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MINI-REVIEW

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Enslaved bacteria as new hope for plant biotechnologists

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Abstract The most distinguishing feature of the plant cell is a DNA-containing organelle that sets plants apart from all other organisms: the chloroplast. Compelling evidence supports an endosymbiotic origin for chloroplasts. According to this theory, chloroplasts are descendants of formerly free-living cyanobacterial ancestors which entered an endosymbiotic relationship with a pre-eukaryotic cell and were ultimately integrated into the metabolism of the host cell. Chloroplasts retain many prokaryotic features and their gene expression system still closely resembles that of their eubacterial ancestors. During the past decade, our knowledge about chloroplast biology has benefited immensely from a most remarkable methodological breakthrough: the development of transformation technologies for chloroplast genomes. Moreover, recent advances in the manipulation of higher plant chloroplast genomes have created unprecedented opportunities for the genetic engineering of plants and promise to overcome many of the problems associated with conventional transgenic technologies. This review describes the state of the art in genetic engineering of higher plant chloroplast genomes and highlights the tremendous potential of these technologies for the biotechnology of the future.

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Introduction

Plastids and mitochondria as prokaryotic systems of endosymbiotic origin

The making of the eukaryotic cell is one of evolution's most remarkable feats, and scientific understanding of this process has itself been evolving at a rapid pace (Martin and Müller 1998; Brocks et al. 1999). The rise of eukaryotes, possibly as early as 2,700 million years ago, is closely connected to the acquisition of two cell organelles: the mitochondrion and the plastid (best known in its green differentiation form, the chloroplast). The structural complexity of eukaryotic cells and the fact that prokaryotes had existed at least one billion years before the first eukaryotes appeared led to the idea that eukaryotic cell organelles could have originated from formerly free-living prokaryotes. An attractive mechanistic explanation for such an evolutionary conversion of prokaryotes into mitochondria or plastids has been provided by the endosymbiosis theory (for review, see e.g. Gray 1993; Gray et al. 1999): a bacterium was engulfed by a pre-eukaryotic host cell and, instead of being digested, became domesticated. This process involved the gradual integration of the endosymbiont into the metabolism of the host cell by establishing a division of labor and inventing sophisticated regulatory networks to coordinate the host's gene expression with that of the endosymbiont. Genetically, the evolutionary optimization of the endosymbiosis was accompanied by the loss of dispensable or redundant genetic information and the massive translocation of genetic information, particularly from the endosymbiont to the host genome (Martin and Herrmann 1998). Contemporary organellar genomes are greatly reduced and contain only a small proportion of the genes that their free-living ancestors had possessed.

Using molecular methods, the origins of organelles have been traced back to specific taxa of Eubacteria: whereas Cyanobacteria were identified as the presumptive

ancestors of plastids, α-Proteobacteria are most closely related to mitochondria (Gray 1993). The present-day organelles are believed to be of monophyletic origin, in that all extant lineages of eukaryotes harbor mitochondria originating from one and the same endosymbiosis event (Lang et al. 1997; Andersson et al. 1998). Likewise, plastids in all lineages of plant evolution have a common cyanobacterial ancestor (Ozeki et al. 1989; Bhattacharya and Medlin 1998; Tomitani et al. 1999).

Gene expression in chloroplasts

The chloroplast genome of higher plants is a circular molecule of double-stranded DNA, typically in the size range 120–160 kb (Fig. 1). Whereas the genome of the cyanobacterium *Synechocystis* contains more than 3,000 genes (Kaneko et al. 1996; Kaneko and Tabata 1997), chloroplast genomes of higher plants harbor only approximately 120 genes (Sugiura 1992), illustrating the dramatic reduction that the endosymbiont's genome has suffered during evolution.

The picture that has emerged from the complete sequencing of several plastid genomes over the past decade is that the chloroplast has retained a largely prokaryotic system of gene organization and expression. Most plastid-encoded genes are organized in operons and hence produce polycistronic mRNAs by co-transcription. This is in striking contrast to gene expression in the plant nuclear genome, where almost all genes are transcribed as monocistronic mRNAs. The gene order in several chloroplast operons is remarkably conserved and still closely resembles that of Cyanobacteria and other Eubacteria (Stoebe and Kowallik 1999). For example, the genes for the ribosomal proteins L23, L2, S19, L22, S3 and L16 are part of an operon in *Escherichia coli* (S10 operon) and are found in exactly the same order within the *rpoA* operon of chloroplasts (Fig. 1).

