructed. In rice, for example, this region is almost entirely absent from the plastid genome (Hiratsuka *et al.*, 1989; Maier *et al.*, 1995) indicating that it is non-coding. In addition, this region of the maize ptDNA is large, allowing for insertion of the foreign gene(s) in a safe distance to the regulatory elements of the neighboring genes. Therefore, this region seems to be particularly suitable for accommodating foreign genes.

Two reliable selection systems have been established for efficient plastid transformation in dicotyledonous plant species: (i) *aadA*/spectinomycin-resistance selection system, and (ii) *nptII*/kanamycin-resistance method (for review see Bock, 2001; Bock and Khan, 2004; Maliga, 2004). The *aadA* gene encodes an aminoglycoside adenylyltransferase, conferring resistance against spectinomycin, whereas the *nptII* gene encodes a neomycin phosphotransferase enzyme which inactivates kanamycin. However, the *aadA* gene product does not only confer resistance to spectinomycin, but also inactivates streptomycin. Likewise, the *nptII* gene product is active against both kanamycin and geneticin. Both genes, when fused to plastid-specific expression signals (plastid-specific promoter, 5' UTR, 3' UTR), allow the recovery of plastid transformants in dicotyledonous species at high efficiencies (Bock, 2001; Maliga, 2004).
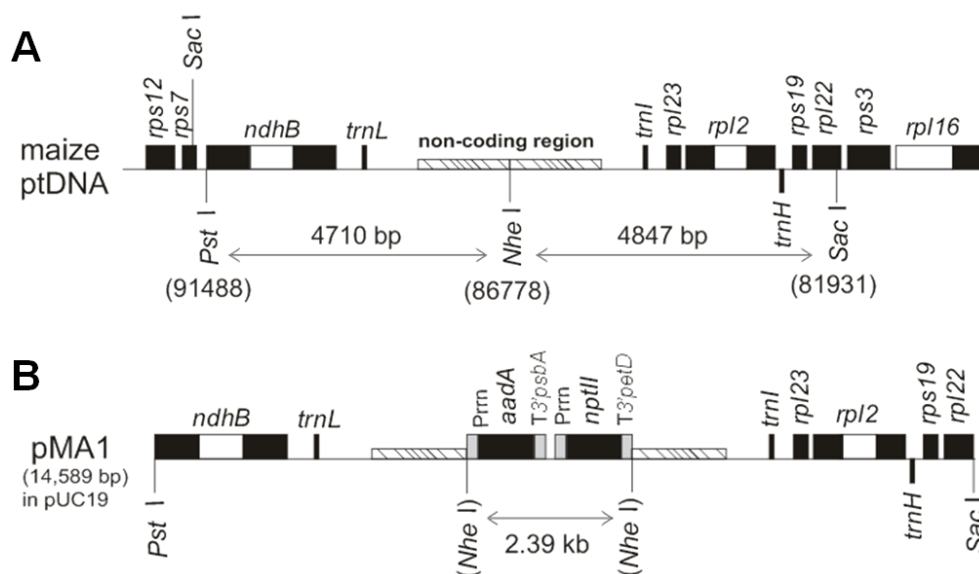
Figure 3.11 Physical map of the vector constructed for plastid transformation in maize. (**A**) Map of the maize plastid DNA (ptDNA) region used for constructing a maize-specific plastid transformation vector. The nucleotides positions in the NCBI database are indicated for all restriction sites used for cloning. (**B**) Map of the maize-specific plastid transformation vector pMA1. First, a *Pst* I/*Sac* I fragment (9.55 kb) from the maize ptDNA was cloned into the pUC19 basic vector (*Pst* I/*Sac* I). The DNA fragment including two selectable marker gene cassettes, *aadA* and *nptII*, was then cloned into the non-coding *trnI-trnL* intergenic region of the maize ptDNA using a unique NheI restriction site and Klenow polymerase treatment. Note that genes above the line are transcribed from the left to the right and genes below the line are transcribed from the right to the left.

In order to improve the efficiency of selection for plastid transformants, a novel approach involving the *aadA* and *nptII* marker genes in tandem was used. The advantage of double selection for both streptomycin and kanamycin can be twofold: (i) it can increase the efficiency of isolating plastid-transformed lines by eliminating false positive clones (spontaneous antibiotic-resistant mutants), and (ii) it suppresses the possible background of antibiotic-resistant lines resulting from transgene integration into the nuclear genome. Both selectable marker genes were fused to chloroplast-specific expression signals. The *aadA* gene is under control of a modified tobacco ribosomal RNA promoter (Prrn; Svab and Maliga, 1993) and *psbA* terminator (T3'*psbA*). The *nptII* gene is controlled by the maize Prrn promoter and the *petD* terminator (T3'*petD*; Fig. 3.11). The maize Prrn promoter which has recognition sites for both the plastid-encoded RNA polymerase (PEP) and the nuclear-encoded RNA polymerase (NEP) (Hajdukiewicz *et al.*, 1997), was fused with the short leader sequence (g10L) of the gene 10 from bacteriophage T7, which contains a perfect plastid ribosome-binding site (RBS) (see 2.1.7; Fig. 6.3) and has been documented as an excellent leader sequence for the expression of various heterologous proteins (Olins *et al.*, 1988; Staub *et al.*, 2000). In addition, the neomycin phosphotransferase enzyme was translationally fused

with 14 N-terminal amino acids of the green fluorescent protein (GFP; Fig. 6.3), which has been demonstrated to enhance translation rate in plastids (Maliga, 2003b).

3.1.5 Evaluation of the sensitivity of maize leaf-derived calli to the aminoglycoside antibiotics kanamycin and streptomycin

Spectinomycin and kanamycin are two frequently used antibiotics in chloroplast transformation experiments. Selection for spectinomycin resistance provides an efficient selection principle for plastid transformation in dicotyledonous plant species (Svab *et al.*, 1990; Svab and Maliga, 1993). In contrast, many grass species such as maize, are naturally resistant against spectinomycin (Fromm *et al.*, 1987). However, the *aadA* gene product is also active against streptomycin. Thus, the effects of streptomycin and kanamycin on the conditions required for selection in the embryogenic system were determined. This information is of particular relevance because, in the biolistic transformation method, embryogenic cultures were utilized as target tissues. Therefore, several experiments were conducted to determine the effects of these two antibiotics (kanamycin and streptomycin) on the development of embryogenic cultures of maize when selection was applied under propagative (callus culture medium, in the dark) or regenerative (regeneration medium, in the light) conditions. To this end, small embryogenic callus pieces (of identical size and age) were cultured on antibiotic-containing ML1C2 propagative or ML1R3 regenerative media. The results showed that in general, application of the selection under regenerative conditions is considerably more stringent than under propagative conditions. For example, a complete inhibition of callus growth could not be obtained in the dark even at high levels of streptomycin (500 mg/l) and/or kanamycin (250 mg/l), whereas 100 mg/l streptomycin and/or 50 mg/l kanamycin completely blocked callus greening and regeneration in the light (Fig. 3.12). When occasionally plantlets appeared, they were totally white.

3.1.6 Direct induction of embryogenic callus from maize leaves in the light

Our finding that maize embryogenic cultures are less sensitive to streptomycin and kanamycin under propagative conditions (which support embryogenesis in the dark), suggests that selection for chloroplast transformants with these antibiotics is more effective in the light. Although ML1R3 regenerative medium is suitable for efficient germination of somatic embryos, but it does not support embryogenesis or organogenesis from single cells. Thus, development of a regeneration medium with high embryogenesis/organogenesis capacity is expected to facilitate the recovery of transformation events at higher efficiency by allowing transformed cells to proliferate, and ultimately regenerate into plants via organogenesis or
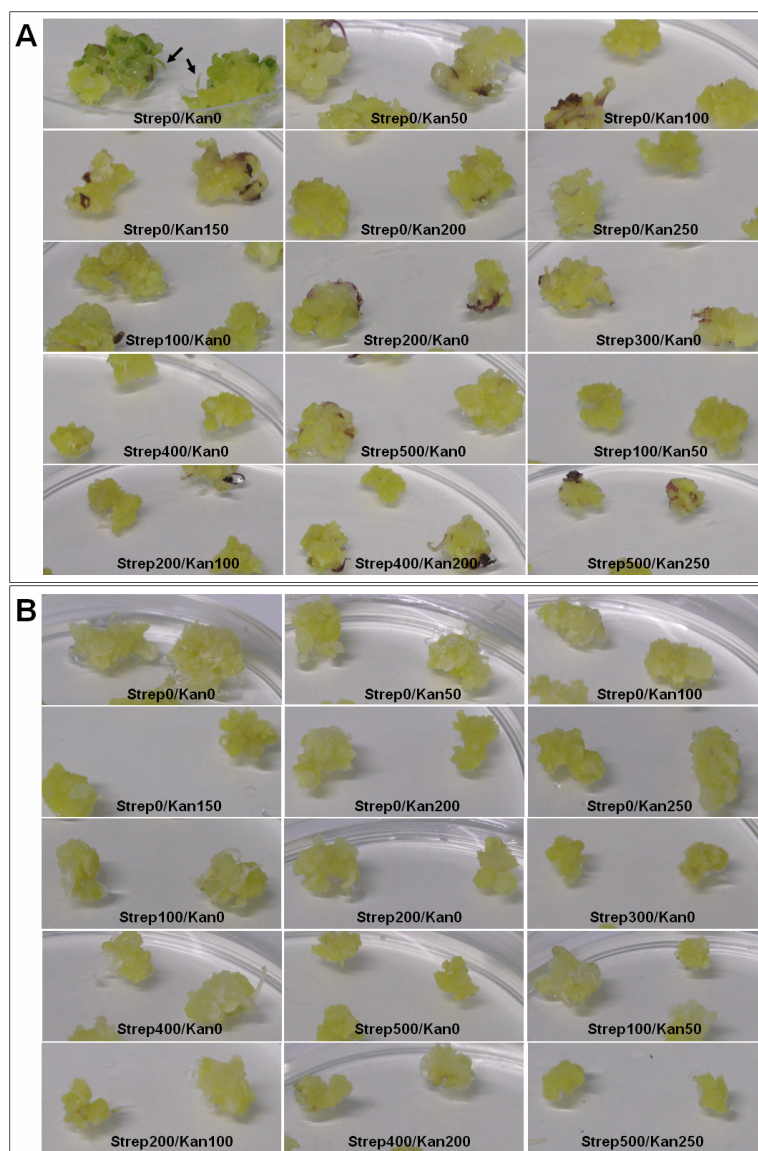
Figure 3.12 Effects of streptomycin (Strep) and kanamycin (Kan) on embryogenic-callus growth and regeneration under propagative (on Ml1C2 medium in the dark) or regenerative (on ML1R3 medium in the light) conditions. Small embryogenic callus pieces (of the same size and age) were incubated on streptomycin- and/or kanamycin-containing media for 40 days in the light (**A**) or in the dark (**B**). The numbers in each picture indicate streptomycin and kanamycin concentrations (in mg/l). Addition of 100 mg/l streptomycin and/or 50 mg/l kanamycin blocked callus greening and regeneration under regenerative condition (**A**), while a complete inhibition of callus growth could not be obtained under propagative condition, even at high levels of streptomycin (500 mg/l) and/or kanamycin (250 mg/l) (**B**). Arrows show shoot formation from calli incubated on antibiotic-free medium under regenerative condition.

somatic embryogenesis. Thus, we attempted to modify the leaf-based tissue culture system in a way that it will increase effectiveness of the selection for transgenic cell lines in the light. Independent of inclusion or omission of the light-sensitive ingredient silver nitrate, the optimized callus induction medium (ML1G1) was not suitable to support embryogenic callus induction from leaf segments in the light. Therefore, a series of modified media were tested

for their suitability to induce regenerable calli from maize leaves in the light and in the absence of silver nitrate. It was found that addition of 30 to 75 nM phytosulfokine-alpha (α-PSK) to a silver nitrate-free culture medium (ML1C2) strongly stimulated embryogenesis as well as organogenesis from maize leaf explants in the light (Fig. 3.13). This version of the leaf-based tissue culture and regeneration system for maize should prove useful when embryogenesis and/or selection of transgenic plants in the light is either favored or an unavoidable step.
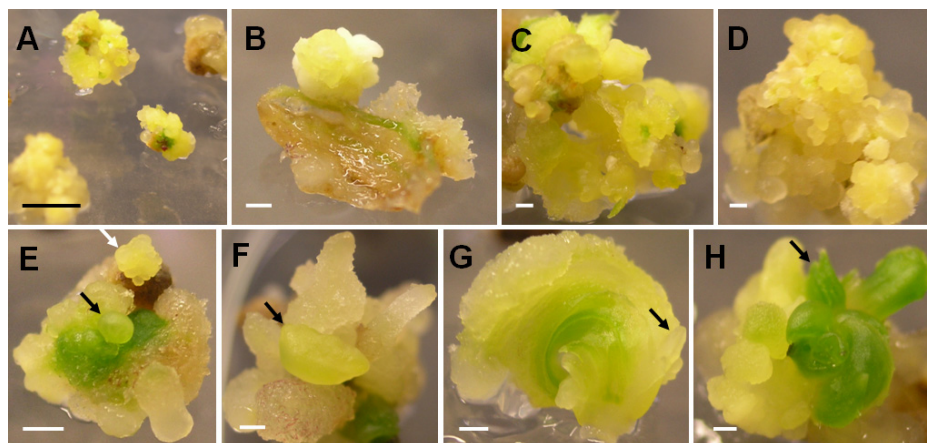
Figure 3.13 Induction of embryogenic/organogenic tissues from maize leaf material in the light. (**A-C**) Induction of type I-like calli from leaf discs. Photographs were taken after six weeks of incubation on α-PSK-containing medium. (**D**) Type II-like friable callus induced from leaf tissue in the light. (**E**) A small globular embryo (black arrow) and type I-like callus induction (white arrow) from leaf tissue. (**F**) A green delta-shaped (Δ) embryo induced from leaf tissue (indicated by the arrow). (**G,H**) Organogenesis and leaf tissue formation (indicated by arrows) from leaf base segments. White scale bars: 1 mm, black scale bar: 1 cm.

3.1.7 Attempts towards development of plastid transformation in maize

The developed tissue culture method provided an efficient nuclear transformation system indicating that particle gun-based foreign DNA delivery to leaf base-derived embryogenic callus cells occurs at high frequency. Therefore, several series of plastid transformation experiments were conducted using the pMA1 vector (see 3.1.4) followed by selection on different antibiotics under various light conditions (summarized in Tables 3.7 and 3.8). In the experiments using kanamycin and/or streptomycin as selection agent (Table 3.7), the direct selection on ML1R3 regeneration medium in the light did not result in the recovery of any candidate plastid transformants. Likewise, when the transformed calli were pre-selected on the propagative medium (ML1C2) containing different levels of these antibiotics (Table 3.7) in the dark and then transferred onto selective regeneration medium, no resistant candidate lines could be obtained. A prolonged incubation of the calli following biolistic bombardment on high osmotic medium did not change the outcome either. This might be due to the low

sensitivity of the wild-type cells to these two antibiotics in the dark as evidenced by the results of the sensitivity tests (see Fig. 3.12). Consequently, the wild type cells might overgrow any transformed cells and inhibit their growth and regeneration. The *nptII* gene product also detoxifies the antibiotic G418 (also called geneticin). Thus, we carried out a series of experiments to test the sensitivity of maize calli to G418 and found that, unlike kanamycin and streptomycin, addition of 50 mg/l G418 to ML1C2 callus propagation medium inhibits wild-type callus growth in the dark completely (Fig. 3.14A). Thus, three series of maize plastid transformation experiments were conducted based on selection on G418-containing medium in the dark (table 3.8).

