Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions and products

Lawrence Bogorad

Plant genetic engineering via the nucleus is a mature technology that has been used very productively for research and commercial biotechnology. By contrast, the ability to introduce foreign genes at specific locations on a chloroplast’s chromosome has been acquired relatively recently. Certain limitations of nuclear genome transformation methods might be overcome by the site-specific introduction of genes into plastid chromosomes. In addition, plastids, mitochondria and other subcellular organelles might provide more favorable environments than the nuclear–cytoplasmic compartment for certain biochemical reactions and for accumulating large amounts of some gene and enzyme products.

Nuclear transformation

There are several steps involved in A. tumefaciens-mediated insertions of DNA into a plant: (1) a foreign gene is inserted between the borders of the T-DNA cloned in a small plasmid; (2) this construct is introduced into an A. tumefaciens strain that lacks T-DNA and the engineered T-DNA inserts into the Ti plasmid; (3) the A. tumefaciens bacteria carrying the foreign DNA on the now-disarmed Ti plasmid are put into contact with plant cells; (4) the T-DNA is transferred into a plant cell; and (5) the new gene, as a component of the T-DNA, is incorporated into a chromosome in the nucleus of the plant cell.

The next step after producing a transformed cell is to obtain a fertile transgenic plant. Research into plant tissue culture started at the beginning of the 20th century. One aspect of this work culminated in the demonstration that single carrot cells, cultured on an appropriate medium, can divide, give rise to embryos and grow into entire plants. The culture media used in the initial experiments included complex and poorly characterized components but later, using information acquired in studies of plant hormone action, entire plants could be regenerated by transferring cells in culture through a series of culture media with different hormone contents. Even mature cells from, for example, tobacco leaves could be stimulated to multiply to form clumps of cells from which fertile plants formed.

The results of these two lines of research – investigations of the Ti plasmid and the regeneration of whole plants from cells in culture – were used together in the early 1980s to produce fertile transgenic plants. The most significant discovery for the genetic engineering of plants was that if a gene was introduced via T-DNA into a cell that was then used to generate a fertile plant, that gene would propagate to tobacco progeny through the sexual cycle and seed. The commercial potential for crops was, of course, apparent immediately. At that time, the major limitation to producing a genetically transformed crop plant appeared to be the ability to regenerate that plant from cells or clumps of tissue in culture. Only a few species could be manipulated in this way then; now many can be.

Crop plants

A. tumefaciens-mediated nuclear transformation technology has been used widely to create transgenic dicotyledonous plants, but monocotyledonous plants proved difficult to transform by this method. The results of early experiments led to the view that most monocotyledonous plants – including cereals – were beyond the host range of A. tumefaciens. However, experiments in the late 1980s showed that maize–straw–virus DNA can be delivered to maize via the A. tumefaciens T-DNA system and can be expressed in the plant. It was only in 1993 and 1994, after the processes involved in T-DNA transfer were better understood, that transgenic rice was produced via A. tumefaciens. The efficient transformation of maize via A. tumefaciens was described in 1996 (Ref. 5) and of wheat in 1997 (Ref. 6). Meanwhile, however, and largely driven by the prospect of crop improvement, commercialization and profits, other techniques were developed for introducing foreign
DNA into cells. Among these techniques, microprojectile bombardment has been broadly effective and has been particularly useful for producing transgenic maise. The rapid increase, over the past few years, in the areas planted with Ti-plasmid-transformed cotton and microprojectile-transformed maize attests to the success of the methods for transforming plant nuclei. However, the current techniques have important limitations. One significant problem, which is at best an inconvenience and at worst a blocking impediment, is that the expression level of an introduced gene can vary greatly from one transformed plant to another. Consequently, many transformants with the same gene usually need to be produced and tested to identify a strongly expressing strain, that is to be introduced into a plant. After selecting transformants that express the first gene well, the best strain must be examined to see if the new gene can be expressed strongly and others that produce cell-wall components, chlorophyll, amino acids, and so on. Indeed, most of the characters that are produced and tested to identify a strongly expressing strain, that is to be introduced into a plant in a single T-DNA fragment may be expressed to different relative extents in one transformant than in another. Thus, contrary to what one might have thought, cloning several genes into a single T-DNA does not generally avoid the compounded, variable expression problem.

