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Review

Potential of Chloroplast Genome in Plant Breeding

Jana ŘEPKOVÁ

Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

Abstract: Chloroplast engineering (or chloroplast transformation technology, CTT) is a strategy consisting of inserting a transgene into the chloroplast genome of a plant instead of its nuclear genome. CTT brings advantages such as control of the site of gene insertion, high rates of transgene expression and protein accumulation, lack of transmission of the transgene via pollen due to the fact that plastid genes are maternally inherited and an absence of epigenetic effects. Tobacco remains the species most amenable to CTT to date, although chloroplast genetic engineering has also been achieved successfully in crops such as maize, tomato, cotton, potato, rice and sugar beets. Improving agricultural traits such as herbicide and pathogen resistance, resistance to drought, salt tolerance and phytoremediation potential are all promising applications. Molecular pharming is another area of chloroplast engineering with high potential; the production of a wide range of products such as vaccine antigens, pharmaceutical proteins (human somatotropin, human serum albumin, human interferon, monoclonal antibodies) and industrial proteins (avidin, beta casein, liquid crystal polymers, xylanase, anthranilate synthase) is economically beneficial in comparison with bacterial cultivation or animal cell cultures. This review summarises the current status of CCT and its potential economic impact from the viewpoint of high levels of transgene expression and high accumulation of foreign proteins.

Keywords: agricultural traits; chloroplast engineering; industrial proteins; molecular pharming; total leaf soluble protein

New genomic tools have been extensively used to understand genome dynamics, and their exploitation also ranges into many fields of plant breeding. Next generation DNA sequencing (Deschamps & Campbell 2010), high-throughput DNA markers and their use in marker-assisted selection (Hospital 2009) and -omics technologies have all emerged as powerful tools for understanding genome variation in crop species with the final aim of crop improvement. The primary target of interest has been nuclear DNA.

One of the major features by which eukaryotic cells differ from prokaryotic cells is the presence of various subcellular organelles, including mitochondria (present in almost all eukaryotes) and plastids (present in plants and algae). Plastids and mitochondria are unique because they possess their own genetic systems and protein synthesis machinery. Plastids perform a wide variety of anabolic reactions that include photosynthetic carbon reduction, amino acid biosynthesis and fatty acid production. Plant mitochondria engage in respiration and act with other

organelles to perform photorespiration and gluconeogenesis (Sugiura & Takeda 2000). According to the endosymbiont hypothesis, chloroplasts and mitochondria evolved from prokaryotes that were engulfed by proto-eukaryotic cells during the origin of the eukaryotic lineage about 1.2 million years ago. During evolution, most of the DNA once present in the organelles was transferred to the nuclear genome. This process of gene transfer appears to be ongoing at the present time (Grevich & Daniell 2005). Similar to the genomes of bacteria and unlike the nuclear genomes of eukaryotes, the DNA within these organelles is predominantly circular and does not form complexes with proteins.

The chloroplast genome is a molecule of 120 to 160 kb (Sugiura & Takeda 2000). The number of copies per cell ranges from 1 000 to 10 000 depending on cell age: a typical photosynthetically active plant cell contains approximately 100 chloroplasts, and each chloroplast contains up to 100 copies of DNA. Plant mitochondrial genomes are more variably sized and range from about 200 kb to 2600 kb (Sugiura & Takeda 2000). These genomes exist in multiple sub-genomic circles; small circles are derived from larger ones by means of crossing-over. This makes analyses difficult.

The complete sequences are now available for the plastid genomes of more than 130 plant species, including tobacco, maize, rice, barley, carrot, cotton, papaya, pea, pine, potato and sunflower (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=2759&opt=plastid). For mitochondrial genomes, the complete sequences are known for about 20 plants (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=33090&opt=organelle). Sequencing efforts have revealed that plastid genomes in higher plants are highly conserved. In contrast to plant mitochondria, genome organisation and coding capacity in plastids display relatively little interspecific variation in coding regions (Grevich & Daniell 2005). The known sequences are necessary (for example) for directed transgene insertion.

