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Chloroplast transgenic approach for the production of antibodies, biopharmaceuticals and edible vaccines

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Introduction

The endosymbiotic organelle, chloroplast, harnesses solar energy and provides food and oxygen that ensure the existence of life on this planet. Besides carrying out the life sustaining process of photosynthesis, the recent capability of engineering their genomes has ushered in a new era in the field of agricultural biotechnology. This technology is environmentally friendly in contrast to the current approach of introducing foreign genes via the nuclear genome of higher plants that has led to negative public perception. As an exception to Mendelian inheritance, chloroplast genomes are maternally inherited in most crops. This eliminates the probability of foreign trait dissemination by pollen to other plant species. Since the chloroplast possesses prokaryotic protein synthetic machinery, it is feasible to express genes of prokaryotic origin and also multiple genes without the impediments of position effect and gene silencing observed in nuclear transformation. The most advantageous feature is the potential to amplify the transgene to 5000-10,000 copies per cell depending upon the site of integration into the chloroplast genome. Because of these reasons, chloroplast transformation may be the harbinger of the next revolution in plant biotechnology and transgenic chloroplasts are being viewed as factories for low-cost production of biopharmaceuticals making them available to those who need them most.

The idea of chloroplast transformation, conceived in the mid-eighties, has evolved into a safe and promising technology over the last decade. The technique involves insertion of a foreign gene, flanked by homologous chloroplast DNA sequences that facilitate recombination at the desired site. Earlier efforts in this field were directed towards the development of *in organello* system (McFadden and Daniell, 1987) because of the availability of techniques to regenerate plants from protoplasts that took up foreign chloroplasts. Later, with the advent of gene gun, the focus shifted to transforming intact plant cells. The first semblance of engineering of chloroplast genomes of higher plants came from the transient expression of a reporter gene in dicot chloroplasts (Daniell et al. 1990; Ye et al. 1990) followed by similar investigations with monocots (Daniell et al. 1991). One such study involved the use of a unique expression system that employed autonomously replicating vectors in the chloroplast (Daniell et al. 1990). In the natural sequence of events, stable chloroplast transformation was achieved in higher plants that involved integration of a selectable marker gene into the chloroplast genome (Svab and Maliga 1993). However it is only in the past few years that this technology has been applied towards realizing its real potential of expressing economically important traits.

Several genes of agronomic importance have been expressed via the chloroplast genome. These include genes conferring resistance to insects (McBride et al. 1995, Kota et al. 1999, DeCosa et al. 2001), phytopathogens (DeGray et al. 2001) and drought tolerance (Lee et al. 2001). Expression levels of the gene products were several hundred-fold higher than the levels achieved by nuclear transformation. Consequently transgenic plants exhibited a greater extent of resistance to the above-mentioned biotic and abiotic stresses. Taking cue from these observations, it is desirable that transgenes are introduced into agronomically important crops in order to improve the overall crop productivity and address the ever-growing food demand of the world.

Recent accomplishments in our laboratory have unveiled a completely new facet of chloroplast biotechnology. Most vital has been the establishment of a selection system based on an edible selectable marker (Daniell et al. 2001a). Confluence of this development and other advantages of chloroplast transformation should play a pivotal role in easing public concern regarding edible transgenic plants. Chloroplast transformation is emerging as a promising alternative for the expression of biopharmaceuticals and this concept had its dawn in the production of a medically important biodegradable protein-based polymer in tobacco chloroplasts. The polymer consists of the repeated amino acid sequence of GVGVP, which is ubiquitously present in all sequenced mammalian elastin proteins (Yeh et al. 1987) and finds use in various medical applications like tissue reconstruction, wound covering and programmed drug delivery to name a few (Guda et al. 2000). Another therapeutically important protein, human somatotropin, used primarily in the treatment of hypopituitary dwarfism in children, was also successfully expressed in the chloroplast (Staub et al. 2000). These two reports lend credence to the concept of employing transgenic chloroplasts for the production of biopharmaceuticals. Other important developments in this field have been the expression of an operon and chaperonin-mediated accumulation of extraordinarily high amounts of protein (DeCosa et al. 2001) as well as hyper-expression of a human blood protein highly sensitive to proteolytic degradation (Fernandez-San Milan et al. 2001). Further, the expression and assembly of functional oligomers of β subunit of cholera toxin in transgenic chloroplasts demonstrated that this sub-cellular compartment facilitates the formation of disulfide bonds and quaternary structure of the protein that are essential for its functionality (Daniell et al. 2001b). The feasibility of expressing therapeutically important proteins in combination with the recent capability to transform chloroplasts of food crops like potato and tomato (Sidorov et al. 1999, Ruf et al. 2001) has opened new avenues for the production of edible vaccines. This article highlights some of these groundbreaking recent achievements.

Materials and Methods

Chloroplast transformation in tobacco was done as detailed by Daniell (1997). Integration and expression of transgenes via chloroplast genomes and characterization of transgenic plants have been described in depth for the β-subunit of cholera toxin (Daniell et al., 2001b), Human Serum Albumin (Fernandez-San Millan et al. 2001), antimicrobial peptide (DeGray et al. 2001) and edible selectable markers (Daniell et al. 2001a).

