

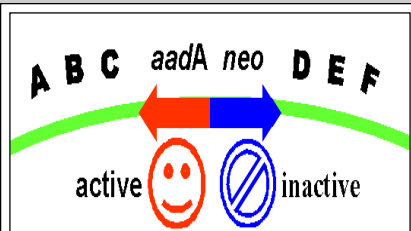
ISB NEWS REPORT

COVERING AGRICULTURAL AND ENVIRONMENTAL BIOTECHNOLOGY DEVELOPMENTS

JUNE 2003

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PLANT RESEARCH

INCREASING VITAMIN C CONTENT OF PLANTS THROUGH ENHANCED ASCORBATE RECYCLING

Daniel R. Gallie

Life likely appeared on earth when the atmosphere contained only low levels of oxygen. The increase in atmospheric oxygen most likely occurred as a byproduct of photosynthesis by cyanobacteria and other photosynthetic species. Although this increase in atmospheric oxygen is thought to have influenced the evolution of oxygen-requiring species, the evolution of land-based life required adaptation to this oxygen-rich atmosphere. Oxygen can be highly damaging to organisms given its reactivity, particularly in its many radical forms. For plants, given the presence of oxygen in the chloroplast in which considerable amounts of energy are absorbed and substantial electron transport activity takes place, the inadvertent production of active oxygen species (AOS) (e.g., O_2^- , H_2O_2 , hydroxyl radicals, and singlet oxygen) occurs as a consequence of normal photosynthetic activity. Exposure to many abiotic stresses can exacerbate the production of AOS, including cold, drought, salt, or high light. AOS can also invade a plant directly from the atmosphere, for instance when a plant is exposed to pollutants such as ozone. Production of AOS can damage the photosynthetic machinery or, under severe conditions, lead to death.

Production of active oxygen species is not always inadvertent. For example, oxygen photoreduction (known as the Mehler peroxidase reaction) results from the transfer of electrons from photosystem I (PSI) to oxygen to form superoxides and eventually hydrogen peroxide (H_2O_2). The Mehler reaction thus serves to maintain electron flow through PSI and sustain its function. AOS is also produced in other cellular compartments such as the mitochondria, which have substantial electron transport activity, and in peroxisomes during the oxidation of glycolate.

To limit the deleterious effects of many active oxygen species resulting from physiological processes or from exposure to environmental pollutants, plants (like other organisms) use antioxidants. Ascorbic acid (vitamin C) is the most abundant antioxidant in plants, present in millimolar concentrations that range from 10 to 300 mM^1 and is used to detoxify H_2O_2 , superoxide, hydroxyl radicals, and singlet oxygen. Glutathione, the other major soluble antioxidant, is typically present at only 10% of the concentration of ascorbate. The concentration of ascorbate often correlates with those environmental conditions that cause photo-oxidative stress. For instance, alpine plants that grow at high altitudes and thus are exposed to a combination of high light, cold temperatures, and elevated levels

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rirwin@vt.edu

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Information Systems for Biotechnology

207 Engel Hall
Virginia Tech
Blacksburg, VA 24061
Tel: 540-231-3747
Fax: 540-231-9070
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of UV-B contain high levels of ascorbate. The high concentration of ascorbate may be required to eliminate the active oxygen species that are produced in high amounts under these growth conditions.

Vitamin C is essential to animals to prevent diseases affecting the connective tissue such as scurvy. It also improves cardiovascular and immune cell function and is used to regenerate vitamin E. In contrast to most animals, humans cannot make vitamin C and therefore must obtain the vitamin regularly from dietary sources. Vitamin C is present at high levels in some fruits such as citrus and kiwi as well as in several green leafy vegetables but is present in low amounts, however, in those crops most important to humans such as grains.

Ascorbic acid (ASC) biosynthesis in plants differs from that in mammals and results from the oxidation of L-galactose to L-galactono-1,4-lactone, which in turn is oxidized to ascorbate by L-galactono-1,4-lactone dehydrogenase². Once used, ascorbate is oxidized to dehydroascorbate (DHA) and is rapidly regenerated by the enzyme dehydroascorbate reductase (DHAR), which requires glutathione as the reductant (Fig. 1). If ascorbate is not salvaged by DHAR, it is quickly lost. Glutathione reductase uses NADPH produced principally from photosynthesis to regenerate glutathione from oxidized glutathione. Consequently, the detoxification of active oxygen species by this ascorbate-glutathione pathway involves the transfer of electrons from PSI to NADPH to glutathione to ascorbate to H₂O₂ in a series of reactions in which there is no net loss of ascorbate or glutathione (Fig. 1). The principal function of DHAR activity, therefore, is to maintain the existing pool of ascorbate in a reduced state and enable an organism to recycle ascorbate.