The chloroplast genome in most flowering plants contains 120 to 130 genes (Sugiura & Takeda 2000) and shows conserved organisation with two inverted repeat regions and two regions (small and large) of single-copy genes. The genes fall into two categories: genes for the genetic system and photosynthesis-related genes. Most plastid DNAs contain all of the rRNA genes, a full complement of tRNA genes and genes for ribosomal proteins and RNA polymerase subunits. They include about 100 single-copy genes, most encoding proteins that are required for photosynthetic functions, such as the *rbcL* gene for Rubisco (enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase required for CO_2 fixation). Many genes in the plastid genome are organised into polycistronic transcription units, i.e., clusters of two or more genes that are transcribed by RNA polymerase from a single promoter. Some of these resemble prokaryotic operons; however, unlike the operons of prokaryotes, plastid genomes also contain polycistronic transcription units composed of functionally distinct genes.

The successful transformation of chloroplasts two decades ago (Svab *et al.* 1990) offered an attractive alternative to nuclear gene transformation due to its advantages in many respects. A single plastid gene is represented 10 000 times within a photosynthetically active cell; taking into consideration the two inverted repeat regions of higher plants, the number of copies can reach up to 20 000. This results in high levels of transgene expression. Foreign proteins have been shown to accumulate to a level as high as 46% of the total leaf soluble protein (tsp) (De Cosa *et al.* 2001), and the accumulation of transcripts is 169 times higher in chloroplasts than in plants after nuclear transformation (Lee *et al.* 2003). Chloroplasts are an ideal place to accumulate proteins or their biosynthetic products that may be harmful in the cytoplasm (Bogorad 2000), such as cholera toxin B subunit, trehalose or xylanase. This trans-plastomic expression is the most common and successful method for the production of recombinant proteins using the chloroplast bioreactor. After insertion of foreign genes into nuclear chromosomes, transcripts require transit peptides, also called chloroplast targeting signals (Comai *et al.* 1988), to target heterologous proteins in chloroplasts to the locations where the proteins function.

Recently, plastoglobules, which are sub-chloroplastic compartments, have been targeted for recombinant protein accumulation (Vidi *et al.* 2007). These are low-density particles associated with thylakoid membranes. Like oil bodies, they are light in weight and contain only a few proteins (Ytterberg *et al.* 2006). Plants have the ability to correctly carry out post-translation modifications such as glycosylation, phosphorylation and amidation. Microorganisms are also used for large-scale industrial applications of recombinant protein production; however, post-translation modification processes such as glycosylation, proper folding, formation of disulfide bonds

and the assembly of complex multi-subunit proteins do not take place.

Chloroplasts are characterised by an absence of epigenetic effects such as gene silencing (De Cosa *et al.* 2001; Lee *et al*. 2003) and position effects (Daniell *et al.* 2001) because transgenes are integrated into site-specific spacer regions of the chloroplast genome. A very attractive feature is the simplicity of transgene assembly into operons (De Cosa *et al.* 2001; Daniell & Dhingra 2002). The efficiency of transformation is significantly higher when complete homology of plastid DNA flanking sequences is retained. Lack of the complete chloroplast genome sequence is still one of the major limitations to extending this technology to useful crops. Chloroplast genome sequences are necessary for the identification of spacer regions used for the integration of transgenes at optimal sites via homologous recombination, as well as to identify endogenous regulatory sequences for optimal expression of transgenes (Grewich & Daniell 2005).

Plastids are inherited in a uniparental, strictly maternal fashion in most angiosperm plant species (Hagemann 2004); therefore, gene containment occurs owing to the lack of transgene transmission by pollen. Although pollen contains plastids, the plastid DNA itself is lost during the process of pollen maturation and therefore is not transmitted to the next generation (Daniell 1999, 2000; Nagata *et al.* 1999).

The reason why this technology has not been extended to many of the major crops is that transgenes are integrated via homologous recombination within the plastid genome, unlike the random integration that occurs in nuclear transformation. Plastid transformation vectors are designed to contain homologous flanking sequences on either side of the transgene for site-specific integration (Daniell 1993, 1997; Kumar & Daniell 2004; Daniell *et al.* 2005). Highly efficient plastid transformation has been recently accomplished via somatic embryogenesis using species-specific chloroplast vectors in carrot, cotton, maize, rice and soybean. Several other crop chloroplast genomes including cauliflower, cabbage, lettuce, oilseed rape, petunia, poplar, potato, tobacco and tomato have been transformed via organogenesis (Daniell *et al.* 2005; Ruhlman *et al.* 2007). Tobacco has been a widely exploited host for chloroplast transformation technology (CTT) because of its easy genetic manipulation. Tobacco plants hold great promise as hosts for the production of useful compounds, as one hectare of tobacco can produce more than 100 metric tons of leaves per year (Verma & Daniell 2007).