Results and Discussion

Establishment of antibiotic free selection system

The use of antibiotic based selection to identify transgenic plants has been one of the hotly debated public concerns. The *aad*A gene that confers resistance to the antibiotics, spectinomycin and streptomycin, has been conventionally employed for chloroplast transformation. However, these drugs are routinely administered against bacterial infections

in humans and animals. The presence of such a gene product at high levels in plants is undesirable. To alleviate this problem, Daniell et al (2001a) used betaine aldehyde dehydrogenase (BADH) as a selectable marker. BADH is a naturally occurring gene in spinach whose product catalyzes the conversion of betaine aldehyde (BA) to glycine betaine. Betaine aldehyde accumulation within plant cells is toxic and has an adverse effect on growth. This enzyme is present only in chloroplasts of a few plant species that are salt or drought resistant (Rathinasabapathy et al. 1994, Nuccio et al. 1999). The introduction of BADH into the chloroplast genome and subsequent selection on BA containing media resulted in 25-fold higher transformation efficiency in comparison to spectinomycin based selection. It was demonstrated that BADH provides both a superior selection system as well as a reduction in the time required for recovering transgenic plants, from 6-8 weeks to less than two weeks (Daniell et al. 2001a). Introduction of an edible selectable marker in the procedure to obtain transgenic plants will certainly make GM foods more acceptable to the public.

Hyper-expression of Human Serum Albumin (HSA)

The attempts to express therapeutically valuable proteins in plants have been difficult so far owing to disappointingly low levels of human protein accumulation via nuclear transformation. We endeavored to address this problem by expressing transgenes in the chloroplast where their products can accumulate up to 46% of total soluble protein (tsp) (DeCosa et al. 2001). HSA finds various therapeutic applications, ranging from blood volume disorders, extensive burns or dehydration and cirrhotic/hepatic illnesses. Its presence is instrumental in maintaining colloidal osmotic pressure within blood vessels and transfer of ligands across organ circulatory interfaces. To optimize gene expression within the chloroplast, this gene was expressed under the control of two different 5' UTRs, *psb*A and *cry*2Aa2 and the chloroplast preferred ribosome-binding site (RBS). It was observed that the level of protein accumulation varied in seedlings and potted plants. In seedlings, the *cry*2Aa2 UTR was found to be more efficient (5.9% HSA of tsp) while in mature plants the *psb*A 5' promoter and UTR were more efficient (8.2% HSA of tsp) as evident from the percentage of HSA accumulation in total soluble protein content. HSA is highly sensitive to proteolytic degradation and accumulated to only 0.02% tsp when expressed using the RBS. In the past, expression of transgenes using the RBS has resulted in very high accumulation of foreign proteins (McBride et al. 1995, Daniell et al. 2001b). Hyper-expression of proteins that are highly susceptible to proteolytic degradation, under the control of the *psb*A promoter and 5' region, should serve as a model for the expression of other such proteins (Fernandez-San Milan, 2001). This study shows the potential use of plants as the proverbial factories for producing biopharmaceutical proteins.

Expression of Anti-Microbial Peptides (AMP) to combat drug-resistant pathogens

There are several human pathogenic bacteria that are drug resistant or have acquired resistance over a period of time. There is an urgent need to explore alternate ways of combating such bacteria. Magainin and its analogues have been investigated as a broad-spectrum topical agent, a systemic antibiotic, a wound healing stimulant and an anticancer agent (Jacob and Zasloff, 1994). Magainin's analogue, MSI-99, a synthetic lytic peptide, has been recently expressed via the chloroplast genome (DeGray et al. 2001). This AMP is an amphipathic alpha helix molecule and possesses affinity for negatively charged phospholipids found in the outer membrane of all bacteria. The probability of bacteria adapting to the lytic activity of this peptide is less likely. The minimum inhibitory concentration of MSI-99 was investigated. Based on total inhibition of bacterial cells or fungal spores, MSI-99 was most effective against *P. syringae*, requiring only 1µg / 1000 bacteria (DeGray et al. 2001). Because the lytic activity of antimicrobial peptides is concentration dependent, the amount of

antimicrobial peptide required to kill bacteria was used to estimate the level of expression in transgenic plants. Based on the minimum inhibitory concentration, it was estimated that transgenic plants expressed MSI-99 at 21.5-43% of the total soluble protein. A multi-drug resistant gram negative bacteria, *Pseudomonas aeruginosa*, that is an opportunistic pathogen for plants, animals and humans was used for *in vitro* assays to test for the effectiveness of the lytic peptide expressed in the chloroplast. Cell extracts prepared from T1 generation plants resulted in 96% inhibition in growth of this pathogen (Figure 1). This result is very encouraging as it may pave the way for an alternative method to combat drug-resistant human pathogenic bacteria. The lytic peptide may also provide a solution for the patients suffering from cystic fibrosis, who are extremely susceptible to P. *aeruginosa* infections.

