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Chloroplast Genetic Engineering

a novel method to produce therapeutic proteins

By Shiv Gaglani

Every year Americans spend billions of dollars on prescription medication. Unfortunately, many are finding it increasingly more difficult to afford these expensive but necessary drugs. The rapidly escalating costs of pharmaceuticals and the prospective need for mass-produced vaccines make it necessary to produce these medicinal compounds more economically and in greater quantity. Recombinant DNA technology, the artificial manipulation and transfer of DNA between species, has begun to tackle both problems by introducing many medicine-producing genes into mammalian and bacterial cells. Genetically engineered mammalian cells are superior to their bacterial counterparts because, unlike bacteria, they contain molecular machinery that can produce proteins that are identical to those formed in the body. Bacterial vectors such as E. coli cannot correctly modify these proteins, so they are often incorrectly folded (1). Therefore, bacterial products require expensive post-processing procedures to form usable proteins; in fact, this accounts for 60% of the cost for the commercial production of insulin using E. coli (2). However, mammalian cells have their own disadvantages: unlike bacteria, they are very expensive to culture, require high maintenance, and are capable of only low levels of protein production. A better alternative to these two systems of production is plant genetic engineering.

Advantages of Plant Genetic Engineering

The production of transgenic plants has been explored since the 1960s with the aim of creating crops with resistance to herbicides, pests, and disease. In fact, in 1994, the first transgenic plant, the "FlavrSavr" tomato (which was modified to have a longer shelf life), was approved for sale (3). Recently, scientists have begun transforming "pharmacrops" to generate pharmaceuticals because this approach has several unique advantages. First, plants do not require industrial bioreactors, vats wherein recombinant proteins are produced, because they can create and store proteins in their cells. Furthermore, the technologies already exist to mass harvest and process these plants (1). Nicotania tabacum (tobacco) is often used for genetic engineering due to its easy genetic manipulation and its considerable growth-rate—it can produce 40 tons of fresh leaf weight per acre and up to one million seeds per plant (4).

A third improvement could make it unnecessary to isolate the desired pharmaceutical. Since the therapeutic compound is produced and stored in plant tissue, it might be possible to receive the benefits of the medicine simply by eating the plant (1). However, stomach acid poses an obstacle to this pathway of delivery as it results in protein degradation. Cellulose, a protein found in plant tissue, could guard against this potentiality because it cannot be digested.

Before the medicinal proteins can enter the blood stream, they need to cross the intestinal membrane. To accomplish this, the proteins can be fused to the protein cholera toxin B-subunit (CTB) derived from pathogenic Vibrio cholerae (5). CTB itself is innocuous and only serves to transfer the cholera toxin into the circulation by binding to a receptor on the intestinal membrane, and it has been experimentally demonstrated that CTB can be used to deliver therapeutic proteins through the intestinal membrane effectively (6).

One study successfully used CTB to deliver insulin orally to diabetic mice (7).

Nuclear Modification

Plants have two main reservoirs of DNA: nuclei and chloroplasts. Therefore, both of these are potential targets for genetic engineering. To modify a plant's genetic information, scientists use the soil microbe Agrobacterium tumefaciens to deliver a therapeutic gene into the nucleus of the plant. This bacterium infects wounded plants and inserts a large plasmid (a circular segment of DNA) into the plant's cells to promote tumor formation so that the bacteria can feed off of the growing plant. Scientists can modify the plasmid so it expresses the gene of interest and not the harmful genes, and use this natural infection process to induce the plant cell to produce the desired protein (8).

Transforming the nuclear DNA of these plants, however, has provoked a great deal of controversy due to its potentially harmful ecological effects. If recombinant genes (a.k.a. transgenes) were to be disseminated through pollen and integrated into other plants, invasive species and widespread ecological damage could result. For example, an herbicide-resistance gene in a genetically modified (GM) crop that transferred to a weed could lead to its ceaseless proliferation.

Nuclear transformation can also be harmful to the individual plant itself because the transgenes are integrated into the plant's nucleus at random locations. This can deactivate other important genes and cause deleterious changes in the host organism. Also, nuclear modification of plants is not very efficient because there is only one nucleus per cell and, at most, a few copies of the recombinant gene, producing relatively low levels of protein.

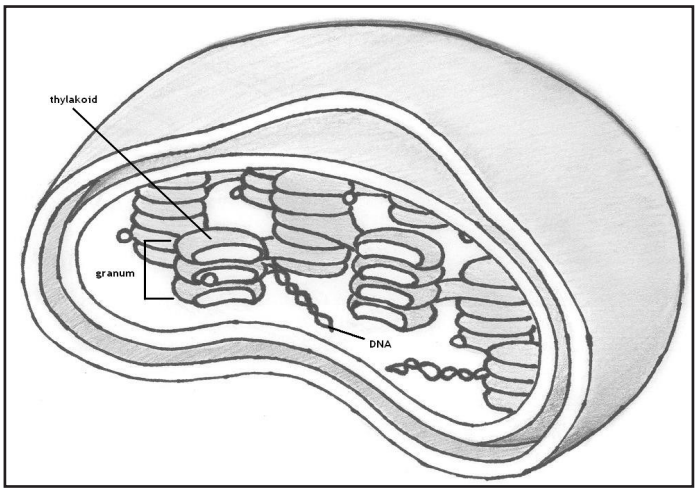

Figure 1. The inner details of a chloroplast. Notice that multiple chloroplasts are contained within the plant cell. Each chloroplast (bottom) is composed of thylakoid stacks involved in photosynthesis and also contains