Recent advances in plastid engineering have provided an efficient platform not only for the improvement of agronomic traits but also for the production of therapeutic proteins, vaccines and biomaterials. "Molecular farming" is not particularly well-known for most of plant species. It is a method used to integrate a foreign gene into plants in order to use them to produce high-quality pharmaceutical substances or diagnostics. In contrast, neither microorganisms nor yeast are able to correctly assemble and fold complex proteins. The production of pharmaceutical substances in animal cell culture is often associated with the risk that the product is contaminated with pathogens that are dangerous to human health. These include, for example, AIDS and hepatitis viruses, BSE pathogens and carcinogenic substances. Although animal systems have been used for years for the safe production of pharmaceuticals, it is still necessary to apply time-consuming and costly test procedures in order to constantly monitor their quality.

Among the frequent arguments against genetically modified (GM) plants is the fact that they contain an antibiotic resistance gene. There have been concerns about horizontal gene transfer from transgenic plants back to bacteria, which may result in antibiotic resistance. For instance, the *Neomycin phosphotransferaseII* (*NPTII*) gene, which can confer kanamycin resistance to transgenic plants, represents an invaluable tool for plant engineering and belongs to a class of antibiotic resistance genes acceptable for commercial release (EFSA 2004). Nevertheless, the development of techniques to avoid the presence of a selectable marker in transgenic plants could improve public perception of plant genetic engineering. Antibiotic-free selection after plastid transformation can be also accomplished by using the *betaine aldehyde dehydrogenase* (*BADH*) gene.

Agronomic traits expressed via the plastid genome

Many important crops have already been genetically modified for agronomic traits via the nuclear

genome. However, nuclear transgenic plants have drawbacks inherent to their method of transformation because of the risk of transgenes spreading to their wild relatives. Plastomes offer an ideal place for agronomic gene expression because of their level of expression and gene containment via maternal inheritance. Many important agronomic traits have already been engineered via the plastid genome, such as herbicide resistance, insect resistance and tolerance to drought and salt.

Transgenic plants engineered for herbicide resistance are mostly insensitive to the herbicide's effect. The most commonly used herbicide, glyphosate, inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSPS), which is encoded by a nuclear gene and is involved in the biosynthesis of aromatic amino acids. Nuclear transgenic plants are quite effective; however, the possibility of transgenes outcrossing to other related species and weeds is a significant drawback. Because the target of glyphosate resides within the chloroplast, CTT is an ideal strategy here. The first report of a correctly expressed chloroplast-targeted eukaryotic gene was presented for petunia. The *aroA* gene was integrated between the *trn*I and *trn*A genes in the inverted repeat region or between the *rbc*L and *acc*D genes in the large single copy region (Daniell *et al*. 1998). Integration of this gene resulted in resistance to up to 5mM glyphosate, which is 10-fold greater than the lethal concentration. More recently, the *CP4 EPSPS* gene was expressed in tobacco plastids resulting in over 250-fold more EPSPS enzyme in comparison with a nuclear transgenic model (Ye *et al.* 2001). However, these increased levels of glyphosate-resistant EPSPS did not correlate to increased tolerance to glyphosate. Transgenic tobacco plants expressing *bar* genes via the chloroplast exhibited field-level tolerance to phosphinothricin, which was conferred by even the lowest levels of *bar* expression (Lutz *et al*. 2001).

Most approaches have aimed at plants engineered with genes for insecticidal protoxins produced by a variety of *Bacillus thuringiensis* subspecies. The Cry2Aa2 operon, representing such target genes, consists of the genes *Cry2Aa2*, *orf* 1 and *orf* 2. The chaperone activity of orf 2 tobacco chloroplasts was shown to correctly process a bacterial operon and express the Cry2Aa2 protein at levels up to 46% tsp, the highest level ever recorded (De Cosa *et al.* 2001). The expressed foreign protein was protected from degradation by overexpression of the chaperone crystal protein of *Bacillus thuringiensis* that forms a cuboidal crystal structure. Low expression levels of Bt toxins engineered via the nuclear genome increases the risk of developing Bt-resistant pests. Chloroplast transgenic plants were able to kill insects that could withstand insecticidal protein concentrations 40 000 times higher than normal, even at lower levels of Cry2Aa2 protein expression (Kota *et al.* 1999). The high levels of Cry2Aa2 protein accumulation in transgenic chloroplasts offers complete resistance to insects that feed on transgenic plants. Another Cry1Ia5 protein accumulated to levels of up to 3% of tsp in tobacco leaf tissue after protoplast transformation (Reddy *et al.* 2002).