In these experiments, 38 cell lines (out of 24 shots) survived after several rounds of selection on geneticin-containing medium in the dark (Fig. 3.14B). Callus samples from these candidate lines were transferred onto regeneration medium containing 50 mg/l kanamycin. This double selection system was thought to eliminate escapes and spontaneous geneticin-resistant lines. The candidate calli could not be regenerated into green shoots and were sensitive to kanamycin, except one candidate line which turned green on selective medium (Fig. 3.14C). However, it could not be regenerated into shoots. Southern blot analysis was conducted to analyze the restriction pattern of the plastid DNA in putative geneticin-resistant candidate lines. Two different probes were used to investigate whether or not the transgenes had been integrated into the ptDNA: (i) a 2.85 kb *Hinc* II fragment from plasmid pMA1 (Fig. 3.14D), hybridizing to the left flanking region, and (ii) a 0.8 kb *Nco* I/*Xba* I fragment from *nptII* gene sequence (Fig. 3.14D). The enzyme *Sac* I cuts the maize ptDNA in a position outside of the left flanking region and was chosen to eliminate the possibility of integratin of transgenes and flanking sequence into the nuclear genome, an event which should produce a fragment different from the expected size for transgenic ptDNA. When the flanking region-specific probe was used, all putative candidate lines showed restriction patterns similar to the wild type (Fig. 3.14E), suggesting that the candidate lines were not plastid transformants, but either escapes or spontaneous mutations for geneticin resistance. Hybridization with the *nptII* gene-specific probe detected signals in one and the single candidate line which showed greening on kanamycin-containing medium (Fig. 3.14C,F), although most of the plastid DNA was degraded in green tissues. The signals, however, differed from the expected size for the transgenic ptDNA, suggesting that the *nptII* gene is possibly integrated into the nuclear genome. Absence of the transgenes from the ptDNA of putative candidate lines was also confirmed by selection on streptomycin-containing medium in the light, where no green calli or shoot formation was observed.

Table 3.7 Biolistic gun-based (see 2.2.1.6) plastid transformation experiments in maize using plasmid pMA1 (see 3.1.4) and leaf-derived embryogenic calli as target material

Exp. No.	No. of shots	On osmotic medium		First selection round					Second selection round					Third selection round				
		Prior (hours)	After (days)	Strep. (mg/l)	Kan. (mg/l)	Light con.	Period (weeks)	Medium	Strep. (mg/l)	Kan. (mg/l)	Light con.	Period (weeks)	Medium	Strep. (mg/l)	Kan. (mg/l)	Light con.	Period (weeks)	Medium
1	11	0.5	1	500	50	Light	12	ML1R3	-	-	-	-	-	-	-	-	-	-
2	4	0.5	1	100	0	Dark	2	ML1C2	500	0	Dark	4	ML1C2	300	0	Light	8-12	ML1R3
3	6	24	7	500	0	Dark	4	ML1C2	100	0	Light	4	ML1R3	100	0	Light	8	ML1R3
4	6	24	7	0	150	Dark	4	ML1C2	0	50	Light	4	ML1R3	0	50	Light	8	ML1R3
5-1	3	0.5	3	0	50	Light	4-8	ML1R3+PSK	-	-	-	-	-	-	-	-	-	-
5-2	2	0.5	3	100	0	Light	4-8	ML1R3+PSK	-	-	-	-	-	-	-	-	-	-
6	16	0.5	3	0	50	Light	4	ML1PSK	0	50	Light	4	ML1PSK	0	50	Light	4-8	ML1R3

Various parameters, including incubation time on osmotic medium (ML1TC1; see 2.2.1.6.2) prior and after transformation, light conditions, antibiotic [streptomycin (Strep.) and Kanamycin (Kan.)] combinations and concentrations, incubation media and multi-step selection systems have been tested. For details on media composition, see Materials and Methods (2.1.10). The ML1PSK medium is a modified ML1C2 medium in which silver nitrate was omitted, 1.1 mg/l 2,4-D was used and 30 nM filter-sterilized α-PSK was added after autoclaving. For dark experiments, all callus materials from each selection round were transferred to the next selection cycle while in light experiments, only the callus pieces which showed greening were carefully isolated (using a stereomicroscope under sterile conditions) and transferred to the next selection cycle. Remaining callus material was kept and evaluated for possible additional candidate transformants. In experiments using α-PSK, remaining callus materials were transferred onto kanamycin-containing ML1R3 medium after 8-10 weeks. The cultures, which were selected in the dark, were subcultured every 2 weeks on fresh medium, and finally transferred onto antibiotic-containing ML1R3 regeneration medium followed by selection in the light.

Table 3.8 Particle gun-based plastid transformation experiments in maize using G418 as selection agent

| Exp. No. | No. of shots | First selection round | | Second selection round | | Third and fourth selection rounds | | Regeneration (ML1R3+Kan50) |
		G418 (mg/l)	Period (weeks)	G418 (mg/l)	Period (weeks)	G418 (mg/l)	Period (weeks)	Period (weeks)
1-1	3	45	4-5	50	4-6	60	3-6	4-8
1-2	3	50	4-5	50	4-6	60	3-6	4-8
2	8	50	4-6	50	6-7	60	4-7	4-8
3-1	1	45	4-8	50	4-6	60	4-6	4-8
3-2	9	50	4-8	50	5-6	60	4-6	4-8

Leaf-derived embryogenic calli were incubated on osmotic medium (ML1TC1; see 2.2.1.6.2) for half an hour and bombarded with pMA1 plasmid-coated gold particles (see 2.2.1.6). Transformed calli were incubated for three days on osmotic medium followed by transfer onto ML1C2 medium containing geneticin (concentrations are given for each selection cycle). After several rounds of selection on geneticin-containing medium, surviving cell lines were transferred onto ML1R3 regeneration medium containing 50 mg/l kanamycin (ML1R3+Kan50). Integration of the *nptII* gene into the genome will confer resistance against both geneticin and kanamycin. This double selection system was thought to eliminate false positive clones resulting form spontaneous mutations for geneticin resistance.
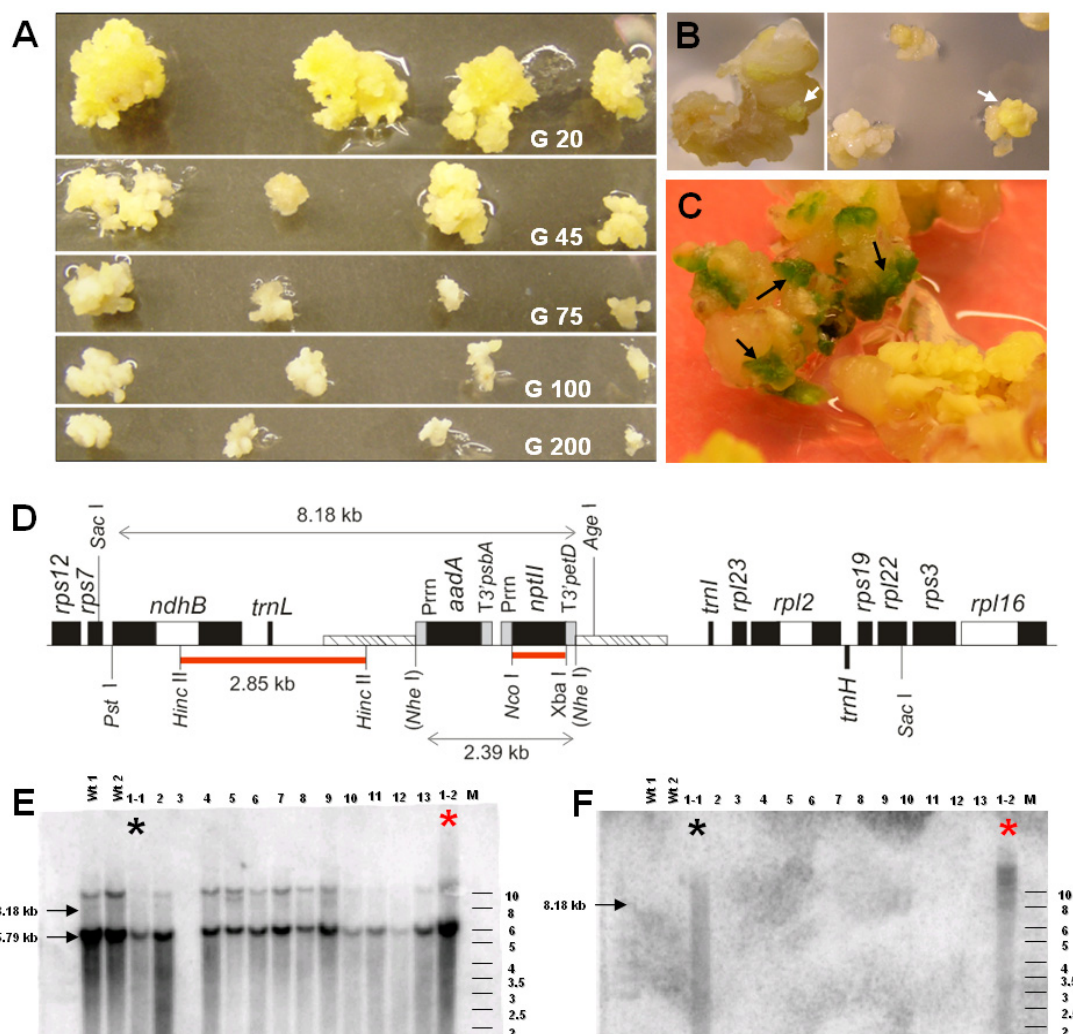
Figure 3.14 Plastid transformation experiments in maize based on G418 (geneticin) selection. (**A**) Evaluation of the sensitivity of maize leaf-derived embryogenic calli against geneticin. Leaf-derived embryogenic callus pieces

of different sizes (from ~2 mm, at the right, to ~6 mm, at the left) were incubated on ML1C2 medium (see 2.1.10) containing different levels of geneticin (as stated in white). After four weeks, callus growth was significantly inhibited on the media containing ≥45 mg/l geneticin. (**B**) Isolation of surviving colonies (indicated by arrows) following biolistic transformation of callus cultures using plasmid pMA1, after six weeks of selection on ML1C2 medium containing 50 mg/l G418. (**C**) Regeneration of isolated G418-resistant callus on ML1R3 medium containing 50 mg/l kanamycin. While dark green callus pieces (indicated by arrows) could be observed after 3-4 weeks incubation in the light, shoot regeneration could not be obtained. (**D**) Expected map of the maize plastid DNA after successful integration of the *aadA* and *nptII* gene cassettes from plasmid pMA1 into the maize ptDNA via two homologous recombination events. Genes above the line are transcribed from the left to the right and genes below the line are transcribed from the right to the left. The size of the integrated DNA sequence (2.39 kb) and the expected fragment size generated by digestion with the restriction enzymes *Sac* I and *Age* I (8.18 kb) are shown. The *Hinc* II fragment (2.85 kb) from thee flanking region and the *Nco* I/*Xba* I fragment (0.8 kb) from the *nptII* gene, which were used as probes for Southern blot analysis, are shown as red bars. (**E**,**F**) Southern blot analyses of surviving cell lines (No. 1 to 13) after several rounds of selection on geneticin-containing medium in the dark following biolistic transformation using vector pMA1. 5-10 μg of total DNA extracted from callus material were digested with *Sac* I and *Age* I, separated in a 0.8% agarose gel, blotted and hybridized to a radiolabled restriction fragment derived from: (E) cloned maize plastid DNA (*Hinc* II fragment; see Fig. 3.14D) and (F) the *nptII* gene (*Nco* I/*Xba* I; see Fig. 3.14D). For candidate line number 1, which showed greening in the light [shown in part (C)], DNA was isolated from green calli induced in the light (1-1; black asterisks) and from dark-grown calli (1-2; red asterisks). (**E**) RFLP with the *Hinc* II probe. The probe, as expected, detects a 5.79 kb fragment from the wild-type plastid genome. While the wild-type signal could be detected in all selected candidate lines, an 8.18 kb fragment could not be detected, indicating that the selected lines were not true plastid transformants. The probe detects second, weak band of bigger size (> 10 kb) which is likely to be a plastid DNA fragment located in the nuclear genome as a result of DNA transfer from the organellar genome to the nuclear genome, an evolutionary event that takes place at relatively high frequency (Martin and Herrmann, 1998; Stegemann *et al.*, 2003). (**F**) RFLP with the *nptII* probe. This probe detects signals from candidate line number 1 which showed greening on selective medium in the light [as shown in part (C)]. However, no fragment of the expected size could be detected indicating that the *nptII* gene has been integrated into the nuclear genome. Molecular weigh markers are in kb. Wt1: green wild-type maize plant, Wt2: wild-type callus incubated on ML1R3 regeneration medium containing 50 mg/l kanamycin for four weeks in the light.

As mentioned (1.4.5), plastid gene expression is regulated by light at the transcriptional and translational levels, and plastid gene expression is drastically higher in photosynthetically active green chloroplasts than in non-green plastids. Thus, several transformation experiments were conducted using green calli as target tissue and/or selection in the light with application of α-PSK. In one set of the experiments (Table 3.7, Exp. No. 5), propagated embryogenic calli were first incubated on non-selective callus culture medium containing 30 nM α-PSK for two weeks. The green embryogenic calli were then collected and used for biolistic transformation using plasmid pMA1. After three days post-transformational incubation on osmotic medium, bombarded calli were transferred on the selective ML1R3 regeneration medium supplemented with 30 nM α-PSK (Table 3.7) followed by incubation in the light. A single green shoot was obtained after 3 weeks selection on kanamycin-containing medium. The regenerated shoot grew for further 3-4 weeks on the selective medium and showed a variegated green/pale-green phenotype (Fig 3.15A). However, the plantlet was not able to grow on antibiotic-containing medium after 8 weeks, and lost its green color over time. Southern blot analysis, using the 2.85 kb *Hinc* II fragment (Fig 3.14D) as a probe, was performed to reveal the plastid genome state of the regenerated candidate line for plastid transformation in comparison to the wild type (Wt). Beside Wt, a white plantlet regenerated from non-transgenic calli on kanamycin-

containing medium was included in the Southern blot analysis as additional control. Similar to the Southern blot analysis for the candidate lines obtained from geneticin selection system (Fig 3.14E), no transgenic plastid genome signal could be detected for the regenerated green plantlet (Fig 3.15C).
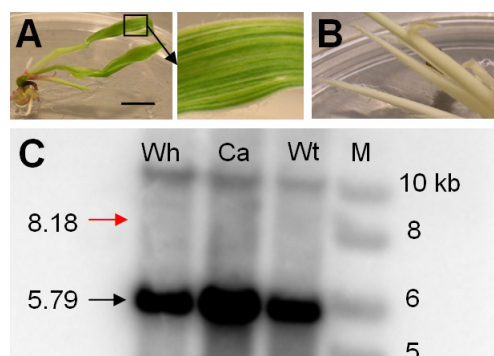
Figure 3.15 Maize plastid transformation experiments based on selection on α-PSK-containing regeneration medium. (**A**) Regeneration of a variegated plantlet from selection of maize calli on α-PSK-containing selection medium (see Table 3.7) after biolistic transformation using plasmid pMA1. Transformed embryogenic calli were incubated on 50 mg/l kanamycin-containing ML1R3 medium plus α-PSK for 4 weeks. A close-up of a variegated leaf segment is shown at the right. Scale bar: 1 cm. (**B**) Completely white plantlets regenerated from Wt calli under the same selection conditions as described in part (A). (**C**) Southern blot analysis of regenerated plants from selection on α-PSK-containing medium. 5 µg of total DNA extracted from variegated leaves of the transplastomic candidate line [shown in part (A); Ca, standing for candidate], a regenerated white plant [described in part (B); Wh, standing for white] and a wild type plant (Wt) were digested with *Sac* I and *Age* I, separated in a 0.8% agarose gel, blotted and hybridized to a radiolabled restriction fragment derived from cloned maize plastid DNA (*Hinc* II fragment; see Fig. 3.14D). Similar to Fig. 3.14E the probe detected a 5.79 kb fragment from the wild-type plastid genome. The 8.18 kb fragment expected for the transplastomic candidate line could not be detected, indicating that the selected line was not a true plastid transformant. The candidate line lost its green color after two more weeks and turned white on continued selection on kanamycin-containing medium. Molecular weighs are in kb.