An interesting single-gene approach for simultaneously introducing several new proteins into a cell via nuclear gene transformation was taken by Dasgupta et al. They constructed and introduced a large single gene encoding a polyprotein containing three enzymes into a plant. The enzyme proteins were separated from one another by the amino acid sequence recognized by the tobacco-vein-mottling-virus N1a proteinase, which was one of the three enzymes in the polyprotein. The three enzymes were detected as individual active peptides in the transformants, showing that the N1a proteinase cut the individual enzymes out of the polyprotein and that all three enzymes were functional.

Table 1. Comparison of the nuclear and plastid genomes of angiosperms

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<tr>
<th></th>
<th>Nuclear genome</th>
<th>Plastid genome</th>
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<tbody>
<tr>
<td>Chromosomes</td>
<td>Two copies of each of many chromosomes; the number of chromosomes per diploid cell is species-specific</td>
<td>~60 copies of a single circular chromosome per cell</td>
</tr>
<tr>
<td>Genes per chromosome</td>
<td>Could be thousands</td>
<td>~120-150</td>
</tr>
<tr>
<td>Arrangement and transcription of genes</td>
<td>Each gene is separate and is transcribed individually</td>
<td>Many genes are in operons and are transcribed together</td>
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In nature, however, the problem is approached in various other ways. Operons (groups of genes with a single promoter), which often encode enzymes of the same biosynthetic chain, are common in bacteria. The single transcript of an entire multicistronic operon carries information required for the production of several proteins. Nuclear genes are transcribed singly; they are not arranged in operons. However, chloroplast genes are often present in operons. Some pertinent features of nuclear and plastid genomes are compared in Table 1.

Chloroplasts for genetic engineering

Plant cells have three gene-containing compartments: the nuclear–cytoplasmic system, mitochondria and plastids. There are several types of plastid including: (1) chlorophyll-containing chloroplasts, (2) yellow, orange, or red carotenoid-containing chromatophores, (3) starch-storing amyloplasts; (4) oil-containing chlorenchyma plastids.
The photosynthetic apparatus of the chloroplast captures light energy and converts it into biologically useful energy that is, for example, available for: (1) manufacturing sugars and amino acids; (2) reducing nitrate to ammonium; (3) making complex organic compounds; and (5) assembling plastid proteins that are required for the chloroplasts themselves to function. (Non-green plastids use energy produced elsewhere in the cell for metabolism.) It is easy to imagine modifying or adding to existing biosynthetic chains, which are already linked to the energy-converting machinery of the chloroplast, to produce and accumulate desirable new products. The plastid genomes of flowering plants encode perhaps 150 genes; the remainder of the hundreds or thousands of plastid proteins are the products of nuclear genes. The compositions of chloroplasts and other plastids can be modified by inserting genes into the nuclear genome; a chimeric gene can be constructed with an N-terminal sequence that targets its protein product to the chloroplast.

**Why plastosids?**

However, why do we need to send a protein into the plastid? Some syntheses other than photosynthesis are only carried out in plastids, probably because one or another feature of the organelle’s environment does not exist elsewhere in the cell. Furthermore, the plastoplasm and chloroplast contain different proteases, and a protein might survive better in one compartment than in the other. A specific type of plastid might be a good place to accumulate certain proteins or their biosynthetic products that would be harmful if they were present in large amounts in the cytoplasm or in a plastid of a different type. The various forms of plastid (amyloplasts, chromoplasts, etc.) have desirable properties as places to conduct reactions and to accumulate proteins or products of enzymes, and they can be exploited using known nuclear gene transformation methods. Nevertheless, the limitations of plant nuclear transformation technology discussed above remain. Fortunately, there is an alternative: new genes can be introduced directly into the plastid genome. This has several advantages, although some technical problems have still to be solved to simplify the procedure.

**Methods for transforming plastids**

In 1988, it was reported that a deletion in the chloroplast genome of the green alga *Chlamydomonas reinhardtii* incapable of carrying out photosynthesis could be corrected by introducing the wild-type gene. The DNA was delivered to target cells as microprojectiles ~1 μm in size and propelled into cells spread on an agar plate using a gunpowder charge. Later, compressed gases were used for biolistic transformations. The DNA became incorporated into the *Chlamydomonas* chloroplast chromosome by homologous recombination. Thus, unlike nuclear transformation, DNA can be directed to a specific site within the plastid chromosome. Blowers et al. showed that foreign DNA bordered by chloroplast DNA sequences is incorporated and stably maintained in the *Chlamydomonas* chloroplast chromosome.