The prokaryotic origin of chloroplasts is also mirrored by the molecular mechanisms of practically all steps in plastid gene expression. Plastid transcription is carried out by a eubacterial-type RNA polymerase, the subunits of which are encoded in the chloroplast DNA (*rpoA*, *rpoB*, *rpoC1* and *rpoC2*; Fig. 1). As in Eubacteria, a set of sigma-like factors interact with this plastid-encoded RNA polymerase, conferring promoter-specific binding and mediating transcriptional regulation in response to environmental cues. Many, but not all, promoters of plastid genes resemble bacterial promoters in that their core sequence consists of the typical −10 (TATA box) and −35 elements (Igloi and Kössel 1992). Recently, a second, nuclear-encoded, RNA polymerase activity could be identified which utilizes non-consensus promoters. The enzyme turned out to be closely related to single-subunit bacteriophage RNA polymerases (Hedtke et al. 1997; Hess and Börner 1999). Interestingly, the phage-like polymerase is predominantly active in undifferentiated plastids and preferentially transcribes plastid genetic system genes (e.g. rRNA genes, ribosomal protein genes) whereas the *E. coli*-like enzyme

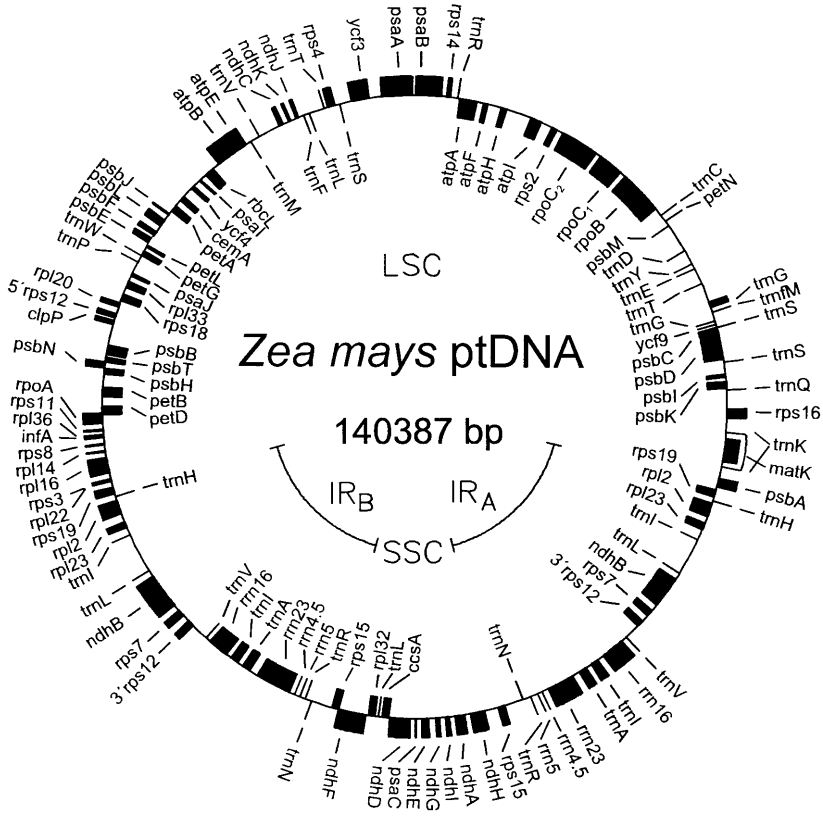

Fig. 1 Structure of a typical chloroplast genome of a higher plant. The physical map of the plastid DNA from maize (*Zea mays*) is shown. The circular genome contains two large inverted repeat regions (IR_A and IR_B) which separate the molecule into a large single copy region (*LSC*) and a small single copy region (*SSC*). Major gene classes are: *rrn* (rRNA genes), *trn* (tRNA genes), *rpl* (ribosomal proteins of the large subunit), *rps* (ribosomal proteins of the small ribosomal subunit), *rpo* (RNA polymerase subunits), *psa* (subunits of photosystem I), *psb* (subunits of photosystem II), *pet* (subunits of the cytochrome b_6f complex), *atp* (subunits of the ATP synthase), *ndh* (subunits of an NAD(P)H dehydrogenase) and *ycf* (conserved open reading frames). Genes located on the outside of the circle are transcribed clockwise, genes located on the inside are transcribed counterclockwise

provides the major RNA-synthesizing activity in mature chloroplasts and is responsible for the transcription of plastid-encoded photosynthesis-related genes (Hajdukiewicz et al. 1997). This interplay of two RNA-polymerizing activities in plastid development illustrates a general principle in organelle evolution: while the basically prokaryotic features of the bacterial ancestors were retained, novel mechanisms were invented to facilitate the concerted expression of nuclear and organellar genomes in a tissue-specific and developmental stage-specific manner.