Because DHAR regenerates ascorbate from DHA and thus rescues it before DHA is further catabolized, an increase in DHAR activity might be expected to improve a plant's ability to

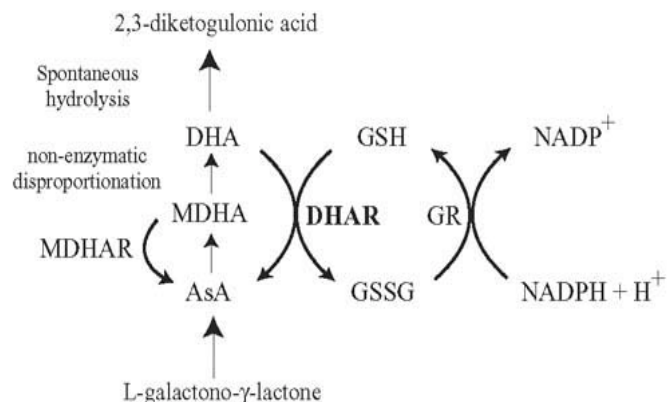


Figure 1.



recycle vitamin C and thus increase its cellular concentration of vitamin C. This would be true particularly if DHAR were limiting. In order to examine whether increasing the amount of DHAR in plants could increase their vitamin C content, we purified DHAR protein to near homogeneity from germinating wheat seedlings by assaying for the reduction of DHA to ascorbate in a glutathione-dependent assay. Antiserum was raised against the purified DHAR protein and was used to screen a wheat seedling cDNA expression library. We were successful in isolating a full-length DHAR cDNA encoding a protein that is 89.5% conserved with rice DHAR. We then introduced the wheat cDNA into corn where expression from the transgene increased the amount of total DHAR up to 100-fold³. This increase in DHAR elevated the amount of ascorbic acid in corn leaves and grain, showing that the vitamin C content of plants can be elevated by increasing expression of the enzyme responsible for recycling the vitamin. Similar results were achieved in tobacco leaves, which were used as a model for a leafy, non-grain crop, where leaves contained up to 4.6-fold more ascorbate relative to the control transgenic plants. Because ascorbate is the most abundant antioxidant in plants, it contributes significantly to the redox state of a cell. Consistent with its function, overexpression of DHAR resulted in a higher ratio of ascorbate (81%) relative to control plants (60%). Glutathione is used by DHAR to reduce DHA to ascorbate. Interestingly, the level of glutathione increased by up to 3.5-fold in leaves from DHAR-overexpressing plants, suggesting that increasing DHAR expression increases the level of ascorbate and glutathione and maintains a more reducing cellular environment.

In contrast to animals, land plants have adopted very different means by which they respond to environmental changes. This is partly a consequence of their more direct dependence on the physical environment for their survival, e.g., their light-driven mode of generating energy as well as their sessile nature. Because land plants cannot escape environmental stress, they must evolve means to minimize the damage resulting from exposure to a stress. Several abiotic stresses result in the generation of active oxygen species, including exposure to ozone, ultraviolet light, drought, salt, and cold. The ability to maintain ascorbate in a reduced state may likely be as important in mounting a rapid response to an acute demand on the antioxidant as is ascorbate synthesis. Because ascorbate is involved in reducing damage imposed by oxidative stress, increasing DHAR expression may well provide broad protection to several types of abiotic stresses, including drought and exposure to ozone. Determining to what extent DHAR expression and function is important in the response program to those stresses that generate active oxygen species

will be critical to understanding the role that oxidative damage plays in these stresses and how DHAR is used to mount an effective response to limit stress-related damage. Engineering greater resistance to oxidative stresses or to drought would be highly advantageous to crop species important to U.S. agriculture, such as cereals, and may significantly improve plant performance in environments degraded by ozone, soils contaminated with salt, or where instances of increases in temperature or drought can occur. With the continued spread of development into agriculturally important regions and the resulting degradation of air quality as well as the continued salt contamination of farmland soils that results from irrigation practices, manipulating DHAR expression and thus the level and redox state of ascorbate may be an important means to improve crop performance under adverse growth conditions. Thus, increasing the vitamin C content in plant species will not only prove to be directly beneficial in improving the human diet but may provide an indirect effect by improving crop productivity in response to those environmental stresses that involve oxidative abiotic stress.

We have therefore developed technology that increases the amount of vitamin C in plants, including grains, by increasing the amount of the enzyme that is responsible for recycling vitamin C. The ability to increase the level of this important vitamin in plant foods will enhance their nutritive value by increasing the number of foods from which the vitamin can be obtained as well as increasing the level of the vitamin in those foods that are already good sources of vitamin C. The current recommended dietary allowance (RDA) of vitamin C for adults is 75 and 90 mg for adult females and males, respectively, which is sufficient to prevent diseases arising from severe vitamin C deficiency such as scurvy. Although the RDA can be obtained through a balanced diet, more than 30% of Americans fail to consume 60 mg a day. In addition, some studies have suggested that up to 200 mg may be necessary to ensure good cardiovascular health and immune cell function, increasing the likelihood that more people would fail to achieve the RDA with their present dietary habits. Our ability to increase the vitamin C content in plant foods such as green leafy crops as well as grains should make it easier for people to obtain enough of the vitamin for their optimal health.