Daniell et al. and Svab et al. took advantage of this knowledge and introduced DNA into tobacco chloroplasts in cultured cells in situ or in leaf tissue. Transient expression was observed and, in addition, transformed cells were regenerated into plants with modified chloroplasts. The usefulness of plastid transformation for biotechnology was shown strikingly by McBride et al., who introduced the gene encoding the B. thuringiensis lepidopteran protoxin into the chloroplast chromosomes of tobacco plants using the microprojectile method. In the resulting plants, this protoxin constituted 2–3% of the soluble leaf protein. This very high level of transgene expression was attributed to the presence of ~50 chloroplasts per leaf cell and 60–100 chloroplasts per plastid. Each leaf cell contains ~500 copies of each gene, present once in each plastid chromosome. However, the large number of copies of the gene in the cell is unlikely to be the entire explanation: some chloroplast genes encode proteins that are amongst the most abundant in the cell, but others are much less highly expressed. Also, the mRNAs for some nuclear genes that are present in ~10 copies per cell are as abundant as the most abundant plastid-encoded mRNAs.

Two additional methods for delivering DNA into plastids have recently been developed: a polyethylene glycol-mediated method and direct injection of DNA into cells. In the resulting plants, this protoxin constituted 2–3% of the soluble leaf protein. This very high level of transgene expression was attributed to the presence of ~50 chloroplasts per leaf cell and 60–100 chloroplasts per plastid. Each leaf cell contains ~500 copies of each gene, present once in each plastid chromosome. However, the large number of copies of the gene in the cell is unlikely to be the entire explanation: some chloroplast genes encode proteins that are amongst the most abundant in the cell, but others are much less highly expressed. Also, the mRNAs for some nuclear genes that are present in ~10 copies per cell are as abundant as the most abundant plastid-encoded mRNAs.

**Operons**

Many chloroplast genes are grouped in operons. Multicistronic mRNAs transcribed from these operons can be processed to produce mRNA for single protein. Introducing blocks of foreign genes in a single operon would avoid the complications inherent in putting one gene at a time into random locations in the nuclear genome. Introducing genes in a single block would be the easiest but, because the position on the plastid chromosome is not known, it would be advantageous to be able to control the rate of transcription of a gene, there might be some disadvantage...
to having transgenes with the same kind of promoter at separate locations on the chromosome. Because insertion is at a specified site on the chromosome, if particular sites are found to affect the transcription of an inserted gene (e.g. through effects on DNA conformation that affect transcription[25]), the site of insertion of transgenes could be selected in order to take advantage of these effects. This is better than being at the mercy of undirected insertion and position effects. (Of course, uniform transcription does not assure uniform stability or equal translation of transcripts. But these are separate problems and are faced in whichever cellular compartment the gene is transcribed.)

The plastid genome of some plant species, as in the case for some nuclear genes, is regulated by environmental factors (e.g. light) and other genes are expressed only in specific types of plant and cell. Such specificity can be taken advantage of in genetic engineering. There is a lot of information available about plastid-gene DNA-dependent RNA polymerases, promoters and enhancers, and all of this knowledge can be exploited for genetically engineering the plant's relatively simple plastid genome.

Inheritance

The chloroplasts of angiosperms are generally transmitted only by the maternal parent. This means that chloroplast genes are not present in pollen. Consequently, a trait introduced by genetically engineering the chloroplast genome would not be unintentionally transferred to sexually compatible relatives of the crop that might be growing nearby. Furthermore, the plastid-chromosome-encoded protein would not be produced in the pollen and thus would not affect insects that feed on pollen or pollen-coated plant tissues.

Pros, cons and uncertainties

Plastids are important alternatives to the cytoplasm as sites in which to engineer enzymatic reactions or to make, process or store proteins or enzyme products. The internal environments of some differentiated forms of plastids (e.g. chloroplasts, chromoplasts and amyloplasts) might be more favorable than those of the cytoplasm for certain enzymatic reactions, for modifying or processing a particular protein and so on. A protein might be more stable in the plastid than in the cytoplasm because of differences in proteases, ions and other constituents. It might also be advantageous to place energy-intensive biochemical reactions close to the site of photosynthesis. In addition, if the protein that accumulates or if the product of an enzymatic reaction is deleterious to the cell if present in the cytoplasm, it might not be harmful in some form of plastid. However, the plastid could be the wrong place for a process that might not be harmful in some form of plastid. How can we ensure that the plants do not lose all the DNA sequences, all in a single transformation. Third, foreign genes will not disperse to related species growing within pollination range of the crop because chloroplast genes are not present in most angiosperm pollens. In addition, locating genes in plastids might offer different possible expression patterns in the plant than nuclear localization (e.g. in all cells containing proplasts or chloroplasts or amyloplasts).