Control of chloroplast gene expression is not only exerted by regulating the activity of the two RNA polymerases but has been shown also to be primarily regulated by post-transcriptional events including transcript stability, translation and protein turnover (Rochaix 1996; Sugita and Sugiura 1996). In plastids, primary transcripts undergo a series of RNA maturation steps: processing of the 5′ and 3′ ends (''RNA-trimming''), cleavage of polycistronic into monocistronic mRNAs, splicing of group I and group II introns (*cis*- as well as *trans*-splicing) and RNA-editing, a post-transcriptional process that changes the identity of single nucleotides by cytidine-to-uridine convertions (Smith et al. 1997; Bock 1998). Whereas some of these RNA maturation processes are clearly ancestral, others (e.g. RNA-editing) appear to be evolutionarily recent acquisitions and do not occur in Bacteria.

Also the translational apparatus of plastids very much resembles that of prokaryotes, in that tRNAs, rRNAs, ribosomal proteins and the initiation and elongation factors exhibit strong similarity with their counterparts in *E. coli*. However, whereas in Eubacteria almost all mRNAs possess a Shine-Dalgarno sequence capable of binding to the 3′ end of the 16 S rRNA and thereby mediating accurate translation initiation, only about 40% of chloroplast mRNAs contain Shine-Dalgarno-like sequences in reasonable distance upstream of the initiator codon. This suggests that alternative pathways for translation initiation exist in chloroplasts which may be entirely independent of direct rRNA–mRNA interactions (Gillham et al. 1994; Stern et al. 1997).

In the course of evolution, the chloroplast has undergone a shift from transcriptional regulation (the predominant gene regulation level in prokaryotes) to predominantly translational control of gene expression. Why did that happen and why is translational control the favored mechanism for regulating gene expression in plastids? Plants are exposed to rapid changes in environmental conditions, most notably rapid and dramatic changes in light intensity. Transcriptional regulation is a relatively slow response, in that it takes rather a long time before it becomes phenotypically effective. For example, a light-induced increase in transcription rate would have to be followed by transcript processing and translation, before finally the increased demand for photosynthetic protein complexes could be satisfied. In contrast, translational regulation has the advantage that protein biosynthesis can start immediately from a pre-existing pool of mature transcripts. In chloroplasts, such a fast response is of particular importance, since light energy which is not faithfully converted into chemical energy by photosynthetic electron transport can cause severe photo-oxidative damage of plastid proteins and membranes. It has recently become evident that chloroplasts utilize redox signals generated by photosynthesis in order to effectively and rapidly switch on or off the translation of plastid messenger RNAs (Danon 1997; Bruick and Mayfield 1999).

Whereas translational regulation is clearly the predominating regulatory level in plastids, there is additional fine-tuning at practically all other steps in gene expression, including transcription, RNA processing and differential RNA stability (Hayes et al. 1999). The sophisticated interplay of all these control mechanisms allows the cell to successfully cope with changing environmental conditions and to tightly coordinate the organellar gene expression with that in the nucleocytoplasmic compartment.

The increased understanding of the mechanisms of gene expression in chloroplasts has eventually paved the way for the successful development of technologies allowing the genetic manipulation of plastid genomes. The tamed chloroplasts have opened new horizons in basic plant research and ultimately also have attracted plant biotechnologists.

Generation of plants with transgenic chloroplasts

To introduce genetic changes into plant genomes is of great interest for both basic and applied research. Transformation of the plant nucleus is nowadays a routine in many species and a variety of techniques for delivering foreign DNA to the plant nuclear genome is available (including e.g. *Agrobacterium*-mediated gene transfer, particle bombardment, microinjection, polyethylene glycol-mediated transformation, electroporation, virus-mediated gene transfer; for review, see e.g. Potrykus 1991). The chloroplast genome of higher plants represents a particularly challenging target for genetic transformation due to its enormously high ploidy level (=copy number per cell; Bendich 1987): A single leaf cell may contain more than 100 chloroplasts. The organization of the chloroplast DNA in nucleoids is yet another prokaryotic feature that was retained during evolutionary conversion of the cyanobacterial endosymbiont into present-day plastids. Each chloroplast contains several such nucleoids and each nucleoid again harbors several copies of the plastid genome. Thus, the genome copy number in an individual chloroplast can easily reach 50 or even 100. Taking into account the above mentioned high number of chloroplasts per cell, there can be in total up to 10,000 (identical) plastid DNA molecules present in a single cell. The success of any technology aiming at the stable introduction of foreign genetic material into the plastid genome of higher plants will ultimately depend on how efficiently the

experimental strategy can cope with this extremely high copy number of the chloroplast DNA.