Next, a light selection system (using a selective medium which supports embryogenesis in the light) was tested to develop plastid transformation in maize (Table 3.7, Exp. No. 6). In these experiments, transformed dark-grown calli were selected in the light for 4 weeks on embryogenic medium (ML1PSK; a silver nitrate-free ML1C2 medium in which the 2,4-D amount was halved and 30 nM α-PSK was added to promote embryogenesis) containing 50 mg/l kanamycin. Green sectors were then isolated using a stereomicroscope under sterile conditions, transferred to fresh selective medium and further incubated in the light (Fig. 3.16).
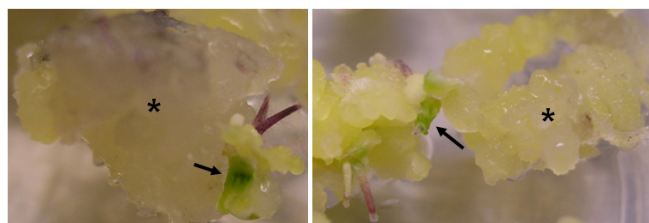
Figure 3.16 Isolation of the green callus tissues (indicated by arrows) from light-selected maize calli after plastid transformation using plasmid pMA1. The transformed calli were incubated for 4 weeks on ML1PSK medium (see Table 3.7). The isolated green sectors were transferred onto fresh medium for a further selection round and the remaining calli (shown by asterisks) were maintained for continued selection.

After 4 weeks, the green color disappeared from most of the isolated candidate lines while a few of them were still light-green. The isolated candidate lines as well as the remaining calli (continuously selected on the first selective medium) were then transferred onto ML1R3 regeneration medium containing 50 mg/l kanamycin. Unfortunately, no shoot regeneration could be observed after 4-8 weeks incubation on the selective medium in the light.

3.2 Modification of the carotenoid biosynthesis pathway in maize by expression of a recombinant daffodil *PSY* cDNA in the nuclear genome

3.2.1 Construction of vectors for engineering the carotenoid biosynthesis pathway in maize

The PMI/mannose selection system and the novel transformation method were used to integrate a recombinant daffodil phytoene synthase (*PSY*) gene into the maize genome. Thus, plasmids pMA5 and pMA6 (for details on the cloning strategy, see 2.1.7) containing *manA* (*pmi*) as a selectable marker gene and *PSY* (Fig 3.17), were constructed. Two different promoters were used to control *PSY* gene transcription in transgenic maize plants: (i) the constitutive CaMV 35S promoter, and (ii) the seed endosperm-specific 27 kDa zein gene promoter (PzmZ27) which is active in the endosperm from the early stages of seed development (Russell and Fromm, 1997). The endosperm-specific expression of *PSY* using PzmZ27 was chosen to minimize possible negative effects of the constitutive expression of the *PSY* on plant growth and development.
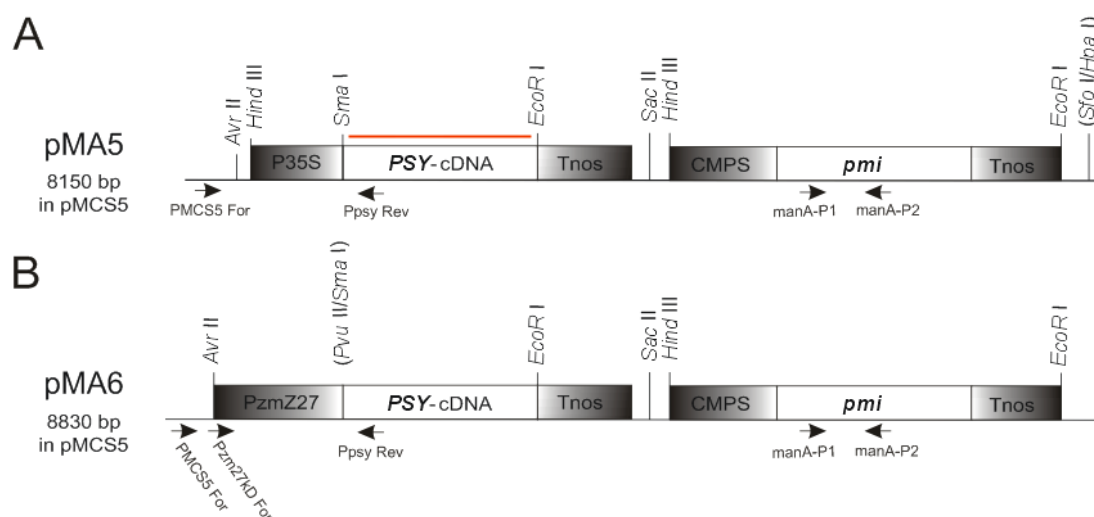
Figure 3.17 Physical maps of the nuclear transformation vectors used for modification of the carotenoid biosynthesis pathway in maize. (**A**) Map of the transformation vector pMA5. The daffodil phytoene synthase cDNA under control of the CaMV 35S promoter and the CMPS+pmi+Tnos (including the CMPS promoter, *pmi* sequence and the nopaline synthase gene terminator) cassette were cloned into the basic vector pMCS5 (for

details on the cloning strategy, see 2.1.7). (**B**) Physical map of plasmid pMA6. The 35S promoter of the phytoene synthase (*PSY*) gene in vector pMA5 was replaced by the PCR-amplified full-length (1.1 kb) maize seed endosperm-specific 27 kDa zein gene promoter (PzmZ27) (for details, see 2.1.7). The relevant restriction sites, the fragment used as a probe for nucleic acid gel blot analysis (labeled by a red line) and the primers used are shown. The orientation of the primers (used for PCR analysis) is indicated by arrows.

3.2.2 Creation of transgenic maize plants using plasmids pMA5 and pMA6

To demonstrate the applicability of the developed leaf-based transformation system for biotechnological purposes, we aimed to manipulate the carotenoid biosynthesis pathway in maize by expression of a recombinant daffodil PSY. In these experiments, one transgenic callus line was isolated from three shots per construct. Small callus pieces were then transferred to regeneration medium containing mannose as carbon source. The regeneration efficiency was comparable to that in the wild type and multiple shoots could be readily regenerated (Fig. 3.18).

Figure 3.18 Regeneration of maize plants transformed by the biolistic system using plasmids pMA5 (35S-PSY) and pMA6 (Z27-PSY). Transgenic lines were selected using the newly developed leaf-based transformation method on mannose-containing medium. Putative transgenic lines could be readily regenerated into shoots and were phenotypically comparable to the regenerated plantlets from wild-type (Wt) calli.

After root induction on mannose-containing medium, regenerated plants were transferred to the greenhouse for growth to maturity and seed production.

3.2.3 Molecular analysis of putative transformants to confirm the physical presence and expression of the recombinant *PSY*

Different assays were used to confirm the physical presence and expression of the *PSY* transgene in putative transgenic lines. PCR analysis was performed to demonstrate integration of the *pmi* gene as well as the *PSY* gene (Fig. 3.19). Also, the presence of different promoters controlling *PSY* gene transcription was proven by PCR assays (Fig. 3.19B-C). PCR analysis using a *pmi*-specific primer pair (manA-P1 and manA-P2; see 2.1.4 and Fig. 3.17) resulted in amplification of a 514 bp fragment from transgenic lines (35S-PSY and Z27-PSY) confirming integration of the *pmi* gene into the maize genome (Fig 3.19A). When a PzmZ27 promoter-

specific primer (PzmZ27kD For) in combination with a daffodil *PSY*-specific primer (Ppsy Rev) was used, a 1.31 kb fragment could be amplified from the selected line transformed with plasmid pMA6 confirming that in the line Z27-PSY, the integrated *PSY* gene is under control of the seed-specific PzmZ27 promoter. As expected, the fragment was absent from the line transformed with the plasmid pMA5 as well as in the wild-type (Fig. 3.19B). The primer PMCS5 For (Fig. 3.17) lands upstream of the promoters controlling the transcription of the *PSY* gene in plasmids pMA5 and pMA6. This primer in combination with the primer Ppsy Rev (Fig. 3.17) resulted in PCR amplification of a 0.66 kb fragment from the transgenic line created using plasmid pMA5, and a 1.35 kb fragment from the transgenic line obtained using plasmid pMA6, confirming the correct sizes of the two different promoters controlling *PSY* gene transcription in transgenic lines (Fig. 3.19C).
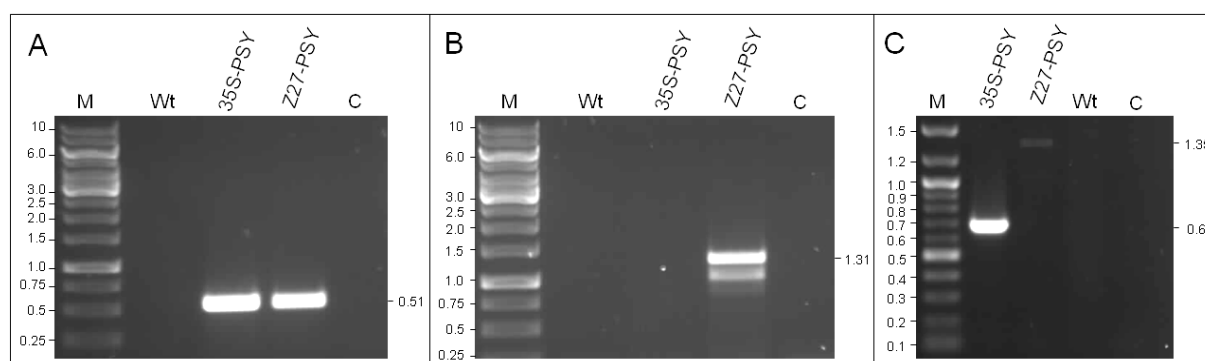
Figure 3.19 PCR analyses of transgenic maize plants to confirm the physical presence of the *pmi* selectable marker and the recombinant daffodil *PSY* genes. (**A**) PCR analysis using a *pmi*-specific primer pair (manA-P1 and manA-P2) resulted in amplification of a 514 bp fragment from both transgenic lines (35S-PSY and Z27-PSY) indicating that the isolated lines are indeed transgenic. (**B**) A PzmZ27 promoter-specific primer (PzmZ27kD For) in combination with a daffodil *PSY*-specific primer (Ppsy Rev) resulted in PCR amplification of a 1.31 kb fragment from the line transformed with plasmid pMA6, indicating successful integration of the *PSY* gene under control of the seed-specific PzmZ27 promoter into the maize genome. (**C**) PCR analysis using PMCS5 For and Ppsy Rev primer pair resulted in amplification of a 0.66 kb fragment from the transgenic line created using plasmid pMA5, and a 1.35 kb fragment from the transgenic line obtained using plasmid pMA6, confirming the correct sizes of the two different promoters (35S and PzmZ27) controlling *PSY* gene transcription in transgenic lines. Fragment sizes of the molecular weight marker (M) are given in kb. Wt: wild type, C: buffer control.

Southern blot analysis was used to determine the copy numbers of the *PSY* transgene integrated into the maize genome. These experiments revealed the integration of multiple transgene copies (more than 8 copies) in the line 35S-PSY (Fig. 3.20A) using a *PSY*-specific probe. In contrast, the transgene copy number was lower in the line Z27-PSY as evidenced by Southern blot analysis (Fig. 3.20A).

Northern blot analysis was used to investigate transcription of the *PSY* gene in leaves of the transgenic lines (Fig. 3.20B). Total RNA was extracted from leaves of three-week old greenhouse-grown plants (Fig. 3.21) and equal amounts (~10 µg) were used for Northern blot analysis. Using a daffodil *PSY* cDNA probe, recombinant *PSY* transcripts were detectable in both transgenic lines. Although the PzmZ27 promoter has been described as an endosperm-specific promoter, Northern blot results revealed the activity of this promoter in maize leaves. However, differences in transcript abundance in seeds and leaves remain to be investigated. The results also showed that most of the *PSY* transcripts in line 35S-PSY are degraded (Fig. 3.20B). Similar sequence-specific degradation of transgene-derived transcripts has been reported in several studies when multiple copies of a transgene are inserted into the genome of transgenic plants (Vaucheret *et al.*, 1998). Integration of a transgene in particular loci is also known to produce signals initiating sequence-specific RNA degradation (Vaucheret *et al.*, 1998). However, intact *PSY* transcripts (~1.6 kb) could be detected in this line at a comparable level as in the Z27-PSY transgenic line (Fig. 3.20B).
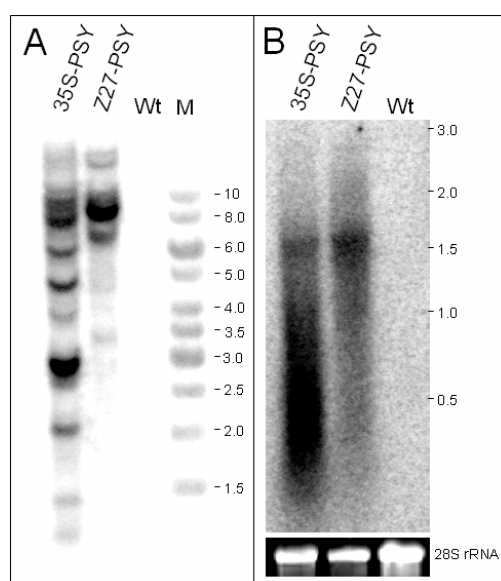
Figure 3.20 Nucleic acid gel blots to confirm integration of the *PSY* gene into the maize genome and analyze its expression. (**A**) Southern blot analysis using a *PSY*-specific probe. Ten micrograms of total genomic DNA digested with the enzyme *Hind* III were used. Multiple transgene copies were detected in both lines, although lower transgene copies have been integrated in the Z27-PSY transgenic line. (**B**) Northern blot analysis to investigate expression of the *PSY* transgene. *PSY* gene transcripts were detected in both transgenic lines indicating that both 35S and PzmZ27 promoters are active in leaves. However, most of the *PSY* transcripts in 35S-PSY line appear to be degraded. Such sequence-specific RNA degradation is known to occur when multiple copies of a transgene are inserted into the genome of transgenic plants (Vaucheret *et al.*, 1998). An ethidium bromide stained agarose gel is shown in the bottom as a loading control, and the 28S ribosomal RNA is represented. Fragment sizes of the molecular weight markers are indicated at the right in kb.

3.2.4 Analysis of carotenoid content in transgenic maize plants expressing daffodil *PSY*

To test whether expression of the daffodil PSY led to altered carotenoid levels in transgenic maize plants, HPLC analysis was performed using three-week old greenhouse-grown plants. These analyses revealed that both transgenic lines accumulate around 17% more carotenoids in leaves in comparison to wild type plants. Importantly, there were no phenotypic abnormalities in morphology and/or development of the transgenic plants (Fig. 3.21).
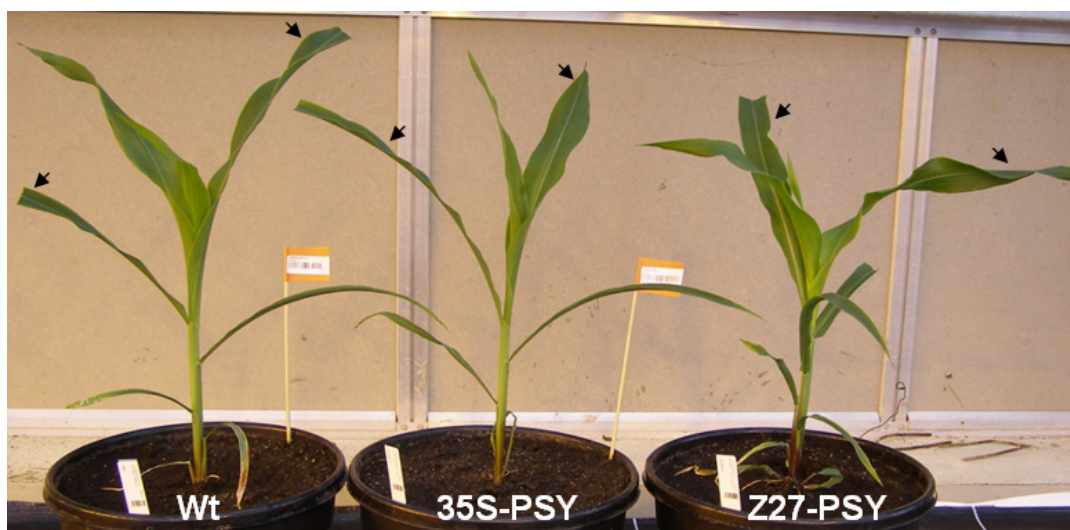
Figure 3.21 Three-week old transgenic maize plants (35S-PSY and Z27-PSY) growing in the greenhouse. A wild-type control is shown at the left. The leaf material used for isolation of RNA and pigments (carotenoids and chlorophylls) is indicated by arrows.