The present range for plastid transformation

Chloroplast transformation has been very successful for Chlamydomonas reinhardtii and tobacco. This has allowed important experiments to be done on the molecular biology of one plant species, as is the case for some nuclear genes, is regulated by environmental factors (e.g. light) and other genes are expressed only in specific types of plant and cell. Such specificity can be taken advantage of in genetic engineering. There is a lot of information available about plastid-gene DNA-dependent RNA polymerases, promoters and enhancers, and all of this knowledge can be exploited for genetically engineering the plant's relatively simple plastid genome.

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Chlamydomonas, by contrast, is a single-celled flagellate, each of whose cells contains a single chloroplast with ~60 chromosomes, and homoplasmic chloroplast transformants can generally be isolated after continued culture under selection. A wild-type plastid chromosome is capable of fusion in a transplastomic crop plant could persist under the ‘protection’ of the product of the introduced gene present in other chromosomes in the same or other plastids and take over in the absence of selection for the transgene.

The current major approach to obtaining homoplasmic plants is repeated selection from transformed tissues in culture, when possible, followed by regenerating plants. Unfortunately, repeated cell culturing puts a further restraint on the kinds of plant material that can be used for regeneration: A different strategy might be to make the transgene (or the transgene product fused to another protein coding sequence) essential for cell survival, thus providing constant selection and rendering the presence of wild-type chromosomes irrelevant. Another course might be temporarily (i.e. reversibly) reducing the number of chloroplasts per cell and the number of chromosomes per plastid. Antisense suppression of one of the genes controlling plastid division in *A. thaliana* results in the number of chloroplasts per leaf cell dropping from 100 to one (34), and Chlamydomonas chloroplast, but not nuclear, DNA is temporarily reduced to one-seventh its normal level (approximately 10 rather than 60 chromosomes per plastid) after 6–7 doublings in the presence of thymidine starvation-induced mutagenesis in bacteria, *T*4 bacteriophage and, probably, Chlamydomonas chloroplast DNA (32), which means that this might be a clue to how to reduce the chloroplast DNA rather than a literal solution.

It remains to be seen whether one or both of these sorts of approaches can be used to address the vexing problem of wild-type chromosomes lurking in the background but ready to ‘take over’. Concerted efforts are likely to overcome the present limitations but technology that can easily solve or circumvent the problem has not yet been developed. It was recognized many years ago that chloroplasts in the giant alga *Acetabularia* might not be entirely independent of one another and, indeed, might be a single fusing and dividing mass (35). The recent observation of connections between chloroplasts in cells of higher plants (36) is a striking reminder of how little we know about plastid biology. As we learn about the subject, we are likely to acquire new outlooks on how to approach the heteroplasmic problem directly and effectively. Some aspects of nuclear and plastid genome transformation are compared in Table 2.

### New and renewed possibilities

The development of multiple sites for genetically engineering plant cells raises new prospects for genetic engineering. It also prompts renewed consideration of two examples of major modifications of plant metabolism involving plastids that could be very important for agriculture: (1) enabling plants to fix N2, and (2) improving photosynthetic CO2 fixation. Although both possibilities might, in reality, still be a long way off, there are reasons to be hopeful. First, we continue to learn more about how to take advantage of multiple sites in the cell for introducing foreign genes and about how to control plant gene expression, and (particularly through proteomics) we should come to know more about the kinds of reactions favored in one cell compartment over others. Second, at the same time, we are becoming increasingly better-informed about the biochemistry and physiology of N2 fixation and natural adaptations for increasing CO2 fixation.

#### Nitrogen fixation

It was first proposed approximately 20 years ago that plants might be suitable and appropriate sites for engineering into plants the capacity for nitrogen fixation (36). Nitrogen fixation requires large amounts of energy in the form of ATP and chloroplasts make this as a product of photosynthetic energy transduction. The sensitivity of the enzyme nitrogenase to O2, a major product of photosynthesis, would appear to preclude N2 fixation by chloroplasts but cyanobacteria also conduct oxygenic photosynthesis despite fixing N2 very efficiently. Some cyanobacteria separate the two processes

<table>
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<th>Table 2. Introducing genes into nuclear and plastid genomes</th>
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<tr>
<td><strong>Nuclear genome</strong></td>
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<tr>
<td>Insertion of foreign DNA into genome</td>
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<td>Transcription of introduced genes</td>
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<td>Current limitations</td>
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**REVIEWS**

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TIBTECH JUNE 2000 (Vol. 18)
CO₂ fixation in C₃ plants