In addition, the development of systems suitable for the genetic transformation of plastid genomes involves:

1. A method to deliver foreign DNA through: (a) the cell wall, (b) the plasma membrane, or (c) the double membrane of the plastid.
2. A plastid-specific selectable marker gene.
3. A mechanism to integrate foreign sequences into the ptDNA.
4. A highly regenerable tissue culture system.

The transformation of chloroplasts was first achieved in 1988 for *Chlamydomonas reinhardtii* – a unicellular alga possessing a single large chloroplast that occupies approximately 60% of the cellular volume. Introduction of the transforming DNA into the chloroplast was accomplished by particle bombardment (Fig. 2): microscopic heavy metal particles (tungsten or gold, 0.4–1.7 μm diameter) can be coated with plasmid DNA and shot into the target cells with a so-called particle gun. Selection of chloroplast transformants was facilitated by using a recipient strain of *Chlamydomonas* that was defective for the chloroplast *atpB* gene and thus incapable of photoautotrophic growth. By delivering a functional *atpB* copy, it was possible to complement the mutation and restore photosynthetic activity. Subsequent molecular analyses revealed that the transforming DNA had integrated via homologous recombination into the chloroplast genome. In later experiments, homologous recombination turned out to be the general principle for incorporating foreign sequences into the plastid DNA. Consequently, any piece of DNA can be inserted into the plastid genome, provided that it is flanked by sequences derived from the target chloroplast genome.

A few years after the initial success with *Chlamydomonas* chloroplasts, plastid transformation protocols also were developed for a higher plant species, tobacco (*Nicotiana tabacum*; Svab et al. 1990). To date, two different delivery methods for foreign DNA into the chloroplast compartment of higher plants have proved successful: (1) particle gun-mediated transformation, commonly referred to as biolistic (= biological + ballistic) protocol (Svab et al. 1990; Svab and Maliga 1993) and (2) chemical treatment of protoplasts with polyethylene glycol (PEG) in the presence of transforming DNA (Golds et al. 1993; O'Neill et al. 1993).

Another prerequisite for successful chloroplast transformation is the availability of a selectable marker gene that facilitates efficient selection of cells with transgenic plastids. Expressed from the chloroplast genome, this marker gene should give resistance to a drug that otherwise would effectively and specifically inhibit gene expression in the plastid compartment. For the first chloroplast transformation experiments in tobacco, a mutant allele of the plastid 16S ribosomal RNA gene