In line 35S-PSY expressing the daffodil *PSY* under control of the 35S promoter, increased levels of several carotenoids, including neoxanthin (28.4%), violaxanthin (20.4%), and lutein (16.8%) were observed. Similar results were also obtained for line Z27-PSY expressing the *PSY* under control of the PzmZ27 promoter (Table 3.9 and Fig. 3.22). In contrast, zeaxanthin levels remained remarkably constant and antheraxanthin contents were almost unaltered (Table 3.9). Similar results, including unaltered levels of zeaxanthin, have been obtained in transgenic *Arabidopsis* plants overexpressing an endogenous phytoene synthase gene (Lindgren *et al.*, 2003).

The chlorophyll levels were slightly increased in transgenic lines (~7% in 35S-PSY and ~5% in Z27-PSY; Table 3.9). Increased chlorophyll levels were unexpected because, geranylgeranyl pyrophosphate (GGPP) provides the substrate for the biosynthesis of phytoene as well as the phytol tail of chlorophyll (Fig. 1.3), and therefore competition between chlorophyll and carotenoid synthesis was expected.

Table 3.9 Carotenoid and chlorophyll contents in leaves of wild-type and transgenic maize plants (35S-PSY and Z27-PSY) expressing daffodil *PSY*. The amounts are represented in µg/g dry-weight. Results are represented as mean (from nine independent measurements) ± standard deviation.

Compound	Wild-type	35S-PSY	Z27-PSY
Neoxanthin	57.9 ± 4.1	74.3 ± 6.7 ***	76.4 ± 7.5 ***
Violaxanthin	279.2 ± 13.6	336.2 ± 20.2 ***	320.4 ± 9.2 ***
Antheraxanthin	213.4 ± 8.4	205.7 ± 6.7 [a]	201.6 ± 37.0 [n.s]
Lutein	651.9 ± 54.6	761.7 ± 44.3 ***	760.0 ± 21.3 ***
Zeaxanthin	102.6 ± 8.9	104.9 ± 5.9 [n.s]	107.2 ± 11.3 [n.s]
Chlorophyll B	3318.0 ± 238.6	3602.6 ± 210.2 *	3541.8 ± 151.6 *
Chlorophyll A	12027.0 ± 795.8	12843.7 ± 538.6 *	12595.9 ± 718.6 [n.s]
β-carotene	1310.7 ± 108.4	1585.5 ± 111.4 ***	1587.6 ± 93.8 ***
Total carotenoid	2615.7 ± 191.4	3068.3 ± 184.6 ***	3053.2 ± 85.4 ***
Total chlorophyll	15345.0 ± 1024.8	16446.3 ± 739.2 *	16137.6 ± 867.8 [n.s]

Values showing a significant increase in comparison to the wild-type levels are labeled by asterisks. "***": P value from T-test < 0.0001; "*": P value < 0.05. "[a]": The amount was significantly (P < 0.05) decreased compared to the wild-type. "[n.s]": Statistically not significant.

Interestingly, leaves from both transgenic lines also contained 21% higher *β*-carotene (pro-vitamin A) content in comparison to the Wt leaves (Table 3.9 and Fig. 3.22). Similarly strongly enhanced carotenoid contents in both transgenic lines correlated with comparable levels of *PSY* transcript accumulation in Northern blot analysis (Fig. 3.20).
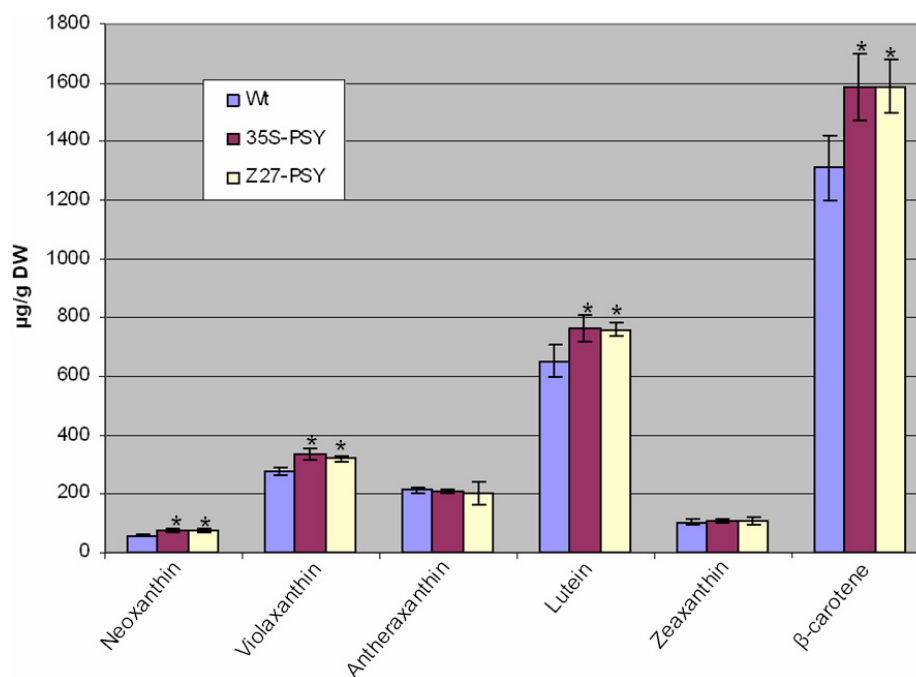
Figure 3.22 Carotenoid contents in leaves of wild-type (Wt) and transgenic maize plants (35S-PSY and Z27-PSY) expressing a recombinant daffodil *PSY*. Most carotenoids, including neoxanthin, violaxanthin, lutein and *β*-carotene, were significantly increased in transgenic lines. Zeaxanthin and antheraxanthin amounts stayed almost constant. "*": significantly (P < 0.0001) higher than the wild-type level.

3.3 Towards development of transformation technologies in *Peperomia*

3.3.1 Development of an efficient leaf-based regeneration system for *Peperomia*

The large size of the chloroplasts in *P. metallica* provides a unique feature to study plastid biology in higher plants. However, the lack of a workable genetic transformation system significantly restricts these studies. Therefore, we attempted to develop a transformation system for *Peperomia*. In this part, an efficient leaf-based regeneration protocol was developed for *Peperomia* species. First, RM medium (see 2.1.10) supplemented with various hormone combinations at different levels (Fig. 3.23) was tested. Leaves from plants grown under aseptic conditions were cut in 0.5×0.5 cm pieces and placed on the medium with their adaxial side. When IAA and zeatin (Zea) were added to the medium, no regeneration was observed. Replacing Zea with BAP resulted in a very poor *in vitro* regeneration response. When GA_3 was applied in addition to IAA and BAP, all leaf segments responded well to *in vitro* regeneration resulting in 100% regeneration efficiency from cultured leaf explants. These results suggest that there is a strong cross-talk among various hormones to stimulate regeneration of plants from somatic cells, and that gibberellic acid plays a significant role in the response of *Peperomia* somatic cells to *in vitro* regeneration.
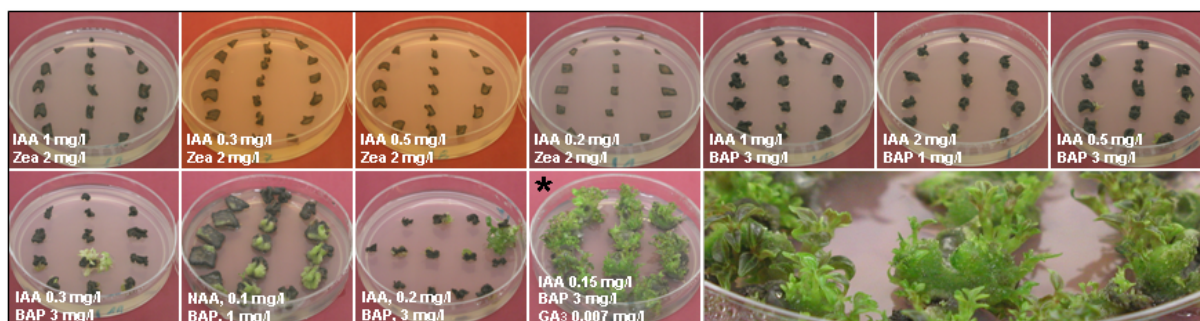
Fig. 3.23 *In vitro* regeneration response of *P. metallica* leaf tissues cultured on different media containing various hormone combinations. The PIBG medium (2.1.10) containing IAA, BAP and GA_3 resulted in 100% regeneration response from leaf segments (indicated by an asterisk). A close-up of the regenerated plantlets from leaf segments is shown in the lower right corner.

3.3.2 Evaluation of the sensitivity of *Peperomia* leaf tissue to kanamycin in *in vitro* cultures

In order to determine the optimal concentration of kanamycin to select transgenic *Peperomia* plants, several experiments were conducted to test regeneration from leaves in the presence of different levels of the selection agent (from 30 mg/l to 200 mg/l). Results showed that, in general, *P. pedunculosa* leaf cells are less sensitive to kanamycin under regenerative conditions than *P. metallica*: While addition of 100 mg/l kanamycin was sufficient to inhibit

regeneration from *P. metallica* leaf cells, shoot formation could be observed from *P. pedunculosa* explants under the same conditions (Fig. 3.24).
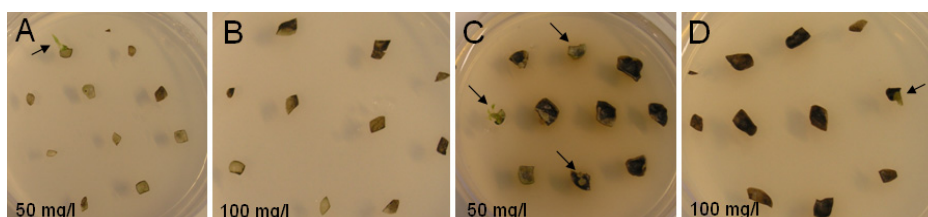
Figure 3.24 Evaluation of *P. metallica* (A,B) and *P. pedunculosa* (C,D) leaves for their sensitivity to kanamycin. Addition of 50 mg/l kanamycin significantly inhibited regeneration from leaf segments (A,C) of both species. However, a few shoots could still be regenerated (indicated by arrows). Regeneration of *P. metallica* was completely inhibited on 100 mg/l kanamycin-containing medium (B), while regenerants (indicated by arrows) could still be obtained from *P. pedunculosa* cultures.

3.3.3   Construction of vectors for developing a transformation system in *Peperomia*

For *Peperomia* nuclear transformation experiments, vectors pMA2 and pMA3 (Fig. 3.25) were constructed, in which the *GFP* reporter gene was fused to the Rubisco small subunit transit peptide to target GFP protein into plastids (to facilitate studying of non-green plastids in different tissues of *Peperomia* species by fluorescent labeling). The reporter gene was controlled by the CaMV 35S promoter and the nopaline synthase terminator. The marker gene *nptII* (conferring resistance against kanamycin) was used for selecting transgenic cell lines.
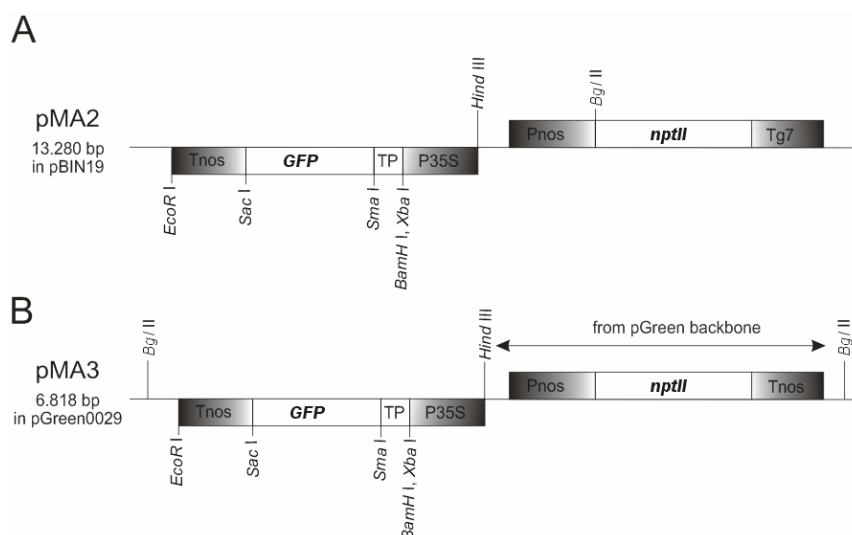
Figure 3.25 Map of the plasmids pMA2 (in pBIN19) and pMA3 (in pGreen0029) used for developing nuclear transformation in *Peperomia*. Two vectors differ in their size and vector backbone. Furthermore, in some experiments, the linear *Bgl* II fragment was excised from the vector pMA3 (not feasible in vector pMA2) and used for transformation. Higher transformation efficiency using linear plasmid DNA has been reported in several studies (Sabri *et al.*, 1996; Lin *et al.*, 1997). The *GFP* is fused to the Rubisco small subunit transit peptide (TP) to target GFP into plastids. The *nptII* gene under control of the nopaline synthase promoter was used as a selectable marker.

3.3.4 Transformation experiments

For transformation, gold particles of 0.6 μm were coated with pMA2 or pMA3 and bombarded onto leaf discs followed by cutting of the leaves in small pieces and placing them on regeneration medium containing kanamycin. When 100 mg/l kanamycin was applied, several hundred plantlets regenerated from leaves of both species, especially *P. pedunculosa* (Fig. 3.26). Most of the regenerated plants were investigated for GFP expression in plastids, but no GFP signal could be observed. In addition, when regenerated plantlets were transferred to root induction medium (RM, see 2.1.10) containing 100 mg/l kanamycin, no root formation could be obtained. This indicated that the regenerated plantlets were not transgenic and that 100 mg/l kanamycin does not inhibit regeneration of Wt cells completely. Therefore, second series of transformation experiments was conducted using higher concentrations of kanamycin (150 and 200 mg/l). For both species, unfortunately, no transgenic line accumulating GFP in plastids could be recovered.

Figure 3.26 Shoot regeneration (indicated by arrows) from leaves of *P. metallica* (**B**) and *P. pedunculosa* (**C**) on kanamycin-containing medium (100 mg/l) following biolistic transformation using plasmid pMA3. A control plate with wild-type *P. metallica* plants regenerated on antibiotic-free medium is also shown (**A**).

3.3.5 Chloroplast size and number in *Peperomia* species

Peperomia metallica has been reported to contain giant chloroplasts in its palisade parenchyma cells (Schürhoff, 1908). However, little is known about plastids in other *Peperomia* species. Furthermore, whether plastid size and number is controlled in a tissue-specific or plastid type-specific manner, is not yet understood. There is also no report about other plastid types, such as root amyloplasts, in *Peperomia*. Thus, although biolistic gun-based transformation of the *Peperomia* genome failed, we analyzed plastid size and number in several species of the *Peperomia* genus and in different tissues.