In C₃ plants (including most crop species), CO₂ is fixed to the five-carbon compound ribulose-1,5-bisphosphate. Two molecules of 3-phosphoglycerate (a C₃ compound) are produced from the product of this fixation. The phosphoglycerate is reduced to 3-phosphoglyceraldehyde using the ATP and reduced nicotinamide adenine dinucleotide phosphate (NADPH) that are produced photosynthetically. All chloroplasts in all the cells of a C₃ leaf contain ribulose bisphosphate carboxylase–oxygenase (Rubisco), the enzyme that catalyses this fixation of CO₂. However, as well as fixing CO₂ to ribulose-1,5-bisphosphate to produce two molecules of phosphoglycerate, Rubisco can catalyse the oxygenation of the same five-carbon compound using O₂ to produce one molecule of phosphoglycolate; O₂ and CO₂ compete for the active site on Rubisco. As the rate of photosynthesis increases (e.g. as the light intensity increases), so does O₂ production, resulting in more competition with CO₂ for Rubisco. As a result, C₃ photosynthesis saturates at about one third of the maximum light intensity under which many crop plants grow.

CO₂ fixation in C₄ plants

C₄ plants can take advantage of higher light intensities. In C₄ plants, such as maize and sorghum, there are two types of photosynthetic leaf cell: bundle-sheath cells, which surround the vascular bundles, and mesophyll cells, which occupy the space between the cylinders of bundle-sheath cells and the upper and lower epidermis of leaves. Bundle-sheath cells contain Rubisco; mesophyll cells do not. Carbon dioxide is fixed first in mesophyll cells by a reaction catalysed by a carbonic anhydrase to form oxaloacetate (a C₄ compound). Then, in maize, for example, oxaloacetate produced in mesophyll cells is reduced to malate, which is transferred to the adjacent bundle-sheath cells. In the bundle-sheath cells, CO₂ is released and reduced by Rubisco in a low-O₂ environment, because bundle-sheath chloroplasts, like cyanobacterial heterocysts, have little or no oxygen-evolving capacity.

Improving CO₂ fixation

Could C₃ plants be converted into C₄ plants? C₄ plants are better adapted to more arid conditions and to using higher light intensities than C₃ plants. New information about the molecular basis for suppressing Rubisco production in the mesophyll cells of maize has allowed plants to be genetically engineered that have reduced Rubisco production in these cells. It has also been shown that Rubisco in C₃ plants can be replaced by a Rubisco homologous enzyme, Rubisco-like oxygenase (RLO), which has a lower affinity for O₂ and is therefore more active when photosynthesis is low. This suggests that it might be possible to convert C₃ plants into C₄ plants by genetically engineering them to express an RLO enzyme in their mesophyll cells.

Future prospects

Relatively few genes have been introduced into plants for commercial purposes. The importance of the availability of separate, metabolically unique compartments in cells will become increasingly evident as more diverse metabolic products and enzymatic reactions are introduced into plants. The possibility that a non-cytosolic environment, such as the mitochondrion or a particular form of plastid, will favor a particular enzymatic reaction and/or the accumulation of a protein or metabolic product is being investigated in more detail. For example, oxaloacetate produced in mesophyll cells is released and refixed by Rubisco in a low-O₂ environment, because bundle-sheath chloroplasts, like cyanobacterial heterocysts, have little or no oxygen-evolving capacity.

Important knowledge has been gained about the genes and enzymes of nitrogen fixation since the first suggestions were made that bundle-sheath plastids and cyanobacterial heterocysts were excellent models for genetically engineering bundle-sheath plastids to be good sites for engineering N₂ fixation; this has been the subject of very thoughtful discussion by Dixon et al.

Box 1. Additional considerations in targeting genes, biosynthetic paths and enzyme products

- The stability and accumulation of a particular RNA or protein may be greater or lesser in the nuclear-cytoplasmic compartment than in a plastid compartment or vice versa.
- Conditions for a particular enzymatic reaction may be more or less favorable in the nuclear-cytoplasmic compartment than in a plastid compartment or vice versa.
- More of the product of an enzymatic reaction or of a chain of reactions may accumulate and not affect the enzyme in the nuclear-cytoplasmic compartment than in a plastid compartment or vice versa.
- If gene containment is important, the maternal transmission of plastids may be advantageous.
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Acknowledgments

The work in the author's laboratory and the preparation of this paper were supported in part by research grants from the National Institute of General Medical Sciences of the National Institutes of Health of the USA.

References