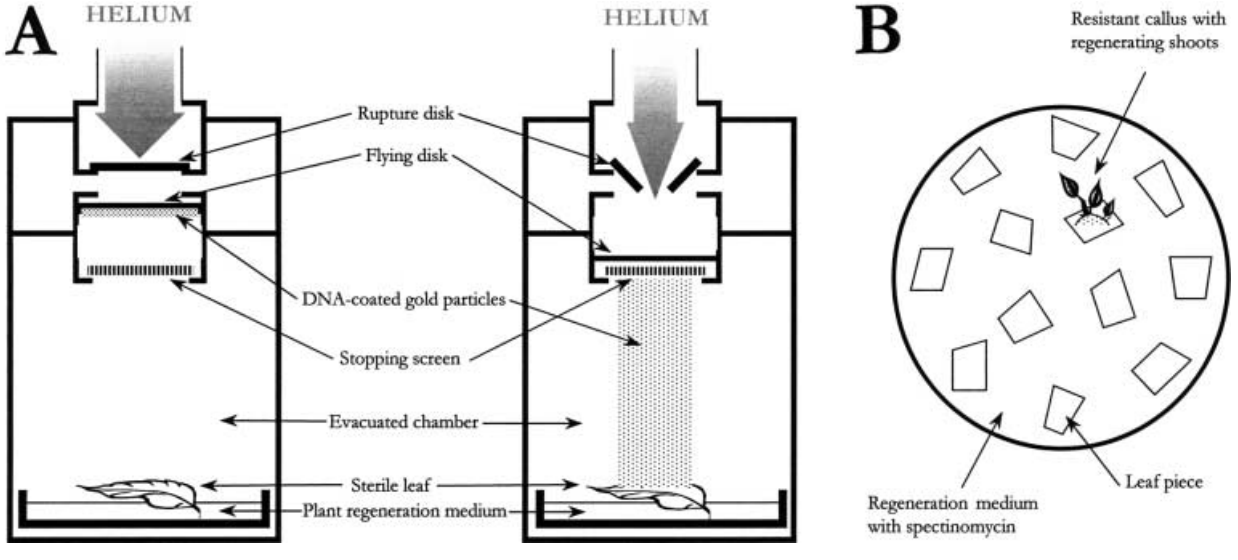

Fig. 2A, B Generation of transplastomic plants. **A** Schematic overview of a helium-driven particle gun. A plastic *rupture disk* seals the gas tube and allows for building up a defined gas pressure inside the tube (*left*). The gold particles coated with the transforming DNA are attached to the lower side of a second plastic disk, the *flying disk*. A metal grid, the *stopping screen*, stops the *flying disk* but not the particles. When the helium pressure reaches the burst pressure of the *rupture disk*, the disk breaks and the inflowing gas accelerates the *flying disk* with the gold particles on its lower side. The DNA-coated particles pass the *stopping screen* and penetrate the cells of the *sterile leaf* (*right*). **B** The bombarded leaf sample is cut into small pieces that subsequently are placed onto the surface of a plant regeneration medium containing spectinomycin. Cells with non-transformed plastids are sensitive to the antibiotic spectinomycin, which effectively impairs plastid protein biosynthesis. Hence, sensitive tissue will eventually bleach and turn yellow. Due to expression of the chimeric *aadA* gene, transplastomic cells are able to divide in the presence of spectinomycin and give rise to small green calli. Typically, after 3–4 weeks incubation, resistant shoots will form from the calli. Initially, these resistant shoots (primary chloroplast transformants) are heteroplasmic and may still contain significant amounts of wild-type plastid genomes. Several additional rounds of regeneration under selective conditions are required to ultimately obtain homoplasmic plantlets. Homoplasmic transplastomic plants are then rooted on phytohormone-free medium, subsequently transferred to the soil and maintained under greenhouse conditions (see text for details)

was employed as a plastid-specific selectable marker gene. This allele carried two point mutations conferring resistance to two aminoglycoside antibiotics known to act as translational inhibitors, both in prokaryotic systems and also in chloroplasts: spectinomycin and streptomycin. This mutant rRNA allele is a ''recessive'' selectable marker in that it provides antibiotic resistance only to those chloroplast ribosomes that have incorporated a 16S rRNA molecule transcribed from a transformed genome copy. Consequently, the transformation frequencies obtained with this selectable marker gene were rather low (Svab et al. 1990; Staub and Maliga 1993). The construction of dominant selectable marker genes specifying antibiotic-inactivating enzymes has led to much higher chloroplast transformation frequencies. Their dominant mode of action is due to the fact that few enzyme molecules are already sufficient to detoxify the entire chloroplast. Currently, the most efficient selectable markers for chloroplast transformation are chimeric *aadA* genes. The encoded enzyme, aminoglycoside 3″-adenylyltransferase, catalyzes the covalent modification, and thereby the inactivation, of aminoglycoside-type antibiotics (e.g. spectinomycin or streptomycin; Table 1). In order to convert the originally bacterial *aadA* gene into a chloroplast-specific selectable marker gene, its coding region was tethered to plastid expression signals: a promoter and 5′ untranslated region taken from a chloroplast gene (including a Shine-Dalgarno sequence for efficient translation initiation) as well as a plastid 3′ untranslated region typically forming a stable stem-loop-type secondary structure at the RNA level and thereby conferring high transcript stability. The development of such chimeric *aadA*-based selectable marker genes not only significantly enhanced the chloroplast transformation efficiency, but also facilitated the incorporation of practically any foreign sequence into the chloroplast genome by physically linking it to the resistance gene.

Our current perception of the chloroplast transformation process is that the transforming DNA is delivered to only one out of the hundreds of chloroplasts within the target cell where it integrates via homologous recombination into a single (or at most a few) of the approximately 100 DNA molecules harbored by the chloroplast. Consequently, cells that carry successfully transformed chloroplasts are initially heteroplasmic (or ''heteroplastomic''), i.e. their population of chloroplast DNA molecules is not homogeneous but consists of both recombinant and wild-type DNA molecules. Heteroplasmy is not a stable state and can convert spontaneously into homoplasmy by randomly sorting the two genome types. Experimentally, homoplasmy for the transformed genome is actively promoted by the constant application of a selective pressure, making the presence of a high amount of recombinant ptDNA molecules essential for the survival of the cell. This is achieved by repeated regeneration of transformed tissue on a synthetic plant regeneration medium containing high concentrations of the selecting antibiotic(s). In this way, all genome copies that are not transformed are gradually sorted out. To be stable, a cell line with transgenic chloroplasts (commonly referred to as ''transplastomic'') must contain exclusively transformed copies of the chloroplast genome; which means that each individual chloroplast must be homoplasmic (''homoplastomic'') with respect to the transgenome. Both for the unambiguous interpretation of research data and for the long-term stability of the transplastomic lines, it is absolutely essential that the plants are in a homoplasmic state.