First, chloroplast size and numbers were investigated in four different species of *Peperomia* (Figs. 3.27 and 3.28; Table 3.10). Results showed that one of these species, *P. pedunculosa*, harbors giant chloroplasts in palisade parenchyma cells (Figs. 3.27D and 3.28D), as has been reported previously for *P. metallica* (Schürhoff, 1908; Fig. 3.28E-F). Interestingly, palisade cells from *P. serpens* contained normal chloroplasts that were comparable in size and number

per cell to chloroplasts in tomato (Figs. 3.27A,B and 3.28A,B; Table 3.10). Another *Peperomia* species, *P. argyreia*, represents an interesting intermediate in that chloroplast size is similar to that in a typical higher plant (Figs. 3.27C and 3.28C), but yet the chloroplast number in palisade cells is similarly low as in the two species with giant chloroplasts, *P. pedunculosa* and *P. metallica*. This striking finding is readily explained by the fact that *P. argyreia* has much smaller palisade cells than the other species (Fig. 3.27A-D; Table 3.10). It thus seems reasonable to assume that *P. argyreia* compensates for its smaller palisade cells by a reduction in chloroplast number while organelle size is kept constant.
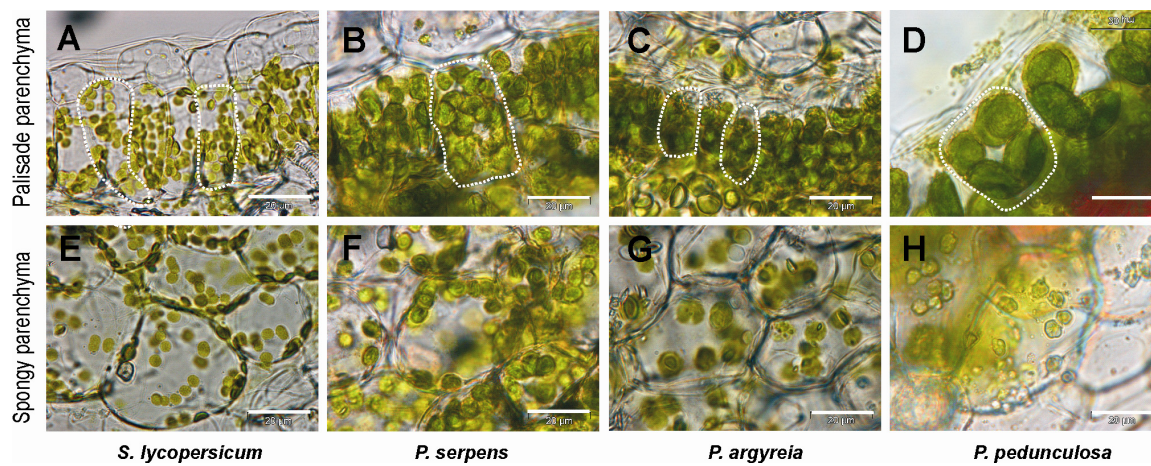
Figure 3.27 Chloroplasts in leaf mesophyll cells of tomato (*Solanum lycopersicum*), *Peperomia serpens*, *P. argyreia* and *P. pedunculosa*. (**A-D**) Chloroplasts in palisade cells of tomato and the three *Peperomia* species. For clarity, the cell walls of single palisade cells are marked by white dotted lines. (**E-H**) Chloroplasts in spongy mesophyll cells of the four species. Images were taken from thin leaf cross sections using high-resolution light microscopy. Scale bars: 20 μm.

Interestingly, when chloroplast size in spongy parenchyma cells was investigated, no correlation was found with chloroplast size in the palisade parenchyma (Fig. 3.27; Table 3.10). In fact, chloroplasts in spongy parenchyma cells had comparable sizes in all *Peperomia* species (Fig. 3.27F-H; Table 3.10) suggesting that chloroplast size (and perhaps division) are differently regulated in different leaf cell types.

Results of the comparison of chloroplast numbers per cell in the four investigated *Peperomia* species are shown in Fig. 3.29 and Table 3.10. The data reveal several interesting aspects: (i) While *P. serpens* has similar chloroplast numbers in palisade and spongy parenchyma cells, this is not the case in the three other *Peperomia* species. (ii) The low number of palisade cell chloroplasts in *P. pedunculosa* and *P. metallica* is compensated for by a more or less proportional increase in organelle size thus keeping the volume occupied by the plastid compartment per cell fairly constant. (iii) Although *P. argyreia* has similar-sized palisade

Table 3.10 Cell area and chloroplast (CP) sizes (diameter) and numbers in leaf mesophyll cells of tomato (S.lyc.), *Peperomia serpens* (P.ser.), *P. argyreia* (P.arg.), *P. pedunculosa* (P.ped.) and *P. metallica* (P.met.)

	S.lyc.	P.ser.	P.arg.	P.ped.	P.met.
Cell area in palisade parenchyma (μm^2)	883.63 ± 29.08	1142.33 ± 65.43	195.63 ± 9.64	975.62 ± 34.89	987.19 ± 50.12
CP size in palisade parenchyma (μm)	6.25 ± 1.14	7.32 ± 1.04	6.69 ± 1.66	16.65 ± 3.39	16.55 ± 4.74
CP size in spongy parenchyma (μm)	5.10 ± 0.65	6.05 ± 0.77	5.72 ± 0.74	5.93 ± 1.17	5.90 ± 1.22
CP No. in palisade parenchyma	28.59 ± 9.54	16.77 ± 3.33	2.90 ± 1.0	2.62 ± 0.80	2.24 ± 0.61
CP No. in spongy parenchyma	19.13 ± 6.30	17.13 ± 3.90	8.85 ± 1.72	7.39 ± 2.0	6.52 ± 2.24

chloroplasts as tomato and *P. serpens*, it shows the same chloroplast numbers in spongy and palisade parenchyma cells as the species with giant chloroplasts, *P. pedunculosa* and *P. metallica* (Fig. 3.29).
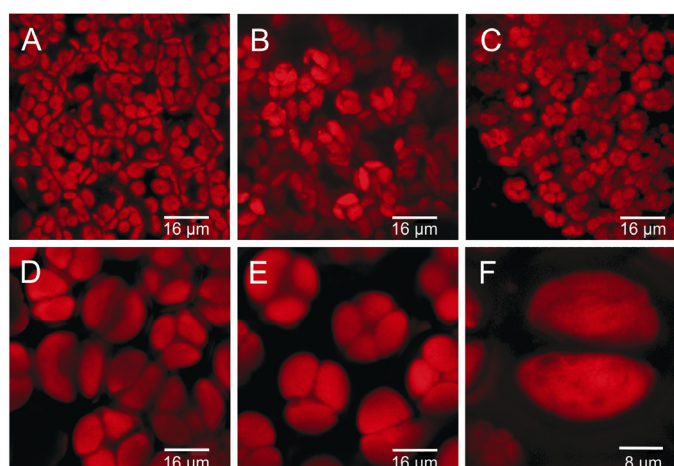
Figure 3.28 Visualization of chloroplasts by confocal laser-scanning microscopy based on chlorophyll autofluorescence in leaf palisade cells of tomato (**A**), *P. serpens* (**B**), *P. argyreia* (**C**), *P. pedunculosa* (**D**), and *P. metallica* (**E**). While *P. argyreia* has the same low number of chloroplasts per cell as *P. pedunculosa* and *P. metallica*, the chloroplasts are much smaller (and comparable in size with tomato chloroplasts). (**F**) Close-up of a single leaf palisade cell from *P. metallica* containing two giant chloroplasts.

This may suggest two possible explanations: (i) a mechanistically similar mode of regulating plastid division operates in *P. argyreia*, *P. pedunculosa* and *P. metallica* and the potential of *P. argyreia* to develop giant chloroplasts in palisade cells is masked by the small cell size in the palisade parenchyma, or (ii) the chloroplast division mechanism in palisade cells of *P. argyreia* is similar to that in tomato and *P. serpens* palisade cells and the small palisade cell size has limited the ability of *P. argyreia* to produce chloroplasts in a higher number.
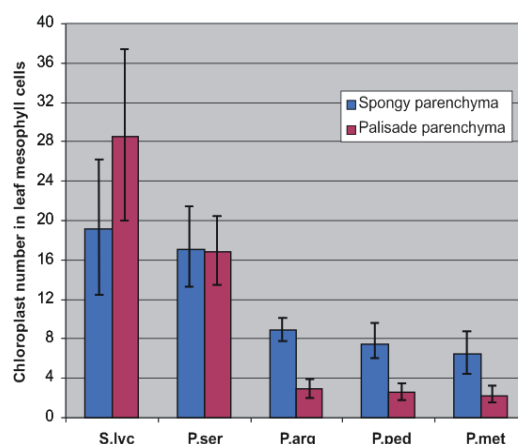
Figure 3.29 Chloroplast numbers in leaf mesophyll cells of tomato (S.lyc.), *P. serpens* (P.ser.), *P. argyreia* (P.arg.), *P. pedunculosa* (P.ped.) and *P. metallica* (P.met.). While chloroplast number in spongy parenchyma cells of *P. argyreia*, *P. pedunculosa* and *P. metallica* is already significantly lower than in *P. serpens*, it is drastically reduced in leaf palisade cells.

3.3.6 Amyloplasts in *Peperomia* species

Light microscopy studies were also conducted to analyze non-green amyloplasts in root cells. One species from each group of plants showing a similar pattern in their chloroplast size and number in mesophyll cells, were analyzed. With high-resolution light microscopy studies of thin root sections (~100 µm), amyloplasts could be visualized without any treatment or staining technique (Fig. 3.30A-C). To demonstrate that these compartments are indeed amyloplasts, iodine staining was performed to label starch grains as a marker for amyloplasts. As expected, the observed compartments were stained with iodine indicating that they are starch-storing amyloplasts (Fig. 3.30).
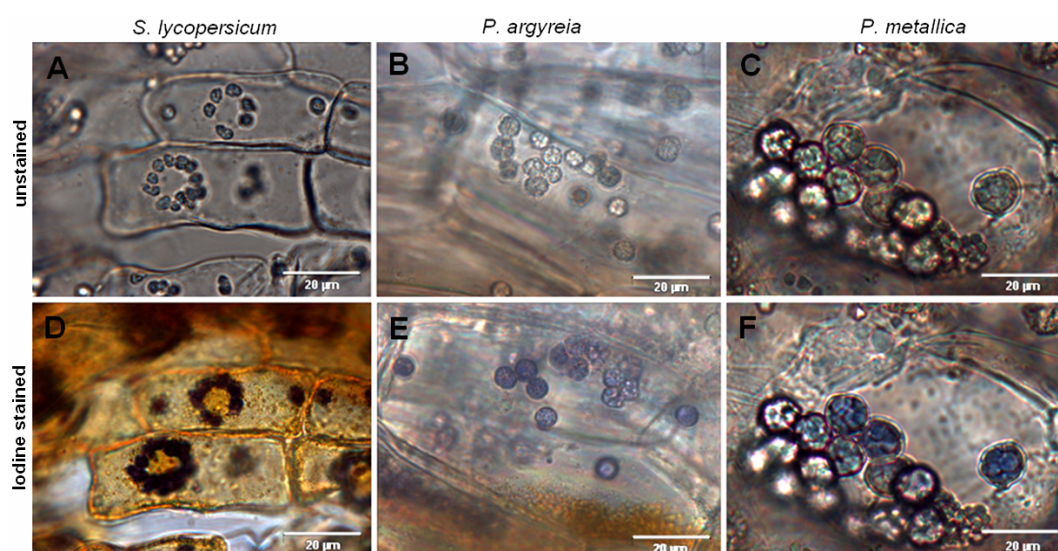
Figure 3.30 High-resolution light microscopy images from root cells of *S. lycopersicum* (A,D), *P. argyreia* (B,E) and *P. metallica* (C,F). Amyloplasts in unstained root cells could be observed using light microscopy (A-C) as confirmed by iodine staining of the starch grains (D-F). Scale bars: 20 µm.

Interestingly, a correlation was observed between chloroplast size in the palisade cells and amyloplast size in root cells (Table 3.11; Fig. 3.31): *P. metallica* with giant chloroplasts in the palisade parenchyma also had big amyloplasts in roots, whereas *P. argyreia* with normal-sized chloroplasts in palisade cells had amyloplasts of similar size as in tomato root cells.

Table 3.11 Amyloplast size (diameter) in root cells and chloroplast (CP) size (diameter) in leaf mesophyll cells of tomato, *P. argyreia* and *P. metallica*. Results are represented as mean of 100-330 measurements for chloroplasts and 150-250 measurements for amyloplasts. Standard deviations are also indicated.

	S. lycopersicum	*P. argyreia*	*P. metallica*
Amyloplast size in root cells (μm)	4.24 ± 0.63	5.52 ± 0.70	11.65 ± 2.43
CP size in palisade parenchyma (μm)	6.25 ± 1.14	6.69 ± 1.66	16.55 ± 4.74
CP size in spongy parenchyma (μm)	5.10 ± 0.65	5.72 ± 0.74	5.90 ± 1.22

The good correlation between chloroplast size in the palisade parenchyma and amyloplast size in roots becomes obvious when chloroplast sizes in spongy parenchyma and palisade parenchyma cells are compared with amyloplast size (Fig. 3.31). While all species had chloroplasts of similar size in their spongy parenchyma cells, the sizes of giant chloroplasts in *P. metallica* and *P. pedunculosa* palisade cells corresponded well to the sizes of big amyloplasts in root cells.
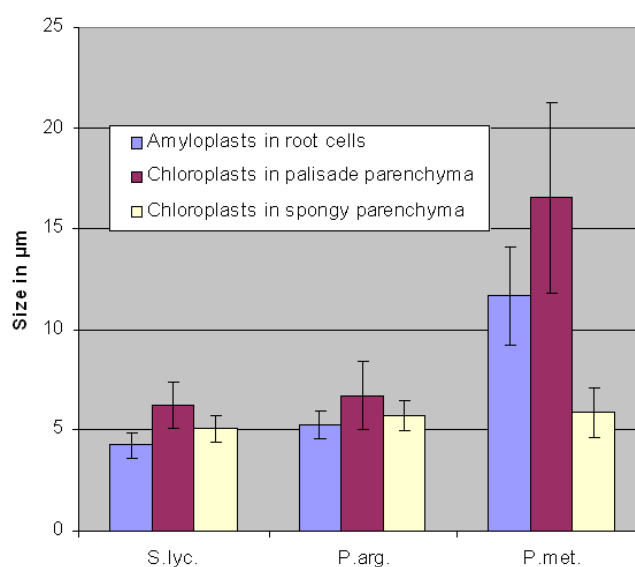
Figure 3.31 Correlation between chloroplast size in the palisade cells and the size of amyloplasts in roots. Data are shown for tomato (S.lyc.), *P. argyreia* (P.arg.) and *P. metallica* (P.met.).

3.3.7 Detection of chloroplast stromules by light microscopy

Stromules are stroma-filled tubules that originate from chloroplasts as well as non-green plastids (reviewed e.g. in Natesan *et al.*, 2005). In some cases, stromules can connect neighboring plastids and thus mediate the exchange of at least some proteins, such as GFP, between plastids (Kwok and Hanson, 2004a), although some studies have suggested that such an exchange may not occur on a regular basis (Shiina *et al.*, 2000). Mainly, stromules have been detected by visualizing them via GFP labeling and confocal laser-scanning microscopy (Kwok and Hanson, 2004a, b; Pyke and Howells, 2002; Waters *et al.*, 2004). Although recent data obtained from video microscopy studies illustrated interaction of stromules with cytoskeletal strands, their exact function remains obscure. The observation that defective chloroplasts lacking the *clpP* gene (encoding an essential protease subunit) cannot be metabolically complemented by wild-type chloroplasts present in the same cell has been interpreted as strong evidence against a functional relevance of stromules (Shikanai *et al.*, 2001). Therefore, we took advantage of the presence of giant chloroplasts in palisade cells of *Peperomia metallica* and attempted to visualize stromules in the absence of GFP expression or tissue fixation for electron microscopy. When giant chloroplasts in palisade cells were investigated by high-resolution light microscopy, neighboring chloroplasts being interconnected by tubular structures were indeed observed (Fig. 3.32). This proves unambiguously that stromules are a genuine morphological feature of higher plant chloroplasts and provides light-microscopic confirmation for stromules forming plasmatic connections between chloroplasts.
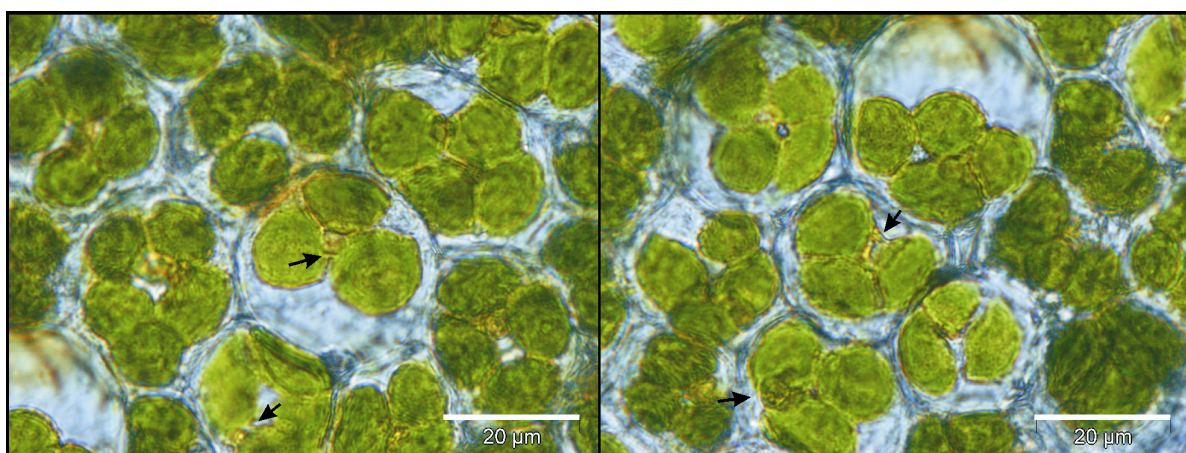
Figure 3.32 Chloroplast networks in *P. metallica* palisade cells as visualized by high-resolution light microscopy. Arrows are indicating examples of interchloroplastic connections between chloroplasts (stromules).