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Daniel R. Gallie
Department of Biochemistry, University of California
Riverside, CA
drgallie@citrus.ucr.edu



CHLOROPLAST EVOLUTION, GENETIC MANIPULATION AND BIOSAFETY

Jeremy N. Timmis

The higher plant chloroplast (cp) has become an attractive arena for genetic manipulation because diverse proteins, or sets of proteins, may be produced by growing transplastomic plants on a field scale¹. Chloroplast genes are often highly expressed to produce large amounts of protein, and they are partially contained through stringent maternal inheritance that operates in many of the most important crop plants. However, our recent experiments² indicate that chloroplast genes occasionally transfer to the nucleus and therefore may escape in pollen. This transfer process has led to both popular and scientific discussion regarding the evolution of eukaryotic cells and the impact of the gene transfer process on the level of biological containment that has been claimed by proponents of chloroplast biotechnology³. Both sorts of discussion contain a mixture of fact and fiction.

The extant genomes of cytoplasmic organelles are far too small to encode the full spectra of different proteins that are required for the functions of chloroplasts and mitochondria. Since the advent of early eukaryotes, organellar genomes have evolved, throughout hundreds of thousands of years of endosymbiosis, from those of ancestral free-living prokaryotes. During this long period many ancestral organellar genes have been transferred to the nucleus and been deleted from mitochondrial and chloroplast DNA.

The circular chloroplast genome of higher plants—the plastome—is approximately 150 kb in size and it encodes about 120 products. The remaining several thousand genes required to make functional chloroplasts are located in the nucleus. Although the plastome is small, each of several hundred chloroplasts within a mature leaf cell, in most species, contains tens of copies of an identical plastome. Therefore a chloroplast gene will usually be present thousands of times per cell with the result that chloroplast genes produce the most abundant proteins on the planet. The large number of chloroplast genomes per cell also

means that cpDNA, despite its genetic simplicity, may contribute a very large proportion (up to 40%) of total cellular leaf DNA. A further interesting feature of organelle genes is that they are uniparentally inherited, with offspring usually inheriting only the maternal chloroplast genotype. This contrasts with Mendelian inheritance that characterises nuclear genes. The chloroplast genotype must therefore be considered relatively simple and homogeneous but present, in most species, in thousands of copies per cell.

A wide variety of studies has shown that most if not all of the nuclear genes that now encode chloroplast proteins were transferred from an ancestral prokaryote during endosymbiosis and then deleted from the plastome. This sounds like a long shot because a prokaryotic gene would not readily be expressed even if it were transferred to a nuclear environment. Neither would it be able to give rise to a product equipped to enter the organelle and usurp the function of, and eventually oust, its progenitor chloroplast gene. However unlikely this appears, we know that such events have happened repeatedly during endosymbiotic evolution⁴. The relocation of both chloroplast and mitochondrial genes can be tracked in multiple independent events in separate branches of phylogenetic trees. Some of the means by which such itinerant genes establish functionality in the nucleus have been characterized and the process occurs in a variety of different ways.

The ongoing nature of chloroplast-to-nucleus gene relocation is supported by the presence of tracts of present-day cpDNA in the nuclear genome of many plants^{2,5}. It has been concluded that the first step in the relocation to the nucleus of ancestral chloroplast genes involves the transfer of DNA, and that this process still continues even though the gene content of extant higher plant plastomes is relatively stable and invariable. It appears that most of the chloroplast genes that are able to transfer beneficially to the nucleus have already done so, leaving behind a minority that are apparently better placed in the chloroplast. Experimentally it is possible to move some of this latter group to the nucleus, suggesting that there are selective advantages that require the maintenance of separate genetic compartments. One obvious advantage of becoming a nuclear gene is the availability, not accessible in the chloroplast, of meiotic recombination and sexual reproduction that have been the most powerful drivers of evolution on earth.

We set out to measure the frequency with which cpDNA transfers to the nucleus, assuming that it would have to occur at an experimentally approachable rate to facilitate the evolutionary relocations described above². We designed



a gene, *neo*, which confers resistance to kanamycin, so that it would be efficiently expressed only in the nucleus (Fig. 1). Using the chloroplast transformation vector pPRV111A, we placed this gene into the two inverted repeats of the tobacco plastome. This vector contains the chloroplast-specific selectable marker gene *aadA*, which allows spectinomycin selection to identify cpDNA transformants. Prolonged spectinomycin selection pressure also leads to homoplasmy for the integration, meaning that regenerated plants become homogeneous for the additional cp transgenes. We aimed to render the *neo* gene inoperative in the chloroplast by adding an intron that we expected could only be spliced within the nucleus from a precursor mRNA transcript. Thus this gene was disabled in the chloroplast but poised to confer kanamycin resistance if it moved to the nucleus (Fig. 1). It became clear that even this blocked gene could be inefficiently expressed from its location in the chloroplast, so we backcrossed two independent homoplasmic plants to wild type female plants. We then scored 250,000 seedlings of this cross for DNA jumping events that had occurred at some time during the life cycle of the transplastomic male parents. To our surprise, 18 kanamycin resistant seedlings were obtained in this screen, suggesting that one in 16,000 pollen grains of the transplastomic parent contained a piece of DNA that had jumped into the nucleus.