The antimicrobial peptide MSI-99 (an analogue of maganin-2) confers protection against many prokaryotic organisms due to its high specificity for negatively charged phospholipids, which are typically found in the outer membranes of bacteria and fungi (Houston *et al.* 1997). Upon contact, peptides aggregate to form pores, resulting in cell lysis. In *in planta* assays, chloroplast transgenic tobacco leaves inoculated with the pathogen *Pseudomonas syringae* pv. *tabaci* showed no sign of necrosis even when 80 000 cells were used for inoculation, whereas a large necrotic area was seen in wild-type (wt) plants inoculated with as few as 800 cells (DeGray *et al.* 2001). Additionally, transgenic chloroplasts showed resistance to the destructive fungal pathogen *Colletotrichum* in the same assay. These data suggest that MSI-99 expressed in tobacco chloroplasts can offer significant protection from both bacterial and fungal pathogens. By contrast, when the same MSI-99 was expressed via the nuclear genome, it did not confer adequate disease resistance against these pathogens due to low levels of expression (Li *et al.* 2001).

Other environmental stresses such as drought, salinity and freezing can severely limit plant growth and development. All these stresses are often accompanied by osmotic stress resulting in severe cellular dehydration. Many plants are capable of expressing low molecular-weight compounds called osmoprotectants (Glick & Pasternak 1998) that act as osmolytes and help organisms survive extreme osmotic stress. Plants accumulate in cells solutes such as betaines, amino acids and sugars in response to stress and thus help to maintain turgor at low water potential and protect cellular macromolecules and cell membranes from damage due to high salt levels.

Trehalose phosphate synthase encoded by the *TPS1* gene mediates a reaction that forms the os-

moprotectant trehalose. Because trehalose has been found to accumulate under stress conditions such as freezing, heat, salt and drought, it is thought to play a role in protecting cells against damage caused by these stresses. In contrast to nuclear transgenic plants that exhibited pleiotropic effects at low levels of *ATP*1 expression including stunted growth and male sterility, chloroplast transgenic plants grew normally and accumulated trehalose at a level 25-fold higher (Lee *et al*. 2003). Chloroplast transgenic plants showed a high degree of drought tolerance by remaining green and healthy in 6% PEG (polyethylene glycol), whereas wt plants were completely bleached. Chloroplast transgenic plants also recovered after they were not watered for 24 days and subsequently rehydrated for 24 h.

The increasing use of irrigation and fertilisers in agriculture has led to unnaturally high salinity in soil. The stress associated with salt can result in a reduction of leaf exchange and photosynthesis capacity. Plants are able to produce osmoprotectants that confer resistance to several stresses, including drought. Glycine betaine (GB) is a quaternary ammonium compound and a highly effective osmolyte found in at least ten flowering plant families and also in marine algae. The synthesis of GB in plants has been studied mainly in species of *Chenopodiaceae* (spinach, beet, and others). GB is extremely soluble in water. Important crops do not accumulate betaine. The spinach *badh* (*betaine aldehyde dehydrogenase*) gene transferred into the nuclear genome resulted in only moderate levels of tolerance to salt stress. However, *badh* overexpressed in carrot plastids resulted in the accumulation of GB and subsequently conferred a significant amount of salt tolerance (Kumar *et al.* 2004). Transgenic lines grew in up to 400mM NaCl in the media, the highest level of salt tolerance ever reported in transgenic plants and equal to levels of tolerance seen only among halophytes.