By altering the primary sequence of a plastid gene in vitro and subsequently delivering the mutant allele into the chloroplasts of a living cell, it has now become possible to directly manipulate the encoded gene product in vivo. This approach is known as ''reverse genetics'' and provides a powerful tool for the elucidation of the function of plastid-encoded genes (Ruf et al. 1997; Hager et al. 1999). In addition, chloroplast transformation technologies have also paved the way for detailed in vivo studies of practically all steps in higher plant plastid gene expression, such as transcription (Allison and Maliga 1995; Hajdukiewicz et al.

Table 1 Foreign genes successfully expressed to date from higher plant plastid genomes

Gene	Gene product	Function	References
aadA	Aminoglycoside 3″-adenylyltransferase	Positive selectable marker (spectinomycin and streptomycin resistance)	Svab and Maliga 1993; Zoubenko et al. 1994
nptII	Neomycin phosphotransferase	Positive selectable marker (kanamycin resistance)	Carrer et al. 1993; Carrer and Maliga 1995
uidA	β-Glucuronidase	Reporter of gene expression	Staub and Maliga 1993; Bock and Maliga 1995
gfp	Green fluorescent protein	Reporter of gene expression	Khan and Maliga 1999; Sidorov et al. 1999
cry1A	Crystal toxin from *Bacillus thuringiensis*	Insecticidal protein (protoxin)	McBride et al. 1995
cry2A	Crystal toxin from *B. thuringiensis*	Insecticidal protein (protoxin)	Kota et al. 1999
codA	Cytosine deaminase	Negative selectable marker (5-fluorocytosine sensitivity)	Serino and Maliga 1997
EPSPS	5-Enol-pyruvyl shikimate-3-phosphate synthase	Herbicide resistance (glyphosate)	Daniell et al. 1998
hST	Human somatotropin	Therapeutic protein (human growth hormone)	Staub et al. 2000

1997), RNA processing (Bock and Maliga 1995), editing (Bock et al. 1996; Chaudhuri and Maliga 1996) and translation (Staub and Maliga 1993, 1994). Moreover, a number of extraordinarily attractive features associated with transplastomic plants recently also have caught the attention of plant biotechnologists.

Attractive features of transplastomic plants for plant biotechnologists

The transformation of higher plant chloroplasts is an extraordinarily powerful tool in plant biotechnology. Compared to "classical" transgenic plants generated by transformation of the nuclear genome, it combines various important advantages.

High levels of transgene expression

The genetic system of chloroplasts is highly polyploid with up to 10,000 copies of the plastid genome in a single cell. The resulting very high copy number of plastid-encoded genes per cell offers an enormous potential for expressing foreign genes to maximum levels. Indeed, with many of the transgenes expressed thus far from the plastid genome (Table 1), protein accumulation rates of 1–10% of total cellular protein content could be achieved (e.g. McBride et al. 1995; Staub et al. 2000). Typically, the expression levels are at least several-fold higher than those obtained upon expression of the same transgenes in the nucleocytoplasmic compartment. Whether or not additional factors besides the polyploidy of the plastid genetic system (e.g. a lower degradation capacity for foreign proteins) also contribute to this very high expression levels of chloroplast transgenes is currently unknown.

Absence of epigenetic effects in chloroplasts

Nuclear transformation experiments in higher plants frequently suffer from epigenetic gene-silencing mechanisms ultimately resulting in metastable transgene expression or complete loss of transgene activity (for review, see e.g. Kooter et al. 1999). Epigenetic transgene inactivation can be brought about either by turned-off transcription (transcriptional gene silencing) or by inducing rapid mRNA decay (post-transcriptional gene silencing). The risk of epigenetic transgene inactivation appears to be particularly high when overexpression is attempted. By contrast, gene silencing phenomena have not been observed in chloroplasts; and it appears highly likely that epigenetic effects are entirely absent from plastids. This makes transformation experiments in plastids much more predictable and reliable than in the plant nucleus.