There were many questions that needed to be answered at this stage of the experiments. Was this DNA incorporated into the nuclear genome? Was kanamycin resistance in the 18 plants inherited as would be expected if it were encoded by a nuclear gene? Did all the 18 plants result from a single rare incident or were they all independent events? It turned out that each event was indeed independent and that, in most cases, the relocated gene behaved exactly as would be expected of a diploid nuclear chromosomal gene. In some instances we were also able to confirm at the sequence level that the transposed DNA from the transplastome was linked to tobacco nuclear DNA. Most of the integrants contained the *neo* and *aadA* genes and extensive flanking native chloroplast genes that were not present in the construct (genes A, B, E and F in Fig. 1). We took pains to emphasize² that in no case did we observe the expression of the *aadA* gene after it was transposed to the nucleus. We concluded that, under our experimental conditions, there was watertight evidence that DNA transposes from the plastome to the nucleus at a frequency that was higher than most biologists, including ourselves, had thought possible. Of course there was absolutely no scientific basis for any such expectations!

More questions immediately arose from these results. Was

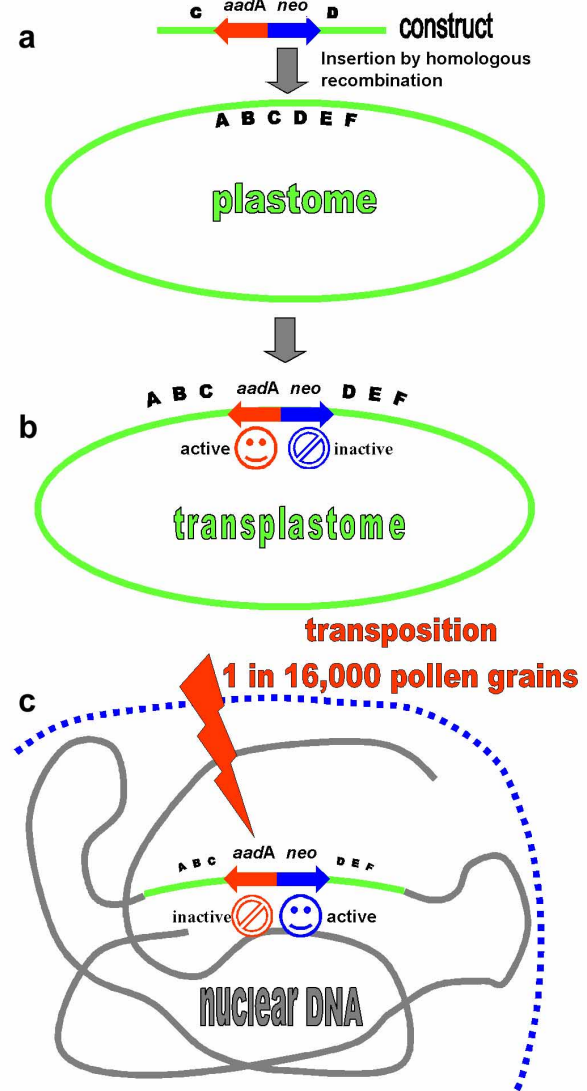


Figure 1. Movement of DNA from the plastome to the nucleus.

- a) The tobacco plastome was transformed with two selectable marker genes, *aadA* and *neo*, using a vector containing sequence similarity (regions C and D) to the plastome.
- b) The transgenes were incorporated into the plastome by biolistic transformation and homologous recombination within regions C and D of the vector and the corresponding regions of the native plastome. The *aadA* gene expressed (☺) to confer spectinomycin resistance that allowed selection for homoplasmy of the transplastome. The *neo* gene was inefficiently expressed (☹) in the transplastome.
- c) The *neo* gene was efficiently expressed (☺) only after transposition to the nucleus in 1 in 16,000 pollen grains, at which time the co-transformed *aadA* gene become inactivated (☹).

this frequency we measured artificially high because of the techniques of the experiment and the material used? Was the plastome destabilised by the added transgenes? Is tobacco atypical because it is an allotetraploid? When and how does transposition occur? We cannot answer many of these questions at this time, but the results, in the context of

our clearly defined and fully controlled experiments, are unequivocal.

What is the importance of these results for chloroplast biotechnology? Before addressing this question we need a little background to the chloroplast GM debate. We have known from strong evidence that has been reinforced time and again over the last 20 years that most plant nuclear genomes contain DNA that is clearly very recently derived from the plastome, necessarily implying that chloroplast transgenes are able to enter pollen nuclei and be dispersed. Plastid transgenes are not entirely contained for a second reason. Although the pollen of a transplastomic plant does not transmit the male plastome, weedy relatives (or non-transgenic crops) may act as male parents to produce hybrid seed. These hybrids would contain the transplastome and potentially could cause an environmental problem, though spread of the chloroplast transgenes would clearly be slower than their nuclear counterparts. Therefore there was never any doubt that chloroplast transgenes were not fully contained by uniparental inheritance, though they did enjoy a higher level of containment than nuclear genes. Our results demonstrate that, in tobacco, which is the major species targeted for chloroplast engineering, the level of potential escape is significant, particularly when large areas of cultivation are envisaged. It must be appreciated that we tracked the relocation of cpDNA by monitoring the expression of a tailor-made nuclear reporter gene. In none of the kanamycin-resistant plants screened using this selectable marker did we also observe spectinomycin resistance, despite the fact that *aadA* was concomitantly relocated in every instance. This is because the *aadA* gene is under the strict control of genetic elements that restrict expression to the prokaryote-like chloroplast compartment. It is possible that *aadA* could find a niche in nuclear DNA where it would be expressed, but we would expect this to be a very rare event, probably orders of magnitude less frequent than the transposition of DNA *per se*.