Nuclear Chloroplast Genetic Engineering

A novel system that appears to circumvent the concerns about nuclear modification is genetic engineering of chloroplast DNA. In chloroplast genetic engineering, the recombinant DNA plasmid is bound to small gold nanoparticles that are then injected into the chloroplasts of a leaf using a "gene gun." This device uses high pressure to insert the plasmid-coated particles into the cell. These plasmids contain multiple important genes: the therapeutic gene, a gene for antibiotic resistance, a gene that increases expression of the therapeutic gene, and two flanking sequences that ensure that the plasmid is not randomly integrated into the chloroplast genome (9; 10). In brief, the flanking sequences guide the human recombinant DNA into a specific place on the chloroplast genome by binding to corresponding parts on the genome. The leaf is then grown on a plate containing an antibiotic, which ensures that the only surviving plant cells will be those that contain the gene for antibiotic resistance and—therefore—contain the therapeutic gene as well. These cells are then exposed to regenerative factors that induce them to start sprouting shoots and grow into full plants that express the desired protein. (Figure 2 provides a schematic detailing the procedure of

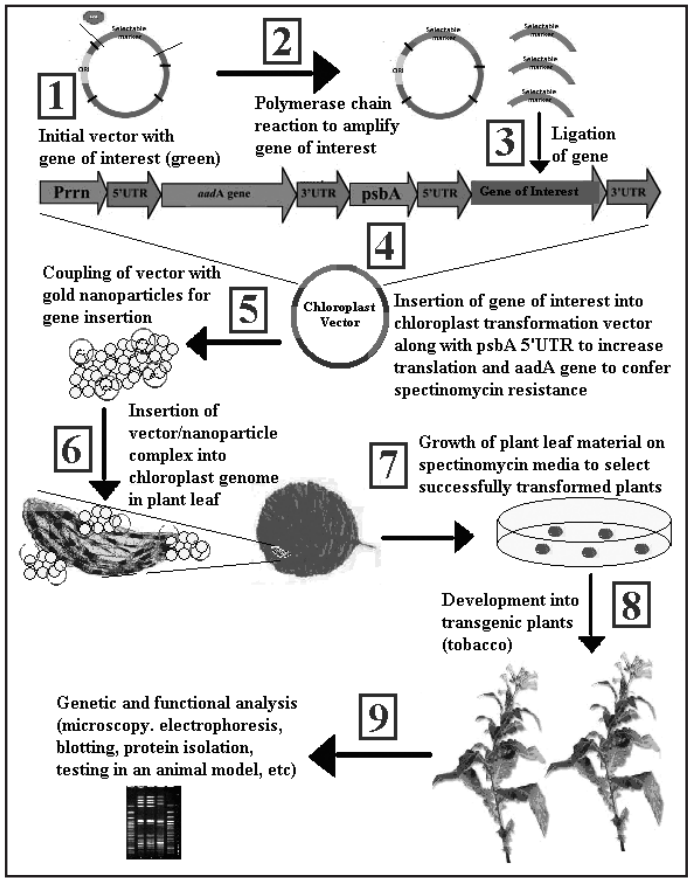

Figure 1. Procedural schematic of chloroplast genetic engineering. Depicts the general procedure used to insert transgenes into chloroplasts of tobacco plants. Designed by Shiv Gaglani.

credit: Grzegorz Babiarz, HSR

chloroplast genetic engineering).

Unlike nuclear transformation, this method ensures that the recombinant transgenes are contained within the chloroplast and therefore will not spread to other plants. Chloroplasts (and the genes they contain) are not passed in the sperm (i.e., pollen) of a plant, so they cannot be spread by pollination. Researchers demonstrated that, even though chloroplasts in leaves were modified to express an insecticidal protein, called CRY, at very high levels (47% total soluble protein), the pollen did not contain any traces of the protein (11). This signifies that the recombinant genes and proteins are contained within the chloroplast so this technique is environmentally friendly.

Chloroplast engineering also allows for large-scale protein production. Scientists reported that the levels of pharmaceutical proteins produced in nuclear-modified plants are less than 1% of the levels needed for the purified protein to be commercially feasible (12). Each plant cell contains approximately 100 chloroplasts and each chloroplast contains about 100 copies of its genome. So, chloroplast genetically-engineered plants have high levels of integration of transgenes—up to 10,000 copies per cell—which elevate expression levels of recombinant proteins (up to 47% of the plant's total soluble protein; 13).