3.3.8 Detection of bacteria-like microorganisms in cells of *Peperomia* species

During light microscopy analysis, small bacteria-like bodies were observed frequently in the mesophyll cells of *P. metallica* and *P. pedunculosa* (Fig. 3.33). Using confocal microscopy, it was confirmed that these microorganisms lack chlorophyll autofluorescence. The microorganisms appear to undergo division (via binary fission) as revealed by light microscopy studies (Fig. 3.33). However, when leaf extract from *Peperomia* plants were cultured on various minimal or rich bacterial media, bacteria-like microorganisms could not been cultured. Bacterial endophytes have been identified in several plant species including maize (reviewed in Rosenblueth and Martinez-Romero, 2006). However, nearly all known endophytic bacteria live in the intercellular space of plant tissues and not inside plants cells, while in *Peperomia*, the microorganisms were observed within cells (intracellular endophyte). This striking finding together with interesting observations from our microscopic studies suggests that *Peperomia* species provide a useful model system not only for studying plastid biology but also for investigating plant-bacteria interactions to understand mechanisms involved in infection and colonization of the plant tissues by such microorganisms.
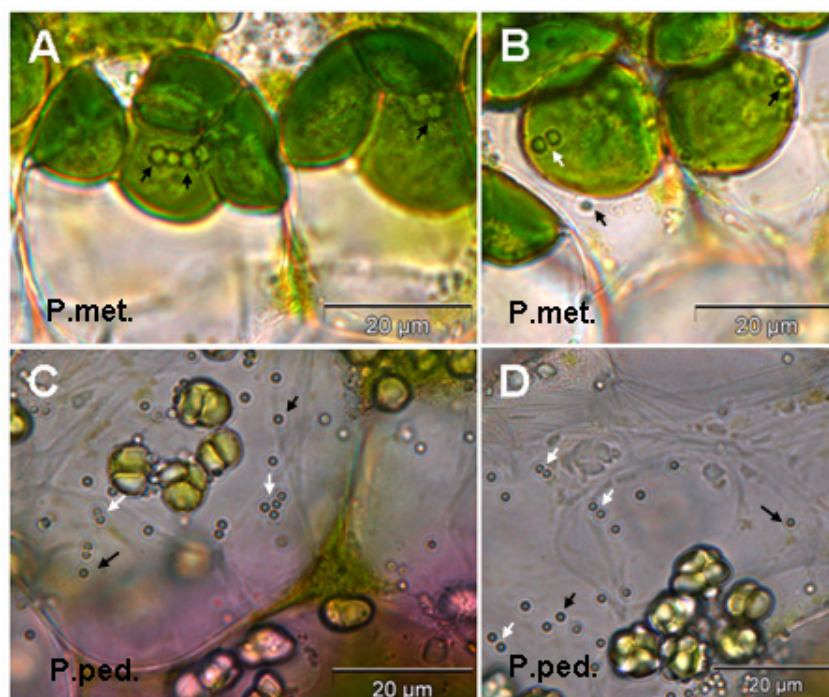
Figure 3.33 Bacteria-like microorganisms (indicated by arrows) located within leaf mesophyll cells of *P. metallica* (A,B) and *P. pedunculosa* (C,D). White arrows show examples of actively dividing microorganisms via binary fission. Scale bars: 20 µm.

4   Discussion

4.1   Development of novel transformation technologies in maize

Plastid transformation provides great advantages over nuclear transformation, such as transgene containment and high levels of foreign protein expression (Bock, 2001; Daniell, 2002). As cereals, such as maize, are the world's most important food crops and the commercialization of transgenic corn expressing herbicide resistances and/or *Bt* genes has raised serious environmental and political concerns (most of which are related to outcrossing, uncontrolled spreading of transgenes via pollen and unwanted contamination of the food chain), the development of a plastid transformation system for cereal species is highly desirable (Bock and Khan, 2004; Maliga, 2004; Daniell *et al.*, 2005). Establishing a stable plastid transformation system in a higher plant requires several important steps. Reliable tissue culture systems allowing repeated rounds of plant regeneration are of particular importance to organelle transformation. In principle, two sources of material can be used for plastid transformation: (i) regenerable callus cultures (Kumar *et al.*, 2004a; Langbecker *et al.*, 2004; Lee *et al.*, 2006), or (ii) leaf tissue (Svab *et al.*, 1990; Carrer *et al.*, 1993; Ruf *et al.*, 2001; Lelivelt *et al.*, 2005; Okumura *et al.*, 2006). Most dicotyledonous plant species, such as tobacco, are able to regenerate efficiently via organogenesis in the light, providing a highly efficient *in vitro* regeneration system to recover transplastomic plants from single transgenic cells. In contrast, monocots, especially *Poaceae* (such as maize), are recalcitrant to regeneration via organogenesis and generally regenerate through somatic embryogenesis, that often requires an incubation phase in the dark.

Chloroplast transformation requires multiple rounds of regeneration and selection to eliminate wild-type plastid genomes and achieve a homoplasmic transplastomic state (Bock, 2001), an essential prerequisite for obtaining genetically stable plastid-transformed plants. These multiple cycles of selection and regeneration are usually conducted by taking leaf explants from primary transformants and subjecting them to a new regeneration cycle under stringent antibiotic selection (Bock, 2001; Maliga, 2004). In fact, the impossibility to carry out these additional regeneration rounds in cereals is considered the major obstacle to the implementation of chloroplast transformation in maize and rice (Khan and Maliga, 1999): While chloroplast-transformed rice cell lines could be obtained at reasonable frequencies, all attempts to stabilize the lines and purify them to homoplasmy have failed (Khan and Maliga, 1999; Lee *et al.*, 2006).

Thus, for developing plastid transformation technology in maize, the use of conventional callus cultures (mainly based on immature embryo culture not allowing repeated rounds of

regeneration) seems impractical. One of the advantages of the leaf-based system (Ahmadabadi *et al.*, 2006) for the development of chloroplast transformation in corn is that primary chloroplast-transformed plants can be subjected to additional rounds of regeneration facilitating the isolation of genetically stable homoplasmic plant lines.

In addition, this method may be an attractive and cost-effective alternative to currently available tissue culture and transformation systems. It eliminates the need for the laborious and time-consuming procedures involved in establishing and propagating callus cultures from immature embryos. Also, it does not require mature maize plants, and regeneration-competent calli can be obtained from leaf segments of the young seedlings which can be produced in a few days. Thus, this system is independent of greenhouse facilities. In addition, for many maize genotypes, available tissue culture systems remain onerous, laborious or even impossible. Therefore, the described tissue culture system may increase the range of maize germplasms responsive to *in vitro* regeneration and genetic transformation.

Successful delivery of the transformation vector into the cell and transgene integration into the target genome is another important step in the genetic transformation of plants. Using phosphomannose isomerase (*pmi*), a well established selectable marker gene for selecting nuclear transgenic maize lines in the dark (Negrotto *et al.*, 2000; Wright *et al.*, 2001), we demonstrated that the embryogenic calli derived from maize leaves are suitable target material for nuclear (and presumably also for plastid) transformation producing up to four transgenic lines per shot (Table 3.6). The transgene was successfully inherited into the T1 and T2 generations as confirmed by different assays (Fig. 3.9).

In this study, dark-grown embryogenic callus material was used as target tissue in most of the experiments towards developing a plastid transformation system in maize. While in dark-grown cultures, plastids are relatively small, undeveloped and dispersed through the cell (Waters *et al.*, 2004), leaf tissues, as a frequently used explant for plastid transformation experiments, contain a high number of developed chloroplasts. This was thought to be an important factor for efficient transgene delivery into the green chloroplast following biolistic transformation, elevating plastid transformation efficiency. However, despite these obvious differences, it has been shown that plastid transformation of tobacco suspension cells (containing undeveloped plastids) is at least as efficient as the chloroplast-containing leaf system (Langbecker *et al.*, 2004). This was also demonstrated in earlier works where plastid transformation in rice became feasible using suspension cells (Khan and Maliga, 1999), although the transplastomic lines remained heteroplasmic. Also, transient gene expression has been reported in photosynthetically active chloroplasts (Sporlein *et al.*, 1991) as well as in

non-green plastid types (Daniell, 1993; Seki *et al.*, 1995; Hibberd *et al.*, 1998). Recently, successful plastid transformation by somatic embryogenesis and the use of non-green cells as target material for biolistic bombardment has been reported for carrot (Kumar *et al.*, 2004b), cotton (Kumar *et al.*, 2004a), and soybean (Dufourmantel *et al.*, 2004). These results indicate that, in general, there is no fundamental barrier to transforming plastid types other than chloroplasts.

The lack of reliable selectable markers seems to be another major obstacle for developing plastid transformation in monocots. Both the *aadA* and *nptII* selectable marker genes were used, allowing to test both selection systems in parallel or in combination to recover transplastomic lines. By far, *aadA* (conferring resistance against spectinomycin and streptomycin) has been the most frequently used dominant selectable marker gene for establishing plastid transformation systems in several dicotyledonous plant species (O'Neill *et al.*, 1993; Svab and Maliga, 1993; Koop *et al.*, 1996; Sidorov *et al.*, 1999; Ruf *et al.*, 2001; Skarjinskaia *et al.*, 2003; Langbecker *et al.*, 2004; Dufourmantel *et al.*, 2004; Zubko *et al.*, 2004; Lelivelt *et al.*, 2005; Kanamoto *et al.*, 2006; Okumura *et al.*, 2006). Generally, primary antibiotic resistant lines are selected on spectinomycin-containing medium and then subjected to streptomycin-containing medium to eliminate spontaneous mutations. However, monocots are intrinsically resistant to spectinomycin (Fromm *et al.*, 1987). Our results show that the application of streptomycin also does not efficiently inhibit callus growth in the dark (Fig. 3.12). On the other side, transformation via somatic embryogenesis often requires selection phase in the dark where proliferation and embryo formation is more efficient, allowing single transgenic cells to produce new regeneration-competent embryos. Thus, when the *aadA*/streptomycin-resistance selection system was carried out to select plastid transformants in the dark, there was a huge background of wild-type (Wt) calli surviving under the selective conditions and, probably for this reason, transgenic lines could not be recovered. This is presumably because, in the absence of an efficient growth inhibitor, no selective advantage remains for transgenic cell lines during the selection phase in the dark. *nptII* is another dominant selectable marker gene which has been used for plastid transformation in tobacco (Carrer *et al.*, 1993). However, it has been rarely used to develop chloroplast transformation systems in other plant species. Our antibiotic sensitivity tests show that kanamycin is also not able to completely inhibit callus growth in the dark (Fig. 3.12) and thus, similar problems occurred when kanamycin was used to select transplastomic lines in the dark. The *nptII* gene product also confers resistance against geneticin (G418). We demonstrated that G418 is lethal for embryogenic calli when applied at >50 mg/l to the culture medium in the dark (Fig. 3.14).

Therefore, a series of experiments were conducted to select transplastomic cell lines based on selection for geneticin-resistance in the dark. Although a number of resistant cell lines could be recovered after several selection rounds (which even survived stringent G418 concentrations of >60 mg/l), most of the resistant lines appeared to be false positives as revealed by Southern blot analysis and regeneration tests on kanamycin-containing medium (Fig. 3.14). In one of the resistant lines, *nptII* integration into the nuclear genome resulting kanamycin resistance was observed (Fig. 3.14). From tobacco chloroplast transformation experiments, it is known that integration of transgenes into the nuclear genome can occasionally occur (Koop *et al.*, 1996). However, the transgenes are often not functional since they are under the control of plastid-specific promoters (Maliga, 2003a). In some cases, these genes can become functional by acquiring the promoter from an upstream nuclear gene (Stegemann and Bock, 2006). Observation of such a rare event in our plastid transformation experiments may indicate that delivery and integration of the transgenes into the nuclear genome occurs at a relatively high frequency. These data also show that, despite its lethality for callus tissues in the dark, G418 results in a large number of spontaneous resistant lines, thus limiting its suitability for selection of transgenic cell lines in the dark.

Yet another possibility for having no success in recovering transplastomic cell lines in the dark could be the less efficient gene expression in non-green plastids. In this study, the plastid ribosomal RNA operon promoter (Prrn) was used to control transcription of the marker genes in transplastomic lines. Prrn is the most frequently used promoter for developing plastid transformation systems in several plant species as well as expressing vaccine antigens and biopharmaceutical proteins via chloroplast transformation (Daniell *et al.*, 2005). It has been supposed that the full length Prrn promoter containing the binding sites for both NEP and PEP RNA polymerases is functional in non-green proplastids as well as green chloroplasts (Daniell *et al.*, 2005). However, in all plastid transformation systems developed so far, selection for transplastomic lines has been carried out in the light and there is no experimental evidence showing the possibility of selecting transplastomic cell lines in the dark. Recent data showing light-inducible accumulation of the PEP polymerase (Allison, 2000; Tsunoyama *et al.*, 2004) may suggest that plastid gene expression is much higher in the light where both NEP and PEP polymerases are functional. Since primary transformants contain only one or a few transgenic plastid genomes, high expression rate may be of great importance to increase the possibility for recovering transplastomic lines from the first selection cycle by efficient inactivation of the selection agent. In the cotton plastid transformation system, when two selectable marker genes, *aphA-2* (*nptII*) and *aphA-6* (both encode enzymes detoxifying kanamycin), were

simultaneously used, the selection for plastid transformants was more efficient than using *aphA-6* alone (Kumar *et al.*, 2004a). This may also suggest that efficient antibiotic detoxification (by expression of the corresponding enzyme either from multiple genes integrated simultaneously or from a single gene under the control of a strong promoter) in the early phase of the plastid transformation process is important for recovering transgenic cell lines. Thus, identification of promoters with higher activities in non-green plastids might be highly useful for developing plastid transformation technologies for dark-grown embryogenic cultures. In our maize plastid transformation vector, the *nptII* gene was further regulated by the T7 gene 10 5'UTR (g10L) to facilitate efficient translation of the *nptII* mRNA. Such translational regulatory elements have been reported to increase translation rates in non-green tissues (Daniell *et al.*, 2005). Therefore, identification of novel 5'UTR and 3'UTR elements enhancing translation of the selectable marker genes in the dark might be very helpful for developing plastid transformation in monocots.