Therefore our results do not radically change what was previously known about chloroplast transgene containment. Genes designed for chloroplast expression are not likely to be functional when they move to the nucleus. This observation, together with the rarity of transfer to pollen and the phenomenon of uniparental inheritance, means that the level of biological containment offered by the chloroplast genetic compartment is high. However, scientists need continuously to evaluate the merits of transgenic technologies for crop improvement and biotechnology.

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Jeremy N. Timmis
The University of Adelaide
Australia
jeremy.timmis@adelaide.edu.au



JUMPING INTO THE NUCLEUS SILENCES PLASTID GENES

Anil Day

Minimizing environmental impact of transgenic crops
Foreign genes that enhance performance, such as herbicide and insect resistance genes, encode the beneficial traits responsible for the success of genetically modified (GM) crops. Whilst foreign trait genes add value to a GM crop, their escape into the environment through cross-pollination with wild relatives or other crops and persistence in hybrid offspring is undesirable. Transgenic crops might also contain excess foreign DNA, such as selectable marker genes, which are by-products of gene transfer protocols and serve no useful purpose in the final crop. Unless steps are taken to exclude excess foreign DNA from transgenic crops, these will accompany trait genes as they are released into the environment. The poor fitness of crop-weed hybrids compared to wild plants provides one effective barrier to reduce the persistence of foreign genes from GM crops in wild plant populations¹. The likelihood of trait gene escape can be reduced further by technologies that remove foreign trait genes from pollen or that reduce the fertility of GM crops. Within the UK, a subgroup of the Advisory Committee on Releases to the Environment (ACRE) have taken a critical, forward-looking approach to refine GM



crop design in order to minimize their environmental impact (<http://www.defra.gov.uk/environment/acre/bestprac/index.htm>). Moving towards best practice in GM crop design requires engineering plants to minimize the risk of gene flow to other crops or wild relatives via cross-pollination; adding as little foreign DNA as possible to plants to exclude all excess DNA; localizing the expression of foreign genes to the tissues and organs where required; and limiting their expression to the times when foreign gene products are needed.

Sequestering foreign trait genes in plastids enhances their containment

Plastids are a family of membrane-bound compartments or organelles found in plant cells. The green chloroplast, which carries out photosynthesis, is the best-known form of plastid. Plastid transformation is a clean gene transformation technology that has the potential to minimize the environmental impact of GM crops (see “Engineered Chloroplasts Snip out Antibiotic Resistance Gene” <http://www.isb.vt.edu/news/2001/news01.feb.html#chloroplasts>, *ISB News Report*, Feb. 2001). The technology inserts genes into plastids rather than the nucleus. Plastid engineering is possible because plastids contain their own individual set of about 100 genes, which are distinct from the 25,000 to 50,000 genes present in the nucleus. The membrane barriers surrounding plastids and nuclei prevent free mixing of their enclosed sets of genes. The expression machineries in the nucleus and plastid needed to make genes talk, resulting in an RNA or protein product, are very different. Whilst nuclear genes are transmitted in equal proportions by sperm and eggs, in most flowering plants the sperm cells from pollen transmit plastids at undetectable or very low frequencies to progeny plants. Plastids are passed on to the next generation by egg cells so that plastid genes are inherited maternally. The efficiency of maternal inheritance is best determined by tracking the inheritance of foreign marker genes transformed into plastids. In tobacco, there are no reports of paternal transmission of the plastid *aadA* marker gene amongst the hundreds of thousands of progeny screened collectively in several laboratories worldwide. This means that when trait genes are placed in plastids the pollen route of dispersal into the environment is prevented. Pollen is known to play a major role in mediating the dispersal of crop genes into the environment (see “Cross-pollination leads to triple herbicide resistance”, <http://www.isb.vt.edu/news/2001/news01.mar.html#mar0102>, *ISB News Report*, March 2001). Reduced dispersal of foreign trait genes and precise insertion of trait genes without excess DNA are key features that minimize the environmental impact of GM crops with transgenic plastids.

Genes can jump from plastids to the nucleus

Plastid genes will also be transmitted through pollen if copies of these genes can relocate or transpose to the nucleus. Movement of plastid genes to the nucleus was expected because insertions of plastid genes have been found in chromosomes within the nucleus but the frequency of these events was unknown. Timmis and co-workers (*ISB News Report*, this issue) describe an elegant experimental scheme to measure the frequency of plastid to nuclear transposition². The experiments measure the rate at which DNA in the plastid, in this case an *nptII* gene endowed with nuclear expression elements, is transferred to the nucleus. It is important to point out that the experiments only measure the frequency of transposition; in other words, the frequency at which a plastid gene can jump into the nucleus without gaining the ability to talk in this new environment.