Absence of position effects due to targeted integration of transgenes

Transgene integration into the chloroplast genome occurs exclusively via homologous recombination. This facilitates targeting of a foreign gene to a specific location in the plastid DNA. In contrast, in nuclear transformation experiments with higher plants, the transforming DNA integrates predominantly by non-homologous recombination, which leads to transgenic lines with largely different copy numbers and integration sites of the transgene. This is associated with widely different expression levels of the transgene, depending on the genomic context and the chromatin structure at the integration site. Consequently, large numbers of nuclear transformants must be generated and screened before a line is identified that displays reasonably high transgene expression. In chloroplasts, the expression level of a transgene is largely independent of its integration site and, instead, is only determined by the choice of the expression signals fused to the coding region (i.e. promoter strength, sequence of the ribosome-binding site, etc.).

"Gene-stacking": expression of operons as polycistronic mRNAs

A particularly attractive feature of the chloroplast transformation technology is the possibility of introducing several transgenes with a single transformation vector into the plant. Polycistronic mRNAs can be efficiently translated in chloroplasts, which allows for the concerted expression of several transgenes driven by a single promoter (Staub and Maliga 1995). This opens up the attractive possibility of expressing entire operons in plastids, e.g. operons from bacterial sources which encode novel biosynthetic pathways.

Ecological safety: containment

Chloroplasts are strictly maternally inherited in most crop species, in that only the egg cell transmits its plastids to the zygote, whereas the sperm cell in the pollen grain is free of plastids. Thus, in contrast to transgenes residing in the nuclear genome, plastid transgenes are excluded from pollen distribution (Daniell et al. 1998; Scott and Wilkinson 1999). This gene containment provided by transplastomic plants reduces to a minimum two of the most important ecological risks potentially associated with transgenic plants: (1) uncontrolled spreading of the transgene from a field with transgenic plants to neighboring fields with non-transgenic plants and (2) outcrossing, i.e. distribution of transgenes through sexual reproduction from the crop plant to its weedy relatives (e.g. from cultivated oilseed rape, *Brassica napus*, to the related wild species, *Brassica rapa*; Scott and Wilkinson 1999).

Techniques for the generation of marker-free transplastomic plants

A general problem with transgenic plants is the continued presence of the antibiotic resistance genes that were used as selectable marker genes in the transformation experiments and will be massively released into the environment upon growing the transgenic plants in the field. Hence, elimination of the marker gene after successful generation of the transgenic plant is highly desirable. Two different strategies have successfully been applied for selectable marker gene removal (= "marker recycling") from transformed chloroplast genomes (Fischer et al. 1996):

1. Use of a selectable marker gene flanked by two direct repeats: following transformation, homologous recombination between the two repeats will result in loss of the marker gene from the plastid genome.
2. Co-transformation: simultaneous delivery into the chloroplast of the transformation vector carrying the foreign gene to be introduced and a second vector containing the selectable marker gene inserted into an essential chloroplast gene. The gene disruption performed by the second vector prevents the transformed plants from becoming homoplasmic for the resistance gene but not for the foreign gene of interest introduced by the other vector. When homoplasmy is achieved for the latter, the heteroplasmic resistance gene will readily be lost by random sorting during plant regeneration on antibiotic-free medium.

Biotechnological applications of chloroplast transformation

In spite of today's advanced agricultural technology and the heavy use of agrochemicals, up to 42% of crop productivity is lost due to pests, pathogens and competition with weeds. In view of a constantly growing world population, the engineering of resistance traits is therefore one of the major challenges currently faced by plant breeders and biotechnologists.