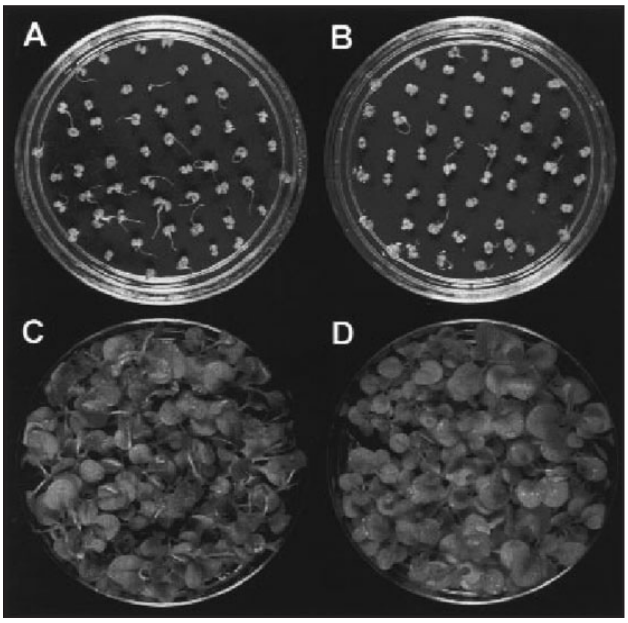

Figure 4. Growth selection of transgenic shoots. The shoots of the plants are grown on antibiotic-containing media. Only the successfully transformed shoots with the antibiotic-resistance gene, along with the therapeutic gene, will grow. (A) control, untransformed shoots, (B) non-resistant shoots, (C and D) transgenic shoots (1).

Figure 3. Phenotype of tobacco plants. An untransformed, control tobacco plant (left) as compared to a genetically modified, transgenic tobacco plant. It is important to note that the whole plants (top), buds (middle), and flowers (bottom) are not phenotypically different (1).

In addition, potentially harmful effects are more likely to be observed in nuclear-transformed plants because of random gene integration. One study showed that even low levels of CTB expression in nuclear-transformed plants significantly inhibited their growth (14). By contrast, chloroplast engineered plants that expressed CTB levels 410-3300-fold higher grew as well as the untransformed control group (5). This is due to the foreign therapeutic proteins being enclosed within the chloroplast organelle and not interfering with other processes within the cell.

Therapeutic Proteins

A number of therapeutic proteins have been produced using the chloroplast genetic engineering system. These include human somatotropin (growth hormone), serum albumin (blood protein), insulin-like growth factor (hormone), antimicrobial peptides (proteins that kill pathogens), interferon alpha/gamma (cytokines in the immune system which are effective against hepatitis and leukemia), monoclonal antibodies (immune system molecules that fight off invading pathogens and toxins), and vaccines to cholera, plague, canine parvovirus (dog virus) and anthrax (15). Each of these proteins is clinically relevant and has not been produced efficiently in nuclear modified plants. Somatotropin, also known as human growth hormone, is used as therapy for stunted growth and even to help maintain muscle mass in patients. Serum albumin is the most widely used intravenous protein that is administered to replace blood volume since it accounts for 60% of blood protein composition (10). Insulin is a crucial hormone that regulates carbohydrate metabolism and therefore energy production. It was the first marketed therapeutic protein produced through genetic engineering (the pharmaceutical company Eli Lilly sold it as Humulin beginning in 1982). Most of the therapeutic proteins produced by chloroplast genetic engineering are still in the developmental stage and need

to be tested in humans. Chlorogen Inc., is a company that is working to commercialize this technology and bring the plant-produced therapeutic proteins to the market. According to Chlorogen's site, their first product will be human serum albumin for the non-therapeutic market (16).

Vaccines are, of course, needed to immunize people from harmful pathogens, such as the polio virus, but many times there is a shortage of the amount of vaccine available. Plague vaccine, which immunizes against Yersinia pestis, has been expressed in transgenic tobacco plants at commercially feasible levels. In addition, the canine parvorvirus vaccine (CPV), which protects dogs against CPV and stomach complications, has been expressed highly. Recently, a team of scientists working on chloroplast genetic engineering reported achieving such high levels of expression of the anthrax protective antigen that, according to their extrapolation, one acre of transgenic tobacco could produce about 400 million doses of the vaccine (4). This is a crucial property of vaccine production due to modern concerns over viral epidemics such as avian flu.

Conclusion

Chloroplast genetic engineering is an exciting technology that has the potential to produce biopharmaceuticals more efficiently and provide them to those who need them most. A potential application of this technology is the production of therapeutic proteins or vaccines in plants indigenous to third-world countries where people do not have access to these medicinal compounds. Unlike other techniques, such as bacterial expression and nuclear genetic engineering or plants, chloroplast modification has succeeded in producing therapeutic proteins and vaccines at commercially feasible levels. In addition, genetically engineering the chloroplast is environmentally friendly since the transgenes are contained within the plant and the proteins they code for do not harm the plant itself (17). However, more work is required before chloroplast genetic engineering can be applied commercially. This work will probably include modifying more types of crops and plants as well as ensuring the functionality of the resultant therapeutic proteins in humans. But it may not be too far in the future when mothers may nag their children not only to eat their broccoli, but to eat their transgenes. H

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