As discussed, selection for transplastomic candidate lines appears to be much more efficient when carried out in the light. Our results show that maize embryogenic calli are highly sensitive to kanamycin and streptomycin in the light (Fig. 3.12). However, a protocol for efficient plant regeneration through organogenesis or embryogenesis from single cells of maize cultures in the light was lacking. Although the regeneration medium supports germination of the embryos formed in the dark, it does not support creation of new embryos from single cells. As the transgene integrates during transformation into the genome of single cells, efficient embryogenesis or organogenesis from single cells is required to recover transplastomic cell or plant lines. Using α-PSK, a tissue culture protocol was developed allowing formation of somatic embryos as well as shoot tissues from segments of young maize leaves (Fig. 3.13; Ahmadabadi *et al.*, 2006). This might be useful to carry out the selection for plastid transformants in the light where gene expression in plastids is more efficient. Using green callus as target material for bombardment and direct selection on regeneration medium complemented with α-PSK, a kanamycin-resistant plantlet was recovered in the early stages of the selection (Fig. 3.15A). However, it did not survive selection after longer incubation on the selective medium and signal for the transgenic plastome (plastid genome) could not be observed in Southern blot analysis (Fig. 3.15C). Since no point mutation conferring resistance against kanamycin has yet been reported (Carrer *et al.*, 1993), spontaneous mutation is unlikely to be the reason for recovering this green plant. Integration of the *nptII* gene into the nuclear genome and subsequent kanamycin resistance has been reported (Carrer *et al.*, 1993). However, the regenerated plantlet showed a variegated

(green and pale-green) phenotype (Fig. 3.15A) which is not observed when the transgene is constitutively expressed from the nuclear genome. Thus, two possibilities remain to explain this observation: (i) It was a true plastid transformant, but the plastid genome contained only a small number of transgenic genomes not detectable by Southern blot and lost during plant growth, or (ii) it was only an escape in the early stages of the selection.

In one set of our plastid transformation experiments, dark-grown callus material was used as target for bombardment and the selection was carried out on the embryogenic medium in the light, using the α-PSK protocol (Table 3.7; Fig. 3.16). This would allow the transgenic cells to proliferate and form new embryos in the light during the primary rounds of selection, followed by plant recovery on regeneration medium. Although several green calli were isolated from the first round of the selection (Fig. 3.16), regeneration of such candidate lines failed. Thus, it seems that several critical factors exist for developing plastid transformation in monocots, most of which are linked to regeneration *in vitro* being based on embryogenesis in the dark.

For future plastid transformation experiments in maize, the use of other vectors [such as vectors containing a plastid-specific origin of replication in their flanking regions, which may support replication of the transformation vector in plastids increasing the possibility of the transgene integration via homologous recombination (Kumar *et al.*, 2004a)] and the combination of visual (such as *GFP*) with selection markers to facilitate identification of the primary transplastomic lines, are suggested. Using multiple selectable marker genes (detoxifying the same antibiotics) under control of different strong promoters in a single transformation vector might also be helpful for recovering primary plastid transformants by efficient inactivation of the antibiotic in the initial steps of the selection. In addition, identification of (i) plastid promoters capable of functioning strongly in non-green plastids, (ii) 5' and 3' regulatory elements supporting efficient translation of transgenes in undeveloped plastids, and (iii) novel selectable marker genes applicable in non-green plastids, seem to be essential if selection of plastid transformants should be carried out in embryogenic cultures in the dark. These can be of particular value, since most crop plants, including cereals, such as maize, are mainly regenerated via embryogenesis in *in vitro* cultures. If these issues cannot be solved, one should consider selecting transplastomic lines in the light. This will require the development of an efficient regeneration system allowing embryogenesis and/or organogenesis in the light. Such tissue culture limitations have often been cited as the major obstacle restricting chloroplast transformation in higher plants (Bogorad, 2000; Daniell *et al.*, 2005). In this work, reliable leaf-based regeneration (in the dark and in the light) and

transformation systems for maize were described. This progress notwithstanding, the development of organelle transformation technologies for recalcitrant species like cereals would benefit greatly from a better understanding of the molecular basis of plant regeneration (Zhang and Lemaux, 2004), the identification of regeneration genes (Nishimura *et al.*, 2005) and the successful molecular manipulation of the underlying pathways (Gordon-Kamm *et al.*, 2002).

4.2 Modification of the carotenoid biosynthesis pathway in maize by expression of a recombinant daffodil *PSY* cDNA in the nuclear genome

In order to demonstrate the biotechnological applicability of the leaf-based transformation system and to better understand the regulation of the carotenoid content in seeds, we aimed to manipulate the carotenoid biosynthesis pathway in maize by expressing a recombinant daffodil *PSY* gene. In this study, transgenic maize plants (35S-PSY and Z27-PSY) were successfully regenerated, which express the daffodil *PSY* gene under the control of two different promoters: the constitutive CaMV 35S promoter, and the seed endosperm-specific PzmZ27 promoter. Physical presence of the transgenes driven by these promoters was confirmed using PCR analysis (Fig. 3.19). In our nucleic acid gel blots, the maize *PSY* genes and transcripts could not be detected using the daffodil *PSY* cDNA as a probe (Fig. 3.20B), indicating that the probe does not efficiently hybridize to the maize endogenous *PSY* sequences. Similar results have been observed in transgenic rice plants expressing a recombinant daffodil *PSY*, where a daffodil *PSY* cDNA-specific probe show no hybridization to the rice endogenous *PSY* in the Southern blot analyses (Burkhardt et al., 1997). This is apparently because of differences in the nucleic acid sequences of the *PSY* genes in different plant species (Gallagher *et al.*, 2004; Fig. 6.4). The PzmZ27 promoter has been previously reported as an endosperm-specific promoter (Russell and Fromm, 1997). These experiments were based on GUS expression under the control of the PzmZ27 promoter. However, using Northern blot analysis, *PSY* gene transcripts could be detected in leaves of the Z27-PSY transgenic maize plants (Fig. 3.20B). These results indicate that the PzmZ27 promoter, in some instances, can also be active in leaf tissue. However, whether or not there is a difference in the activity of the PzmZ27 promoter in seeds and leaves, remains to be investigated by comparative Northern blot analyses. In the 35S-PSY transgenic line, most of the *PSY* transcripts appeared to be degraded. Such transgene-specific RNA degradation has been reported in several studies when high transgene copy numbers were integrated into the genome of the transgenic plants (Vaucheret *et al.*, 1998). In fact, Southern blot analysis confirmed integration of multiple copies of the *PSY* gene into the genome of the 35S-PSY

transgenic line (Fig. 3.20A). In addition, integration of transgenes in particular loci is known to initiate signals triggering transgene-specific RNA degradation (Vaucheret *et al.*, 1998). However, comparable levels of *PSY* transcripts were detected in both transgenic lines (Fig. 3.20B) excluding the possibility that the expression level alone is responsible for the observed mRNA degradation. Interestingly, when carotenoid contents of leaves were analyzed, both transgenic lines showed 17% enhanced carotenoid levels in comparison to the wild-type (Table 3.9). Phytoene synthase has been reported to be a rate-limiting enzyme in the carotenoid biosynthesis pathway (Howitt and Pogson, 2006), and its overexpression has been demonstrated to increase carotenoid content in some plant species (Howitt and Pogson, 2006). Our results confirm the key role of the *PSY* gene in the carotenoid biosynthesis pathway in maize. Interestingly, the *β*-carotene amount was increased by 21% in leaves of both transgenic lines in comparison to the wild-type, without any phenotypic effect on plant growth and development (Fig. 3.21). Lutein, violaxanthin and neoxanthin levels were also significantly increased (Table 3.9; Fig. 3.22). Similar results were obtained in *Arabidopsis* plants by overexpression of an endogenous phytoene synthase (Lindgren *et al.*, 2003). The chlorophyll content in transgenic lines was slightly increased (Table 3.9). Although this unexpected increase was not highly significant, an even higher elevation of the chlorophyll content was reported in *Arabidopsis* mutants overexpressing *PSY* (Lindgren *et al.*, 2003). However, the reason for this increase in chlorophyll amount currently remains unexplained.

Despite significantly increased levels of several carotenoids, the amounts of zeaxanthin and antheraxanthin remained constant in transgenic maize leaves (Table 3.9). Similar observations were made in *Arabidopsis* (Lindgren *et al.*, 2003). However, the reasons for zeaxanthin amount remaining at a constant level are currently unknown. Violaxanthin, antheraxanthin and zeaxanthin are known as the xanthophyll cycle pigments. Violaxanthin levels are usually high in moderate light intensities, whereas antheraxanthin and zeaxanthin levels are increased in response to high light stress (Young, 1991). Moreover, physical stresses like wounding, have been reported to elevate zeaxanthin amounts (Herde *et al.*, 1999). Our results revealed a significant increase in violaxanthin contents in leaves of transgenic maize plants. It is known that, under stress conditions, zeaxanthin can be formed within minutes from violaxanthin (Hager, 1980). Thus, it is possible that the zeaxanthin level is maintained constant by controlled action of zeaxanthin epoxidase and violaxanthin de-epoxidase enzymes under normal growth conditions.

Our results show that there is a significant capacity in maize to increase carotenoid contents, and in particular, pro-vitamin A contents by overexpression of the genes for key enzymes,

such as *PSY*. The similarly elevated carotenoid levels in both transgenic lines correlated well with the comparable levels of intact *PSY* transcripts in Northern blot analyses (Fig. 3.20B). It will be of particular importance to analyze *PSY* transcript levels and their correlation with the carotenoid levels in seeds of both transgenic lines. However, due to the limited timeframe for this PhD work, it was not feasible to wait for seed production from transgenic maize lines and perform these important analyses. In fact, greater differences are expected in carotenoid contents between Wt and transgenic lines in seeds than in leaves: In maize, two *PSY* genes are present in the genome, and transcripts of both genes accumulate in leaves. Our results show that, despite presence of two endogenous *PSY* genes, expression of an exogenous *PSY* results in significantly enhanced carotenoid levels in leaves. However, it has been shown that only *PSY*1 is functional in the maize seed endosperm (Buckner *et al.*, 1996; Gallagher *et al.*, 2004). Therefore the capacity to produce phytoene could be even more limiting in maize seeds than in leaves. Thus, expression of the daffodil *PSY* in seeds of the transgenic lines may lead to even higher levels of carotenoids. In the Z27-PSY transgenic line, the daffodil *PSY* gene is under control of a seed-specific promoter (PzmZ27; Russell and Fromm, 1997). This is expected to specifically increase the *PSY* transcripts (and presumably also PSY enzyme) in seeds of the Z27-PSY transgenic line. Amino acid alignment of the daffodil phytoene synthase with the two maize phytoene synthases revealed several differences in their N- and C-terminal sequences (Fig. 6.4). Whether or not these variable domains affect the catalytic activity of the phytoene synthase and/or the feedback regulation of enzyme activity, and how expression of the daffodil *PSY* will influence the carotenoid contents in maize seed endosperm, remain to be investigated. However, the enhancement of pro-vitamin A content in leaves of transgenic maize plants represents a promising step towards successful engineering of the carotenoid pathway in one of the world's most important crop plants.

4.3 Towards development of transformation technologies in *Peperomia*

Because of the unique feature of having giant chloroplasts in leaf palisade parenchyma, *P. metallica* has become a highly useful model plant to study plastid biology. However, the lack of efficient regeneration and transformation systems has limited these studies towards better understanding the mechanisms controlling e.g., plastid division, size and number in different tissues. In this subproject, we aimed to develop a transformation technology for *Peperomia* to facilitate labeling of non-green plastids for morphological studies on plastid types other than chloroplasts. Using RM medium complemented with different combinations of hormones, a highly efficient *in vitro* regeneration system was developed for *P. metallica* and *P. pedunculosa* (Fig. 3.23). Regeneration occurs through direct organogenesis from leaf

disks at high frequency (Fig. 3.23). Although a few transformation experiments were carried out using biolistic transformation to develop a nuclear transformation system for these species, transgenic lines could not be obtained. One possibility could be that the biolistic transformation method is not very efficient in this plant and other transformation technologies, such as the *Agrobacterium*-mediated method (Tzfira and Citovsky, 2006), would be more efficient. Also, many parameters influence the genetic transformation efficiency via the biolistic gun (Southgate *et al.*, 1995), and optimization of these factors might be necessary for successful transformation of *Peperomia* plants by microprojectile bombardment.

Although the genetic transformation could not be achieved, plastid biology was investigated in wild-type plants and interesting correlations were established between cell size, plastid size and plastid number in different cell types in leaves and roots of *Peperomia* species (Figs. 3.27 and 3.28; Table 3.10). Also, evidence was provided that plastid size and number are regulated in a tissue-specific manner. This regulation seems to be largely independent of the plastid type: Tissues containing the same plastid type (e.g., palisade and spongy mesophyll cells both containing chloroplasts), can exhibit significant differences in plastid number and size (Fig. 3.27; Table 3.10). In contrast, tissues harboring very different plastid types (e.g., palisade cells and root cells) can have similar modes of regulating plastid size (Fig. 3.31; Table 3.11). As it is well-established that chloroplast size and number are inversely correlated and largely determined by the rate of organelle division (Pyke and Leech, 1994; Pyke, 1997; Pyke, 1999; Aldridge *et al.*, 2005), it seems reasonable to conclude that the differences in chloroplast size and number per cell are caused by tissue-specific differences in the activity of the plastid division machinery. However, despite correlation of the amyloplast size in root cells of *P. metallica* with chloroplast size in palisade cells, plastid numbers in root cells were generally higher than in leaf cells (Fig. 3.30). These results, together with data obtained for the correlation of cell size and chloroplast number in palisade cells, provide evidences for cell size also being an important factor that determines plastid number.

So far, investigation of the division of giant plastids has been restricted to mutants impaired in plastid division. Interestingly, analysis of a tomato mutant has provided evidence that, at least in some tissues, division occurs by fragmentation of giant plastids (Forth and Pyke, 2006). In contrast, the naturally occurring giant plastids in palisade cells of *Peperomia metallica* and *P. pedunculosa* seem to follow the normal division mechanism by binary fission: Division intermediates were frequently observed as evidenced by the presence of constrictions resembling PD rings. Also, an increase in the average number of chloroplasts per palisade cell

was noticed during development from young leaves (average number 2.24±0.61) to mature leaves (average number 4.54±1.04) indicating that giant chloroplasts in palisade cells do still undergo division.

With light microscopy studies of giant chloroplasts in palisade cells of *P. metallica*, the formation of chloroplast networks connected via stromules was confirmed (Fig. 3.32), indicating the relevance of these structures *in vivo*. Also, bacteria-like microorganisms were detected within the *Peperomia* cells. This striking finding together with the unusual size of the chloroplasts and amyloplasts recommends *Peperomia* as an interesting model plant for studying plastid biology as well as the mechanisms involved in plant-microbe interactions.