Efficient resistance management has become feasible through the use of transgenic technologies. For example, insect-resistant plants can be developed through the expression of toxin proteins that effectively kill target insects. The most prominent examples for such insecticidal proteins are the crystal toxin proteins (Cry) from *Bacillus thuringiensis* (Bt). Members of this protein family possess potent insecticidal activity against a number of Coleoptera, Lepidoptera and Diptera, by inducing the lysis of epithelial cells in the midgut of the insects. Crystal toxins are classified CryI to CryV, based on their sequence homology and toxicity to the target insects. Many crystal proteins are synthesized as non-toxic protoxins, which are converted into active toxins by proteolytic processing in the insect midgut. Initial attempts to generate insect-resistant plants through the expression of Bt toxin genes in the nucleus resulted in very low expression levels, presumably due to mRNA instability, differences in codon usage and aberrant transcrip splicing. As the Bt toxin genes are AT-rich and plant nuclear genes are usually GC-rich, subsequent attempts focused on the construction of synthetic gene versions, whose sequence was adjusted to the codon usage in the plant nucleus. This, together with testing of a variety of promoters, leader and transit peptides, led to a significant improvement of insect resistance and plants expressing the Bt toxin to levels of up to 0.8% of the total leaf protein (Wong et al. 1992). Altogether, it took 5 years of intensive research (including complete chemical re-synthesis of the gene) before transgenic plants with reasonably high Bt toxin expression and hence insect resistance were developed. In 1995, a single experiment involving chloroplast transformation produced transgenic Bt-expressing plants (McBride et al. 1995; Table 1) that easily beat all nuclear Bt gene transformants ever generated: fusion of a native *cry1A* gene to chloroplast expression signals and incorporation of the construct into the tobacco plastid DNA resulted in plants accumulating the protoxin to 3–5% of the total soluble protein. These plants were extremely toxic to herbivorous insect larvae and, in bioassays, caused 90–100% mortality within 5 days (McBride et al. 1995). Similarly, expression of another Bt toxin gene in plastids, *cry2A*, also conferred high level of insect resistance and resulted in 100% mortality even among insect species resistant to Bt plants generated by nuclear transformation (Kota et al. 1999). In addition, transplastomic Bt plants promise to overcome two ecological problems that recently were found to be potentially associated with transgenic corn plants expressing Bt toxins in their nuclear genome: (1) the toxicity of transgenic pollen to non-target insects (Losey et al. 1999) and (2) the release of Bt toxin into the rhizosphere soil, where it remains active and retains its insecticidal properties (Saxena et al. 1999). Both toxin distribution with the pollen and toxin release from the roots can potentially negatively affect non-target insects or even organisms in higher trophic levels. The risk of damage to non-target species is particularly high because most current Bt crops (mainly corn and cotton) contain a transgene encoding the mature Bt toxin instead of the protoxin. This is because the Cry genes are rather large (~3.5 kb) and transgene size is often the limiting factor for high-level expression in the plant nucleus. In contrast, chloroplast transformation provides containment of both transgenes and transgene products; and it efficiently prevents ecological damage through release of transgenic pollen. Also, in chloroplasts, crystal toxins can easily be synthesized as protoxins, thereby limiting the collateral damage to non-target species.

Transplastomic technologies also proved to be superior in biotechnological weed control. The widely used herbicide glyphosate is a potent inhibitor of the aromatic amino acid biosynthetic pathway in plants. It competitively inhibits the enzyme 5-enol-pyruvyl shikimate-3-

phosphate synthase (EPSPS). Resistance to glyphosate can be achieved by overexpression of EPSPS or, alternatively, through expression of mutant alleles encoding glyphosate-insensitive enzyme variants. Overexpression of EPSPS in transgenic tobacco plastids resulted in very high levels of enzyme accumulation and plants tolerant to remarkably high concentrations of the herbicide glyphosate. Strictly maternal inheritance of the resistance trait confirmed lack of pollen transmission, minimizing the risk of transgene flow from crops to wild weedy relatives.

Recently, the application spectrum of transplastomic technologies was extended to the high-yield production of pharmaceuticals in chloroplasts. Human growth hormone (somatotropin) was successfully expressed from the tobacco chloroplast genome. Soluble recombinant protein accumulated to very high levels (> 7% of total soluble protein). Interestingly, the eukaryotic protein somatotropin could be produced in plastids in its correct, disulfide-bonded form and was shown to be biologically active in bioassays (Staub et al. 2000). This study represents a first promising step towards the use of transgenic chloroplasts as a novel system for the efficient expression of metabolites and pharmaceuticals.

Perspectives

The above described first examples of transgenes accommodated in the chloroplast genome illustrate two particularly attractive features of transplastomic technologies: (1) high level of foreign gene expression and (2) containment of transgenes. However, the full potential of chloroplast transformation for plant genetic engineering has not nearly been explored yet. Transgene stacking and expression as polycistronic messenger RNAs, the introduction of novel biosynthetic pathways, or the controlled manipulation of existing ones provide new attractive challenges to chloroplast biotechnologists. Certainly, future studies will be directed toward these applications.

Chloroplast transformation also offers a great potential in algal biotechnology. Recent examples of successful foreign gene expression in *Chlamydomonas* chloroplasts (e.g. Minko et al. 1999) show considerable promise that the large-scale and cost-effective production of biochemicals and pharmaceuticals in algal chloroplasts will be feasible in the future.

In higher plants, a plastid transformation system was first developed in 1990 for the model plant tobacco. It took almost 10 years before some progress with adaptation of the transplastomic technology for other plant species was made (Khan and Maliga 1999; Sidorov et al. 1999). At present, there are still no chloroplast transformation systems available for most major crop plants. Especially in monocotyledonous plants, the limitations of the currently available tissue culture and plant regeneration systems pose a serious obstacle to the extension of the chloroplast transformation technology to important cereal crops. However, with the current acceleration of research in this field, new strides forward are expected in the near future.

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