For these experiments, it was essential to demonstrate that there were no pre-existing *nptII* genes in the nucleus, as a result of co-transformation of foreign DNA into the nucleus and plastid, before transposition was measured. Arguments to support co-transformation as opposed to transposition to explain the origin of nucleus-localized *nptII* genes have been put forward³. Pre-existing nuclear *nptII* genes would confer kanamycin resistance and be transmitted through pollen to seedlings at high frequencies when crossed with non-transgenic wild type tobacco; about ~50% or more seedlings would be kanamycin resistant. The absence of kanamycin resistance in the T₀ generation and lack of frequent pollen transmission of kanamycin resistance would appear to demonstrate the absence of pre-existing functional nuclear *nptII* genes before transposition was assayed². Low frequency epigenetic activation of silent *nptII* genes co-transformed into the nucleus is also unlikely as an explanation. Only plastid to nucleus transposition can explain the observation that nuclear *nptII* genes are flanked by longer plastid sequences than present in the original vector used for transformation.

Prior to these experiments on tobacco, the possible frequency of plastid to nuclear transposition was discussed by the ACRE subgroup on best practice in GM crop design. Work in baker's yeast (*Saccharomyces cerevisiae*) had shown DNA could escape from the mitochondrion to the nucleus^{4,5}. Mitochondria carry out respiration and are found in animal, plant, and fungal cells. Like plastids, mitochondria contain their own genes. In normal yeast, escape of mitochondrial genes to the nucleus takes place at frequencies between one cell out of every hundred thousand dividing cells to one cell out of every million dividing cells^{4,5}. Clearly tobacco plastids are very different from yeast

mitochondria but a rough comparison between the two systems reveals transposition frequencies that are within ten-fold of each other. Truly meaningful comparisons will require tobacco figures that are calculated as transposition events per cell division. This will require further work on the timing of transposition, which might take place randomly during plant growth and development or be restricted to particular cell types. Estimates of transposition frequency will also depend on experimental design, e.g., the type and size of gene that is tracked.

Jumping plastid genes cannot talk in the nucleus

The majority of plastid genes transposed to the nucleus will not be functional because plastid regulatory elements do not work in the nucleus. If pollen grains with inactive transposed genes fertilize other plants the resulting hybrids will contain but not express the transposed genes. Unlike the original crop, which benefits from a functional trait gene in the plastid, the hybrids will not gain any advantage from defective nuclear-localized trait genes. It remains possible that a very small fraction of transposed genes could be expressed due to chance chromosomal integration events in the nucleus. The frequency of these events will have to be determined by experimentation. If as many as one in a hundred-transposed genes were functional, about one in a million pollen grains would contain a functional transposed gene. Given this low frequency, pollen dispersal of a plastid trait gene into the environment via this transposition route appears unlikely. Seed transmission of a plastid-localized trait gene where crop plants act as female parents is a far more likely dispersal mechanism. To reduce this remote risk of spread through pollen even further, plastid editing sites or plastid-specific introns could be introduced into coding regions to prevent expression of plastid genes transposed to the nucleus.

Questions raised by plastid to nuclear transposition

Much attention has focused on the relevance of intracellular gene transposition to the spread of plastid trait genes from GM crops. Transposition of plastid DNA to the nucleus has far greater consequences for our understanding of the evolution of eukaryotic cells than it does on undermining the containment benefits of localizing genes to plastids. Plastids, which are descendants of ancient cyanobacteria, once contained several thousand genes in the distant evolutionary past. Many genes were lost and others were relocated to the nucleus. Transposition of a plastid gene to the nucleus is the first step in relocating a functional copy of a plastid gene to the nucleus. Rare fortuitous integration events or subsequent acquisition of nuclear expression elements and plastid targeting peptides over evolutionary time would allow the products of trans-

posed genes to function in the nucleus. The finding that plastid genes transpose to the nucleus in real time demonstrates escape to be an ongoing process. This gradual transfer of plastid DNA does not necessarily mean that the amount of DNA in the nucleus will increase with time. The majority of recently transposed plastid genes will not be retained in subsequent generations unless they increase plant fitness. This is because recently transposed genes will be present at very low frequencies within a population and are likely to be eliminated by genetic drift. Also, the DNA in the nucleus is in a dynamic state where turnover of non-coding DNA keeps it in a state of flux. Insertions of plastid DNA will be balanced by their loss from the nucleus at a rate governed by DNA maintenance functions and selection.

Plastid to nuclear transposition provokes questions of general interest to biologists. For example: What is the frequency of the process in other species? Is transposition taking place randomly during development or at particular times in specific cells? What is the mechanism of transposition? Comparisons with work in baker's yeast on mitochondrial DNA escape would indicate that DNA might leak out of plastids when their enclosing membranes become porous, perhaps during faulty plastid division or at plastid senescence. The escaping DNA would then integrate into nuclear chromosomes by a pathway similar to that which takes place when naked DNA is taken up during nuclear transformation of plant cells. No doubt these questions and others will stimulate further investigation of jumping plastid genes.