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6 Appendix

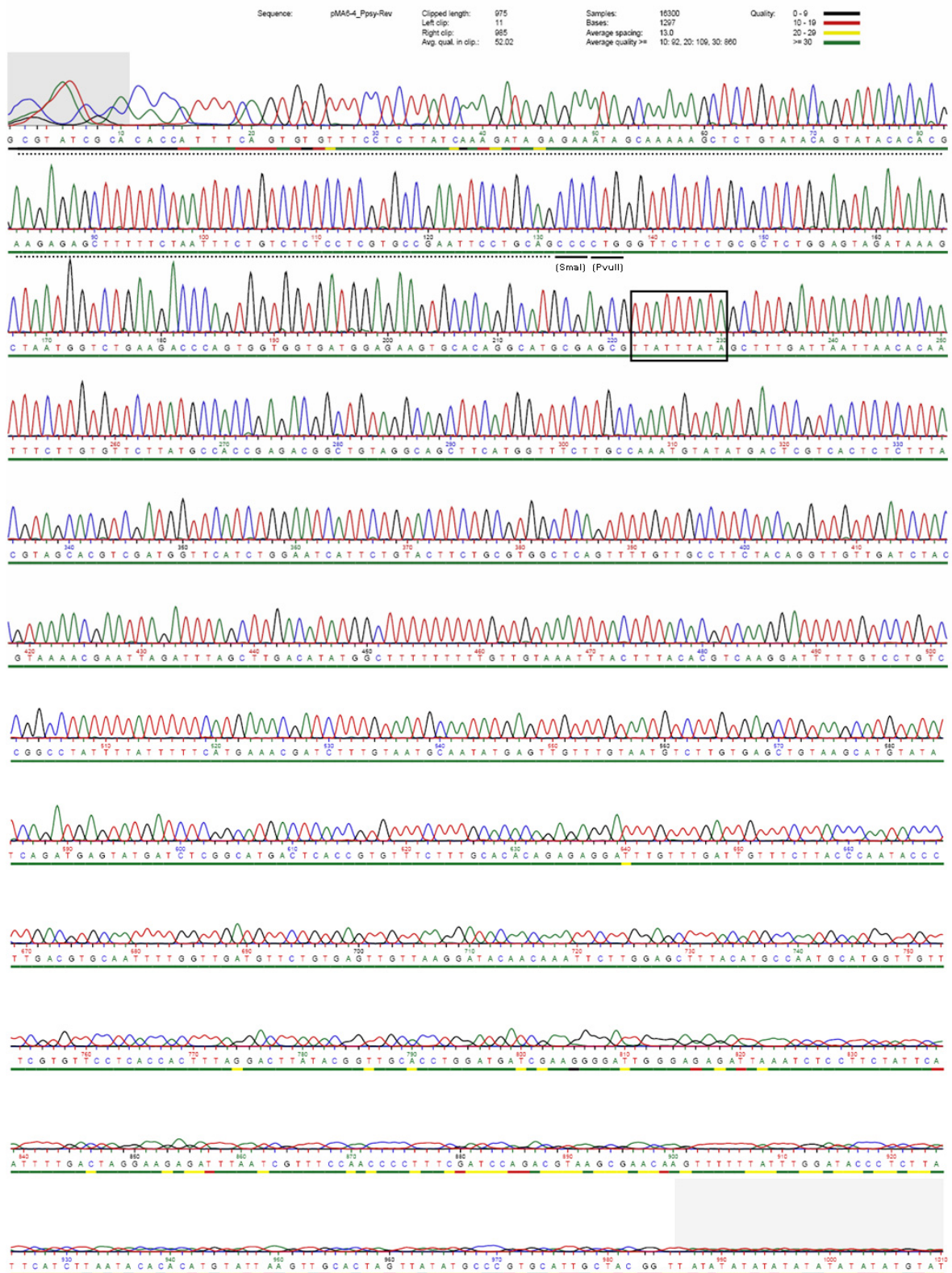**Figure 6.1** Sequence of the PCR-amplified maize 27 kDa zein gene promoter (PzmZ27) which was cloned upstream of the *PSY* cDNA sequence (indicated by the dotted line) generating vector pMA6 (see 2.1.7). Sequencing was performed using primer Ppsy Rev (see 2.1.4) to obtain the sequence information of (i) the 5' end of the daffodil *PSY* gene (~120 bp) and (ii) the PzmZ27 promoter. The three remaining-nucleotides from

digestion of the PCR-amplified PzmZ27 promoter (with the restriction enzyme *Pvu* II) and the cloning vector (pMA5; see 2.1.7) (with *Sma* I) are indicated by black lines. The TATA box, the most common promoter element in eukaryotic genes, is marked with a black box (according to Reina *et al.*, 1990).

```
              10        20        30        40        50        60        70        80        90       100       110       120
PzmZ27 GGGTTCTTCTGCGCTCTGGAGTAGATAAAGCTAATGGTCTGAAGACCCAGTGGTGGTGATGGAGAAGTGCACAGGCATGCGAGCGTTATTTATAGCTTTGATTAATTAACACAATTTCTT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pMA6   GGGTTCTTCTGCGCTCTGGAGTAGATAAAGCTAATGGTCTGAAGACCCAGTGGTGGTGATGGAGAAGTGCACAGGCATGCGAGCGTTATTTATAGCTTTGATTAATTAACACAATTTCTT

             130       140       150       160       170       180       190       200       210       220       230       240
PzmZ27 GTGTTCTTATGCCACCGAGACGGCTGTAGGCAGCTTCATGGTTTCTTGCCAAATGTATATGACTCGTCACTCTCTTTACGTAGCACGTCGATGGTTCATCTGGAATCATTCTGTACTTCT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pMA6   GTGTTCTTATGCCACCGAGACGGCTGTAGGCAGCTTCATGGTTTCTTGCCAAATGTATATGACTCGTCACTCTCTTTACGTAGCACGTCGATGGTTCATCTGGAATCATTCTGTACTTCT

             250       260       270       280       290       300       310       320       330       340       350       360
PzmZ27 GCGTGGCTCAGTTTTGTTGCCTTCTACAGGTTGTTGATCTACGTAAAACGAATTAGATTTAGCTTGACATATGGCTTTTTTTTTGTTGTAAATTTACTTTACACGTCAAGGATTTTTGTC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pMA6   GCGTGGCTCAGTTTTGTTGCCTTCTACAGGTTGTTGATCTACGTAAAACGAATTAGATTTAGCTTGACATATGGCTTTTTTTTTGTTGTAAATTTACTTTACACGTCAAGGATTTTTGTC

             370       380       390       400       410       420       430       440       450       460       470       480
PzmZ27 CTGTTCCGGCCTATTTTATTTTTCATGAAACGATCTTTGTAATGCAATATGAGTTGTTTGTAATGTCTTGAGCTGTAAGCATGTATATCAGATGAGTATGATCTCGGCATGACTCACC
       :::: :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pMA6   CTGT-CCGGCCTATTTTATTTTTCATGAAACGATCTTTGTAATGCAATATGAGTTGTTTGTAATGTCTTGTGAGCTGTAAGCATGTATATCAGATGAGTATGATCTCGGCATGACTCACC

             490       500       510       520       530       540       550       560       570       580       590       600
PzmZ27 GTGTTTCTTTGCACACAGAGAGGATTTGTTTGATTGTTTCTTACCCAATACCCTTGACGTGCAATTTTGGTTGATGTTCTGTGAGTTGTTAAGGATACAACGAATTCTTGGAGCTTTACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: :::::::::::::::::::
pMA6   GTGTTTCTTTGCACACAGAGAGGATTTGTTTGATTGTTTCTTACCCAATACCCTTGACGTGCAATTTTGGTTGATGTTCTGTGAGTTGTTAAGGATACAACAAATTCTTGGAGCTTTACA

             610       620       630       640       650       660       670       680       690       700       710
PzmZ27 TGCCAATGCATGGTTGTTTCGTGTTCCTCAACATTTAAGGACTTATTCGGTTGCACCTGGAT---CGAAGGGGATTGGGAGAAATCGATTAAATCTCCTTCTATTTAATTTTGACTAGGA
       ::::::::::::::::::::::::::::::::::::::  :::  ::::::::  :::::::::::::   ::::::::::::::::::     ::::::::::::::::::: :::::::::::
pMA6   TGCCAATGCATGGTTGTTTCGTGTTCCTCACCACTTTAGGACTTATACGGTTGCACCTGGATGATCGAAGGGGATTGGGAGA----GATTAAATCTCCTTCTATTCAATTTTGACTAGGA

       720       730       740       750       760       770       780       790       800       810       820       830
PzmZ27 AGAGATTTAATCGTTTCCAACCCCTTTCGATCCAGACGTAAGCGAACAAGTTATTTATTTGGATACC-TCTTATTCATCTTAATACACACATGGTATTAAGTTGCCACTAGTCATATGCC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: :::::::::::: :::::::::::::::::::::::::::: ::::::::::: :::::: :::::
pMA6   AGAGATTTAATCGTTTCCAACCCCTTTCGATCCAGACGTAAGCGAACAAGTTTTTTATTTGGATACCCTCTTATTCATCTTAATACACACATG-TATTAAGTTGC-ACTAGTTATATGCC

       840       850       860       870       880       890       900       910       920       930       940       950
PzmZ27 TGTGCATTGCTACGGTTTATATTATATATATATATATATAATATATATATATATATATATATGATAAATTTTGTTTTAATAAAACATATGTTTTCTATTGATTAGGTTGTGTGAATA
       :::::::::::::::::::::::   :::::::::::::::: ::::::::::::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::
pMA6   CGTGCATTGCTACGGTTTATAT---ATATATATATATATATGTATATATATATATAT-GATATATGATAAATTTTGTTTTAATAAAACATATGTTTTCTATTGATTAGGTTGGGTGAATA

       960       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070
PzmZ27 TG-AGCCAACAACCAAGGTCCAGAACACTTATACATAATTTCACCTTATTTTGTACATAAACTCTCTTATTATAGTAGTAAGAGAAGAGATTATAAGAGTGCGGGTTGATTATAAAGAAA
       :: :::::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::::
pMA6   TGGAGCCAACAACCAATATCCAGAACACTTATACATAATTTCACCTTATTTTGTACATAAACTCTCTTATTATAGTAGTA-GAGAAGAGATTATAAGAGTGCGGGTTGATTATAAAGAAA

      1080      1090      1100      1110      1120
PzmZ27 TGTAGGAGTTTTTTAATAATATTGACACGAGACGATCGTTGAACTGGTGCTTT
       ::::::::::::::::::::::::::::: :::::::::::::::::::::::::
pMA6   TGTAGGAGTTTTTTAATAATATTGACGCGGGACGATCGTTGAACTGGTGCTTT
```

Figure 6.2 Alignment (http://www.ch.embnet.org/software/LALIGN_form.html; Huang and Miller, 1991) of the cloned PzmZ27 maize zein gene promoter (pMA6) and the published sequences (PzmZ27; NCBI accession no. X53514). The amplified promoter sequence shows 97.1% identity to the published sequence. The nucleotides which differ from the published sequence (indicated with grey colors) were identical in three independently obtained clones, suggesting that they represent either errors in the published sequence or non-conserved residues among different maize genotypes. The TATA-box region is underlined (according to Reina *et al.*, 1990).

Figure 6.3 Physical map of the ZmPrrnPEP+NEP-G10L-14aaGFP-nptII-petD3' cassette used for constructing the maize plastid-specific transformation plasmid pMA1. The *nptII* selectable marker gene is under control of the maize 16S ribosomal RNA operon promoter (ZmPrrn) and the *petD* gene terminator. The Prrn promoter contains recognition sites for both the plastid-encoded RNA polymerase (PEP) and the nuclear-encoded RNA polymerase (NEP) and was fused with the short leader sequence (g10L) of the gene 10 from T7 bacteriophage, an excellent leader sequence for the high-level expression of heterologous proteins. In addition, the NPTII enzyme was translationally fused with the 14 N-terminal amino acids (14 aa) of the green fluorescent protein (GFP) to enhance the translation rate in plastids.

```
Daffodil_PSY     MVVAILRVVSAIEIPIRLGFSEANWRFSSPKYDNLGRKKSRLSVYSLYTTSKYACVGFE-
Maize_PSY1       MAIILVRAASP-----GLSAADSISHQGTLQCSTLLKTK-RPAARRWMPCSLLGLHPWE-
Maize_PSY2       --------ARP------LPAPTGKFHHLSPSHSHCRPRR--------VLQTPPALPARR-
Rice_PSY         -------MAPP------PPPPCSVRAAGSNPIGCLEVAEP------WSGAAPPPLPPLPG
                 :  :  ::   .  ..:  : :     ..    :    .        :

Daffodil_PSY     AENNGKFLIRSSLVANPAGEATISSEQKVYDVVLKQAALVKDQTKSSRKSTDVKP-DIVL
Maize_PSY1       AGRPS-PAVYSSLPVNPAGEAVVSSEQKVYDVVLKQAALLKRQLRTP--VLDARPQDMDM
Maize_PSY2       SGASPPRASLAAAAPAVAVAVRTASEEAVYEVVLRQAALVEAATPQRRRTRQPRWAEEEE
Rice_PSY         HLHVAAPAAEDDDDALAAAAAAVPSEQRVHDVVLKQAALAAAAPEMR------RPAQLAE
                             *  .   .**: *::***:****           :  :

Daffodil_PSY     PGTVY---LLKDAYDRCGEVCAEYAKTFYLGTLLMTPERRRAIWAIYVWCRRTDELVDGH
Maize_PSY1       PRNG-----LKEAYDRCGEICEEYAKTFYLGTMLMTEERRRAIWAIYVWCRRTDELVDGP
Maize_PSY2       EERVLGWGLLGDAYDRCGEVCAEYAKTFYLGTQLMTPERRKAVWAIYVWCRRTDELVDGP
Rice_PSY         RERVAG--GLNAAFDRCGEVCKEYAKTFYLATQLMTPERRRAIWAIYVWCRRTDELVDGP
                       *   *:*****:* ********.* *** ***:*:****************

Daffodil_PSY     NASHITPSALDRWEARLEDLFAGRPYDMFDAALSDTVSRFPVDIQPFMDMVEGMRMDLKK
Maize_PSY1       NANYITPTALDRWEKRLEDLFTGRPYDMLDAALSDTISRFPIDIQPFRDMIEGMRSDLRK
Maize_PSY2       NASYITPTALDRWEKRLEDLFEGRPYDMYDAALSDTVSKFPVDIQPFKDMVQGMRLDLWK
Rice_PSY         NASHMSALALDRWESRLDDIFAGRPYDMLDAALSHTVATFPVDIQPFRDMIEGMRLDLTK
                 **.:::. ****** **:*:* ****** *****.*:: **:***** **::*** ** *

Daffodil_PSY     SRYKNFDELYLYCYYVAGTVGLMSVPVMGIAPESLAEAESVYNAALALGIANQLTNILRD
Maize_PSY1       TRYNNFDELYMYCYYVAGTVGLMSVPVMGIATESKATTESVYSAALALGIANQLTNILRD
Maize_PSY2       SRYMTFDELYLYCYYVAGTVGLMTVPVMGIAPDSKASTESVYNAALALGIANQLTNILRD
Rice_PSY         SRYRSFDELYLYCYYVAGTVGLMTVPVMGISPDSRANTETVYKGALALGLANQLTNILRD
                 :** .*****:*************:******:..:* * :*:**..*****:*********

Daffodil_PSY     VGEDARRGRIYLPQDELAEAGLSDEDVFTGKVTDKWRSFMKRQIKRARTFFEQAEKGVTE
Maize_PSY1       VGEDARRGRIYLPQDELAQAGLSDEDIFKGVVTNRWRNFMKRQIKRARMFFEEAERGVNE
Maize_PSY2       VGEDARRGRIYLPLDELAQAGLTEEDIFRGKVTGKWRRFMKGQIQRARLFFDEAEKGVTH
Rice_PSY         VGEDARRGRIYLPMDELEMAGLSEDDIFDGRVTDRWRCFMRDQITRARAFFRQAEEGASE
                 ************* ***  ***:::*:* * **.:** **: ** *** ** :**.*...

Daffodil_PSY     LSQASRWPVWASLLLYRQILDEIEANDYNNFTKRAYVSKVKRLAALPLAYGKSLLIPLSL
Maize_PSY1       LSQASRWPVWASLLLYRQILDEIEANDYNNFTKRAYVGKGKKLLALPVAYGKSLLLPCSL
Maize_PSY2       LDSASRWPVLASLWLYRQILDAIEANDYNNFTKRAYVGKAKKLLSLPLAYARAAVAP---
Rice_PSY         LNQESRWPVWASLLLYRQILDEIEANDYNNFTKRAYVPKAKKIVALPKAYYRSLMLPSSV
                 *.. ***** *** ******* ************** * *:: :** ** :: : *  :

Daffodil_PSY     RPPSLSKA-
Maize_PSY1       RNGQT----
Maize_PSY2       ---------
Rice_PSY         RHCSSLTSS
```

Figure 6.4 Amino acid alignment of the daffodil phytoene synthase with the two maize phytoene synthases (PSY1 and PSY2) and the rice PSY enzyme. The alignment was performed using the ClustalW (V 1.83) multiple sequence alignment program (http://www.ebi.ac.uk/clustalw/#). "*" indicates amino acid residues identical in all sequences in the alignment; "**:**" and "**.**" indicate conserved and semi-conserved substitutions, respectively.

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