Oryza sativa L., a non-accumulator of GB, is highly susceptible to abiotic stress. Transgenic rice with chloroplast-targeted choline oxidase encoded by the *codA* gene from *Arthrobacter globiformis* has been evaluated for inheritance of the transgene and water-stress tolerance. Transgenic plants maintained higher photosystem II activity and they showed better physiological performance; for example, enhanced detoxification of reactive oxygen species compared to wt plants under water-stress (Kathuria *et al*. 2009). Metabolic engineering for beta-alanine overproduction in plants is expected to enhance environmental stress tolerance. The *Escherichia coli* enzyme l-aspartate-alpha-decarboxylase (AspDC) encoded by the *panD* gene catalyses the decarboxylation of l-aspartate to generate beta-alanine and carbon dioxide. A constitutive *E. coli panD* expression cassette was co-introduced with a constitutive selectable *aadA* expression cassette into the chloroplast genome of tobacco via biolistic gene transfer and homologous recombination. The *AspD* activity in transplastomic plants expressing *panD* was dramatically induced by high-temperature stress. Beta-alanine accumulated in transplastomic plants at levels four-times higher than in wt plants. The CO_2 assimilation of transplastomic plants expressing *panD* was more tolerant to high temperature stress than that of wt plants, resulting in the production of 30–40% more above ground biomass than wt plants (Fouad & Altpeter 2009).

Plastids engineered for the bioremediation

Phytoremediation is a safe and cost-effective system for cleaning up contaminated environments using plants. However, plants have a limited ability to treat high levels of toxic chemicals. Genetic engineering is aimed at utilising genes to modify the nuclear or chloroplast genomes of plant and thereby enhance their remediation capacity. Organomercurial compounds are the most toxic form of mercury. Since the chloroplast is a primary target of Hg damage in plants, it is an ideal place to engineer resistance and detoxification of organomercurials and metallic mercury (Kupper *et al.* 1996). Two bacterial genes encoding two enzymes, mercuric ion reductase (*merA*) and organomercurial lyase (*merB*), were expressed as an operon in transgenic tobacco chloroplasts (Ruiz *et al.* 2003; Hussein *et al.* 2007). Sixteen-day-old tobacco plants were grown for 14 days in soil treated with various concentrations of organomercurial phenyl mercuric acetate (PMA)-supplemented nutrient solution. The transgenic plants grew well and were resistant to very high concentrations of PMA, up to 400μM, while wt plants barely survived at concentrations of 50μM PMA. Hussein *et al.* (2007) demonstrated that chloroplast transgenic plants expressing *merA* and *merB* can accumulate Hg in roots to levels surpassing the concentration in soil, up to 200 μg/g, without any detrimental effect and could accumulate 100-fold more Hg in leaves than untransformed plants.

Transgenic plastids as a plant factory for biopharmaceuticals

Plants hold the potential for cost-effective, large scale production of recombinant proteins for pharmaceutical uses. Therapeutic monoclonal antibodies are expected to account for at least 40% of the total production of biopharmaceuticals (Galeffi 2009). Plants/crops may offer a flexible system for producing potentially unlimited quantities of biopharmaceuticals for human needs at a relatively low cost. Currently, major application areas for biopharmaceuticals include oncology, anti-infective diseases, blood disorders and vaccines. Since 2001, the Human Genome Project has been generated huge profits obtained from growth hormones, insulin and red-blood-cell stimulating agents. Worldwide, some 80 biopharmaceuticals are on sale and more than 500 are being tested at various clinical levels (Galeffi 2009). However, in Europe, a negative public perception towards transgenic plants could significantly slow down this development. Chlorogen, a biotechnology company, has adopted the CTT as a platform technology for the production of foreign proteins in plants. The company has patented genetic regulatory signals that direct foreign genes to function specifically within chloroplasts and is developing a wide range of protein molecules including vaccines and other therapeutic proteins for industrial applications.

Human somatotropin (hST) is used to treat hypopituitary dwarfism in children, Turner syndrome, chronic renal failure and HIV wasting syndrome. The *hST* gene was integrated between the *trnV* gene and the *rps7/3'-rps12* operon located within the inverted repeat region of tobacco chloroplasts. This expression cassette resulted in high levels of the hST expression, and the biologically active recombinant protein reached 7% tsp (Staub *et al.* 2000). It was shown to contain proper disulfide bond formation through the use of the chloroplast protein disulfide isomerase and it was identical to the native hST protein.