Pharmaceutical companies are exploring the use of lytic peptides as broad-spectrum topical antibiotics and systemic antibiotics. It has been reported that the outer leaflet of melanoma and colon carcinoma cells express 3 to 7 fold more phosphatidylserine than their non-cancerous counter parts (Utsugi et al., 1991). Previous studies have reported that analogues of magainin 2 were effective against hematopoietic, melanoma, sarcoma and ovarian teratoma lines (Baker et al., 1993). Given the preference of this lytic peptide for negatively charged phospholipids, MSI-99 shows potential as an anti-cancer agent.

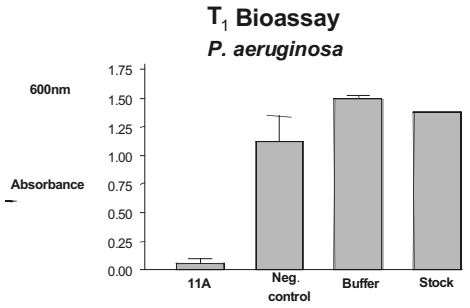

Fig.1 In vitro bioassay for T1 generation (11A) against *P.* aeruginosa. Bacterial cells from an overnight culture were diluted to A_{600} 0.1-0.3 and incubated for 2 hours at 25^0C with 100 µg of total protein extract. One ml of LB was added to each sample and incubated overnight at 26^0C. Absorbance at 600 nm was recorded. Data was analyzed using GraphPad Prism.

Production of edible vaccines

Oral delivery of properly folded and functional biopharmaceuticals is an attractive and cost-effective alternative to traditional methods. Due to this reason, plants have been employed to produce vaccines by engineering their nuclear genome but meager expression levels have made commercial production less attractive. The alternative of edible vaccines or oral delivery of pharmaceuticals is attractive because the cost of purification and *in vitro* processing generally account for 90% of the total production cost (Petrides et al. 1995). Encapsulated delivery of vaccines in plant cells offers protection from digestion and provides an ideal system for oral delivery (Walmsley and Arntzen, 2000). Chloroplast transformation has again come to the rescue. Chloroplast genome has been used to express the β subunit of enterotoxigenic cholera toxin (CTB) of *Vibrio cholerae* (Daniell et al. 2001b). In addition to high levels of accumulation, CTB synthesized within the chloroplast was assembled into functional oligomers and this was antigenically identical to purified native CTB. Evidence for its correct folding and disulfide bond formation came from the *in vitro* assay wherein CTB was found to bind with the intestinal membrane GM-1 ganglioside receptor. The capability to produce high levels of CTB, its assembly within the chloroplasts and its delivery in a bio-encapsulated form sets a precedent for the production of several other edible vaccines. Also, CTB fusion proteins can facilitate the oral delivery of other biopharmaceuticals and make the concept of oral delivery commercially feasible.

Expression of monoclonal antibodies via chloroplast transformation

The most commonplace disease in modern times is dental cavities and colonization of the teeth by *Streptococcus mutans* marks the onset of this condition. The bacterium ferments sucrose to produce lactic acid that erodes the tooth mineral resulting in cavity formation. To counteract this menace, a therapy using topical monoclonal antibody was developed, that prevents the adherence of *S. mutans* to dental surface. This treatment prevented re-colonization of the bacterium for up to two years (Ma et al. 1998). The practice of treating a disease with the help of antibodies is termed as passive immunotherapy. Combating a disease in a local area requires repetitive application of the antibody but it is not a viable practice owing to the high cost of current antibody production methods. As an extension of our efforts to produce low-cost biopharmaceuticals, and encouraged by the assembly of CTB oligomeric functional protein, we have recently expressed the humanized monoclonal antibody, chimeric IgA-G in transgenic chloroplasts (Daniell et al. unpublished). Analysis of transgenic plants has shown that the antibody is expressed and assembled properly within the transgenic chloroplast. This is the first demonstration of expression of a multi-subunit foreign protein joined together by disulfide bridges. Thus chloroplasts contain the machinery for disulfide bond formation as well as chaperonins to fold complex multi-subunit foreign proteins. The production of monoclonals in plants at an agricultural scale would obviously lower the costs and make local passive immunotherapy an inexpensive practice.

Epilogue

It is very fortuitous that in addition to several advantages of chloroplast transformation, the advent of an edible selectable marker and the ability to overexpress functional therapeutic proteins have come together now. The capability of this endosymbiotic organelle to fold and form disulfide bridges in the foreign proteins eliminates the need for post-production *in vitro* modifications, the cost of which amounts to approximately 60% of the total cost of protein production. Another advantage is the absence of contaminating agents, which are unavoidable in case of proteins derived from humans, animals or microbial cultures. These positive accomplishments have made a strong case for transgenic chloroplasts to be used for large scale and low-cost production of pharmaceutical proteins. Moreover, chloroplast transformation of food crops namely potato and tomato, make plants suitable source of edible vaccines. Chloroplast, the light-harnessing green organelle, is providing the guiding light to future research in plant biotechnology, which would finally deliver its promise of benefiting humankind.

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