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Anil Day
 School of Biological Sciences,
 University of Manchester
 anil.day@man.ac.uk



REGULATORY NEWS

CULTIVATING GROUND RULES FOR BIOPHARMING

Phillip B. C. Jones

Almost three years ago, the StarLink™ corn incident inflamed opposition against genetically modified (GM) crops. The GM corn, which produces a *Bacillus thuringiensis* (Bt) insecticidal protein, had been accidentally mixed with conventional corn. In another case of inadvertent commingling, Monsanto Company (St. Louis, MO) announced last spring that conventional canola seeds may contain unapproved GT-200 canola seeds, which mature to Roundup®-resistant plants. This possibility seemed to stimulate little public interest, but the next case of GM crop contamination was different; this case was about biopharming.

The potential benefits of biopharming are considerable. The use of transgenic plants to produce therapeutic proteins could provide a means to manufacture therapeutic compounds at lower cost and in greater amounts. On the other hand, many have been uneasy about the prospect of biopharm crops potentially mixing with conventional food crops. This apprehension was justified by the U.S. Department of Agriculture's November announcement that GM corn produced for ProdiGene, Inc. (College Station, TX) had been mixed with conventional soybeans. ProdiGene had reportedly engineered the corn to produce a protein used in a swine vaccine. The USDA slapped the company with a hefty fine, but some viewed this penalty as an insufficient deterrent to prevent future incidents.

By the end of the year, The Genetically Engineered Food Alert (Washington, D.C.), a coalition of seven organizations, filed a petition requesting that the USDA initiate an immediate moratorium on the planting of food crops engineered to produce pharmaceuticals or industrial chemicals. The coalition also requested the USDA to prepare environmental impact statements for all biopharm crops.

Another coalition requested a similar ban in early 2003. This time, the group represented food manufacturers and restaurants. The Grocery Manufacturers of America, Inc. (Washington, D.C.) took the position that the USDA should create a presumption against the use of food or feed crops for pharmaceutical production unless the company developing the drug clearly demonstrates that it is not feasible to use nonfood crops.

So far, the USDA has not initiated a ban on biopharming. But on March 6, the agency's Animal and Plant Health Inspection Service (APHIS) announced proposed rules for the regulation of field-testing of plants designed to produce pharmaceutical and industrial compounds. Under the new rules, the USDA will increase the frequency of GM crop site inspections, and the agency will prohibit farmers from growing conventional corn within one mile of a field test site for the duration of any field test that involves open-pollinated GM corn. APHIS will also require farmers to operate planters and harvesters dedicated to use in the permitted test sites for the duration of the tests and dedicated facilities for the storage of equipment and regulated articles.

The proposed regulations include a prohibition against the planting of a food crop the year after the land was used for biopharming. This ban might have been inspired by the ProdiGene incident. Following a standard crop rotation practice, farmers had planted conventional soybeans on land previously used to grow ProdiGene's GM corn. The result: corn seed left from the transgenic crop grew into the soybean fields.

During a pre-briefing on the proposed rules, Bobby Acord, the administrator for APHIS, said that the proposed changes are designed "to make absolutely certain that there are no ProdiGenes in the future." Acord assured reporters that APHIS has the resources to meet the challenge of increased inspections, even though APHIS transferred over 2600 of its agriculture quarantine inspectors to the Department of Homeland Security.

For a short time, the Biotechnology Industry Organization had proposed a ban on GM corn biopharming in the Corn Belt. To a certain extent, this geographic isolation approach is built into the proposed rules. Cindy Smith, APHIS's deputy administrator for Biotechnology Regulatory Services, told reporters that the agency anticipates fewer corn field trials in the Corn Belt, because it may be difficult to secure land with the required one-mile isolation distance in an area where land is best suited for corn. Furthermore, the proposed rules would take the most productive land out of use by prohibiting farmers from cultivating conventional food or feed crops on ground used for a GM crop test during the following growing season.

Food industry representatives and biotechnology opponents labeled the proposals inadequate. The rule that the food industry and certain environmental groups wanted was a provision that only nonfood crops could be used to produce pharmaceuticals. The USDA's position is that such a rule

would not prevent contamination of nonfood GM plants with conventional crops. Rather, the agency chose to focus on methods intended to prevent any intermingling of GM plants and conventional plants.

A coalition of 11 U.S. environmental groups, farm organizations, and consumer groups threatened to sue the USDA unless the agency temporarily halts planting of GM crops that produce pharmaceuticals or industrial compounds. The coalition accused the USDA of allowing GM crops to be planted without conducting environmental risk assessments and alleged that the agency violated the National Environmental Policy Act. The groups notified the USDA of their intention to file a suit in federal court by May 5. A copy of the 60 day notice letter of intent to sue is available at the Center for Food Safety's website (<http://www.centerforfoodsafety.org>).

From Biopharming to More Conventional GM Crops

On February 25, the Environmental Protection Agency announced its approval for the commercialization of Monsanto's YieldGard® Rootworm Corn (MON 863). This GM plant produces a *Bacillus thuringiensis* spp. *kumamotoensis* delta-toxin protein (Cry3Bb1), which kills Western corn rootworm—the pest responsible for the greatest use of insecticides in the United States and the number one blight of U.S. corn.