Human serum albumin (HAS) is one of the most widely used intravenous proteins in many human therapies. It is used clinically for the replacement of blood during incidents of trauma and treatment. Plants are an economical, safe and efficient system for many recombinant proteins. Nuclear transformation for the expression of HAS has resulted in only 0.2% tsp in tubers and 0.02% tsp in leaves (Farran *et al.* 2002), far below the levels needed for cost-effective production. It was hypothesised that the AT-richness of the HAS sequence was responsible for its poor translation in the eukaryotic cells of transgenic nuclear plants. On the other hand, this AT-rich composition (57%) is ideal for chloroplast expression. In addition, HAS requires 17 disulfide bonds to be correctly folded and protected from protease activity (Grevich & Daniell 2005). The amount of HAS was found to be up to 8.2% tsp in potted plants of tobacco with the *HAS* gene integrated into the chloroplast genome between the *trnA* and *trnI* genes of the chloroplast *rrn* operon within the inverted repeat regions (Fernandez-San Millan *et al.* 2003). The amount of HAS peaked at 11.1% tsp after 50 h of continuous illumination. The functionality of the recombinant HAS was maintained due to the formation of inclusion bodies.

Human interferon alphas are cytokines of the immune system that are known to interfere with viral replication and cell proliferation. They are potent enhancers of the immune system and are used in various clinical treatments. Interferon IFNα2b has been approved by the FDA for the treatment of leukaemia. The *E. coli* recombinant expression system has been used to manufacture IFNα2b; this approach, however, requires *in vitro* processing and purification. These additional procedures result in high product costs. The expression of IFNα2b in plant plastids should help to reduce its high costs because plastids can correctly fold IFNα2b and form the appropriate disulfide bonds. IFNα2b was expressed at levels up to 18.8% tsp. IFNα2b can be orally delivered after bio-encapsulation and has been proven to be therapeutically effective. The cellulose found in plant cell walls protects the biopharmaceutical in the stomach and enables its gradual release in the gut, avoiding many of its negative side effects (Mor *et al.* 1998). Specific expression cassettes for chloroplast transformation have been used to obtain optimal protein levels in leaves of transgenic plants (Arlen *et al.* 2007).

Human interferon gamma = interleukin-18 (INF-γ or IL-18) is another class of cytokine used therapeutically to prevent viral proliferation, and it plays several roles in the immunoregulatory responses to pathogenic bacteria. Nuclear transgenic plants expressing INF-γ yielded only 0.001% tsp. Chloroplast transgenic tobacco lines produced INF-γ at up to 3% tsp. A protein fusion between GUS and INF-γ increased the expression level, and it

accumulated up to 6% tsp (Leelavathi & Reddy 2003). Protein fusion techniques can enhance the stability of a foreign protein, allowing human insulin to accumulate up to 16% tsp. Fusion with the Cholera toxin B-subunit protein was performed in transgenic plants by Ruhlman *et al.* (2007).

Antibodies (or immunoglobulins, IgGs) are serum proteins that play a central role in the humoral immune response. Immunoglobulins bind and inactivate pathogens or trigger an inflammatory response that results in their elimination. Monoclonal antibodies (mAbs) specific to one epitope have been developed using hybridomas. Because of high capital costs and the inherent complexity of mammalian production systems that are associated with the production of mAbs in mammalian cultures, the use of plastid genome engineering for the production of mAbs in plant-based bioreactors has been implemented. The cost of raw materials for mAb production in plants is more than 3000-fold less than that of systems employing cultured mammalian cells (Dove 2002).

Guy's 13 monoclonal antibody is specific for a surface antigen of *Streptococcus mutans*, a bacterium that most notably causes dental caries. A chloroplast model using a codon-optimised Guy's 13 gene was developed and put under the control of *psb*A regulatory sequences. This model expressed IgA-G, a humanised chimeric form of Guy's 13 antibody, with correctly folded disulfide bonds (Daniell *et al.* 2004).

MSI-99 is a synthetic lytic peptide that has been expressed via the tobacco chloroplast as an antimicrobial peptide. Because bacterial membranes are highly conserved, it is unlikely that bacteria will develop resistance to the mechanism of antimicrobial peptides. MSI-99 and analogues has been shown to be effective against invading human pathogens as well as certain cancerous cell lines. Cell extracts from transgenic tobacco leaves containing MSI-99 were used to determine its effectiveness toward *Pseudomonas aeruginosa*, a multidrug-resistant bacterium that affects plants, animal and humans. Cell extracts yielded 96% inhibition of the growth of *P. aeruginosa*, highly encouraging news for AIDS and cystic fibrosis patients who are highly susceptible to this pathogen.

Plants are being used for the production of chloroplast-derived vaccine antigens against bacterial pathogens (cholera, tetanus, anthrax, small pox, Lyme disease), viral pathogens (canine parvovirus and rotavirus) and meningitis B, and also for the production of recombinant therapeutic proteins. In order for vaccines from plastids to be suitable for oral delivery, two major obstacles must be overcome. First, large quantities of the recombinant protein must be produced in the edible parts of plants, and second, the antibiotic resistance genes used for selecting transformants must be removed.

The cholera toxin B subunit (CTB) is encoded by *Vibrio cholera*. CTB forms a pentamer that binds to the GM_1-ganglioside receptor predominantly found in the intestinal epithelium. Expression of the native CTB gene lacking its leader sequences in transgenic chloroplasts resulted in accumulation of the CTB antigen at up to 4.1% tsp (Daniell *et al*. 2001). Further studies made use of a translational enhancer element and resulted in up to 31% tsp of CTB with properly assembled pentamers (Molina *et al.* 2004). CTB is a powerful transmucosal carrier and is very effective at delivering several vaccine antigens as a fusion partner of CTB (multicomponent vaccines).

For anthrax vaccine, the *PA* gene (*pag*) was cloned into tobacco chloroplasts using *psb*A regulatory signals to enhance translation. This resulted in up to 2.5 mg PA/g fresh weight, enough for 400 million doses of vaccine free of EF (oedema factor) and LF (lethal factor) per acre of transgenic tobacco (Daniell *et al.* 2004). The functionality of chloroplast-derived PA was tested, and it was found to efficiently bind to anthrax toxin receptors, form heptamers, undergo proper cleavage and bind to LF, thereby resulting in macrophage lysis.

Canine parvovirus (CPV) infects dogs and other *Canidae* family members leading to hemorrhagic gastroenteritis and myocarditis. CPV-1 was initially discovered in 1967 followed by CPV-2 in 1978. CPV-2 is a mutated form of the feline parvovirus. Infected animals shed CPV-2 particles, allowing for rapid world-wide distribution. Furthermore, another mutant form called CPV-2a was found in 1979 and proved to be even more virulent. Recently, antigens capable of eliciting a protective immune response against CPV have been found. Molina *et al.* (2004) have expressed the linear antigenic peptide (2L21) from the VP2 capsid protein of CPV in transgenic tobacco chloroplasts. The 2L21 peptide was fused to a KLH carrier protein, and it has been extensively shown to effectively protect animals against parvovirus infection. When the 2L21 peptide was fused to either the CTB subunit or GFP, a ten-fold increase in expression was seen

in the transgenic chloroplast plants compared to nuclear-transformed plants. The maximum levels of expression were seen with the CTB fusion, where levels reached as high as 31.1% tsp, which is 7.49 mg/g fresh weight.

Plastid engineered products of industrial interest

Another example of the effectiveness of plastid transformation for the overproduction of useful compounds is the generation of astaxanthin-producing transplastomic plants through carotenoid pathway engineering. Transplastomic tobacco that expresses both the β-carotene ketolase and β-carotene hydroxylase genes from the marine bacterium *Brevudimonas* sp. strain SD212 accumulated large quantities of astaxanthin at concentrations of up to 5.44 mg/g dry weight, corresponding to 74% of total carotenoids (Hasunuma *et al.* 2008). Astaxanthin is a highly valued red pigment. It has been industrially exploited as a feed dye, particularly as a feed supplement in poultry farming and aquaculture. Its use is also expected in the pharmaceutical, food and feed industries owing to its diverse biological functions including antioxidant, anticancer and singlet oxygen-quenching activities and enhancement of immune responses.

Plastid genetic engineering offers an ideal solution for the cost-effective production of many industrially valuable biomaterials such as enzymes, amino acids and technical proteins for research purposes. For example, avidin is a glycoprotein found in avian, reptilian and amphibian egg white that is primarily used as a diagnostic reagent. Plant-optimised avidin coding sequences have been expressed in maize (Horn *et al.* 2004). Milk proteins such as human β casein have been produced in transgenic tomatoes.