The Bt toxin kills 50 to 80 percent of the rootworm larvae that eat the roots of transgenic corn. Such a system creates selection pressure for larvae that are resistant to the toxin. To reduce the risk of developing a Bt toxin-resistant rootworm population, the EPA is requiring Monsanto to ensure that 20 percent of the planted acreage of YieldGard Rootworm Corn is set aside for growth of conventional corn. These refuge areas will support populations of corn rootworm not exposed to the Bt toxin. Insect populations in the refuges are expected to crossbreed with any insects resistant to Bt toxin, thereby limiting the numbers of Bt toxin-resistant rootworms. This resistance management strategy was developed as a condition of EPA registration.

Environmentalists were not pleased by the 20 percent refuge area requirement. The EPA's scientific panel apparently urged a requirement for farmers to plant refuge areas covering 50 percent of the GM crop acreage, but Monsanto pushed for the lower level. Stephen L. Johnson, the EPA's assistant administrator for prevention, pesticides, and toxic substances, brushed off criticism of the 20 percent level, stating that this requirement would be in effect for only three years while the potential for toxin

resistance is studied further.

The commercialization of GM wheat is also encountering disapproval. A consortium of U.S. agricultural and environmental groups filed a Citizen Petition on March 11 with the USDA seeking a moratorium on the commercial introduction of herbicide-resistant GM wheat, such as Monsanto's Roundup Ready® wheat. The petition seeks an environmental impact statement on the deregulation of GM wheat varieties and requests the listing of GM wheat varieties as noxious weeds. A copy of the Citizen Petition is available from The Center for Food Safety website.

The effect of GM wheat on U.S. exports is a major concern. U.S. wheat exporters currently sell wheat to foreign markets with a USDA-approved statement that no GM wheat is commercialized in the United States. According to the petitioners, GM wheat will cross-pollinate with other wheat, making it impossible for U.S. farmers to export their crops to foreign markets with GM food bans, such as the European Union. Joseph Mendelson, The Center for Food Safety's legal director, promised that the legal petition is the first step to a lawsuit if the USDA did not address the petitioners' concerns.

A few days after the petition was filed, the USDA announced that it may impose strict requirements on Monsanto to ensure that the company abides by its pledges. Monsanto has stated that the company will not introduce Roundup Ready wheat to the marketplace until the demonstration of food, feed, and environmental safety, resulting in regulatory approvals in the United States, Canada, and Japan. The company has also promised to develop grain handling protocols and standardized sampling and detection methods to separate GM wheat from conventional wheat.

The ability to isolate GM crops from conventional crops is a major stumbling block in the progress of agbiotech. The petitioners argue that the events surrounding StarLink corn, ProdiGene corn, and GT-200 canola incidents show that "the current U.S. grain handling system is unlikely to be prepared to sufficiently segregate genetically engineered and non-genetically engineered wheat to the point at which foreign importers will have confidence in the integrity of such shipments."

Selected References

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*Phillip B. C. Jones, PhD., J.D.
Seattle, Washington
phillipjones5939@msn.com*

UPCOMING MEETINGS

More meetings can be found at: <http://www.isb.vt.edu>

GENETICALLY ENGINEERED FOREST AND FRUIT TREES: PUBLIC MEETING

July 8 - 9, 2003

The Animal and Plant Health Inspection Service, USDA has released a notification for parties involved in those fields associated with the environmental release of genetically engineered trees, as well as other interested persons, that a public meeting will be held to provide a forum for discussion on the environmental safety, potential benefits, and risks of genetically engineered trees relative to traditional varieties.

The meeting will be held on Tuesday, July 8, 2003, from 8 a.m. to 4 p.m., and Wednesday, July 9, 2003, from 8:30 a.m. to 4 p.m. at the USDA Center at Riverside, 4700 River Road, Riverdale, MD.

FOR FURTHER INFORMATION CONTACT:
Mr. John Cordts, Biotechnologist, BRS, APHIS
Email: John.M.Cordts@aphis.usda.gov
Tel: (301) 734-5531
Fax: (301) 734-8669
<http://www.aphis.usda.gov/ppq/biotech/>

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UC DAVIS TRANSGENIC ANIMAL RESEARCH CONFERENCE IV

August 10-14, 2003

Granlibakken Conf. Center, Tahoe City, California

This is an international meeting that will bring together representatives from the leading laboratories in transgenic animal research. This meeting is a follow up to the highly successful meetings held at Granlibakken in 1997, 1999 and 2001.

The conference will again focus on state-of-the-art science in the field of transgenic research. Presentations will address cutting-edge methodology, technical improvements, and current progress towards producing transgenic animals for biomedical and agricultural applications. The intent of these meetings is to bring together scientists to discuss progress, problems, and potential application of transgenic technology for animal applications.

Contact: Conference and Event Services

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8TH BIOTECHNOLOGY 2003 EDUCATORS' CONFERENCE

July 16-19, 2003

Virginia Tech, Blacksburg, Virginia

The goal of this conference is to provide technical and content updates for high school and college biology educators. New applications in medicine, agriculture, and the environment continue to be discovered and discussed. Biotechnology 2003 will feature: pre-conference biotechnology boot camp, roundtable discussions, workshops, keynote speakers and much more. The Conference is organized by Fralin Biotechnology Center, Virginia Polytechnic Institute and State University.

Contact: Erin Dolan

Email: BIOoutreach@vt.edu

Tel: 540-231-2692

Fax: 540-231-7126

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