Biodegradable plastics seem to be a viable alternative to synthetic plastics. Biodegradable materials can undergo decomposition into carbon dioxide, methane, water and inorganic compounds with the help of the enzymatic action of microorganisms within a specified period of time. Chorismate pyruvate lyase (CPL), an enzyme encoded by the *ubiC* gene of *E. coli*, catalyses the direct conversion of chorismate to p-hydroxybenzoic acid (pHBA), the principle monomer used in liquid crystal polymers. The conversion of chorismate to pHBA occurs naturally in plants, although it can require up to ten successive enzymatic steps without CPL. When the *ubiC* gene was expressed in nuclear transgenic tobacco, the accumulation of pHBA was 0.52% dry weight (dw), 10- to 20-fold less than what is required for a commercially viable pathway for pHBA production (Viitanen *et al.* 2004). When the *ubiC* gene was expressed in chloroplast transgenic plants grown under normal light conditions, pHBA accumulated steadily, reaching a maximum of ~15% dw after 100 days in soil. Plants continued to accumulate up to 25% dw of pHBA when grown for 22 days under continuous light, an expected increase for a *psbA*-regulated gene. The enzymatic activity of CPL was found to reach a maximum of 50 783 pkat/mg of protein in T_1 lines, which correlates to 30% tsp. The highest levels observed in nuclear transgenic plants were more than 250-fold less.

Polyhydroxybutyrates (PHBs) are the most common co-polymer produced by microorganisms. The polycistronic *phb* operon encodes three enzymes capable of producing PHB (Peoples & Sinskey 1989), which under optimal conditions will degrade completely to CO_2 and H_2O. For transgenic plant-derived biodegradable plastics to be economically feasible, accumulation of at least 15% tsp is required (Scheller & Conrad 2005). When biodegradable plastic-like compounds were targeted to plastids, the expression levels reached up to 40% tsp (Bohmert *et al.* 2000).

Xylanase is used in the paper, fibre, baking, brewing and animal feed industries as an important cellulolytic enzyme. Its routine use is not feasible due to high production costs. The first nuclear transgenic plants suffered cell wall degradation when expressing xylanases. Because of this, xylanase was targeted to the apoplasts, seed oil bodies or secreted through roots into the culture medium. All these nuclear transgenic approaches exhibited expression levels too low for industrial production to be economically feasible. When the *xynA* gene was transformed via the tobacco chloroplast genome, xylanase accumulated to levels of 6% tsp. Enzyme thermostability offers a unique advantage in purification by allowing the use of heat in the first step of purification to reduce proteolytic degradation by denaturing proteases. Chloroplast-derived xylanase was shown to be as biologically active as the bacterial-derived enzyme, and it also retained its substrate specificity.

The majority of the enzymes used for amino acid biosynthesis are encoded by the nucleus and

transported to plastids. Anthranilate synthase (AS) converts chorismate to anthranilate, undergoing feedback inhibition by the end product tryptophan (Trp). Plant AS homoenzymes are made up of tetramers of two α and two β subunits, of which the α subunits bind to Trp and act as a feedback inhibitor. The *ASA2* gene coding for a feedback-insensitive α subunit was cloned and expressed in transgenic tobacco chloroplasts by Zhang *et al.* (2001). The transgenic plants exhibited high levels of ASA2 mRNA, increased expression of the AS α subunit and a four-fold increase in AS enzyme activity that was less sensitive to feedback inhibition by Trp. This resulted in a seven-fold increase in free Trp in chloroplast transgenic leaves (Barone *et al.* 2009). They also tested the *ASA2* gene of tobacco as a new selectable marker.

There are slightly over 165 recombinant pharmaceuticals currently approved for human use. Another 500 protein candidates are in preclinical and clinical development, about 70% of these being glycosylated proteins (Durocher & Butler 2009). A few plant-derived therapeutics have been approved in Europe for topical use in humans, and several products are currently in clinical trials, including interferon alpha (Locteron®) produced in *Lemna minor* and glucocerebrosidase (prGCD) manufactured in carrot cells (Durocher & Butler 2009).

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Corresponding author:

Doc. RNDr. Jana Řepková, CSc., Masarykova univerzita, Přírodovědecká fakulta, ústav experimentální biologie, Kotlářská 2, 611 37 Brno, Czech Republic
tel.: + 420 549 496 895, fax: + 420 549 492 570, e-mail: repkova@sci.muni.cz