Plant Gene Transfer Becomes a Fertile Field

Gene transfer into dicot plants can generate varieties with decreased herbicide susceptibility and can also be used to study plant gene control

Genetic engineering of plants is rapidly becoming a reality, at least for members of one of the two major groups of seed-bearing plants. As numerous presentations at the "First International Congress of Plant Molecular Biology"* made clear, techniques developed over the past few years now make it relatively easy to introduce new genes into dicotyledonous plants, which include potatoes, tobacco, petunias, and tomatoes.

Investigators are already using gene transfer methods to generate plant strains with increased herbicide resistance. In addition, the experiments are producing a better understanding of gene control in plants, thus generating the kind of information needed to ensure that transferred genes function the way molecular biologists want in genetically altered plants.

Genetic manipulation of the monocotyledonous plants, which belong to the second major group of seed-bearers and include the important cereal crops, has not moved as quickly as that of the dicots, although this situation appears to be changing. Investigators have recently begun to develop methods for introducing new genes into monocot cells, including those of maize, the first major cereal to be transformed with new genetic material. Nevertheless, the long-standing inability to regenerate whole plants from transformable cells still remains as an obstacle to the successful genetic engineering of monocots.

Increasing herbicide resistance provides a good target for genetic engineers because a plant's susceptibility to one or another of the chemicals may be determined by a single gene. For example, the herbicide glyphosate, which is the active ingredient in Monsanto Company's product "Roundup," kills plants by suppressing the activity of an enzyme called EPSP synthase that is needed for making the essential amino acids phenylalanine, tyrosine, and tryptophan. The use of this herbicide is currently limited because it kills all plants, including crop plants.

At the Plant Molecular Biology Congress, representatives from two groups, who used somewhat different strategies, described the engineering of plants with increased glyphosate resistance. Luca Comai, David Stalker, and their colleagues at Calgene, Inc., in Davis, California, used a mutant EPSP synthase gene that they had originally isolated from a a glyphosate-resistant strain of *Salmonella* bacteria. The protein encoded by the mutant gene differs in a single amino acid from the normal EPSP synthase of *Salmonella*, but this is apparently sufficient to make it less susceptible to inhibition by the herbicide. When the Calgene workers transferred the mutant gene into tobacco plants, the plants not only made the corresponding *Salmonella* enzyme, but were able to grow when sprayed with glyphosate in amounts equivalent to those that would be used in the field and would normally kill plants.

The experiments illustrate the growing capacity of plant molecular biologists to tailor genes to achieve specific purposes when the genes are transferred into new species.

However, the engineered plants did not grow as well as unsprayed controls. According to Stalker, the plants may not have become completely resistant because the enzyme produced by the transferred gene is located in an abnormal place. "I was surprised to see tolerance at all," he notes. Although the plant EPSP synthase gene is a nuclear gene, the enzyme product is normally transported to the chloroplasts where the other enzymes of the synthetic pathway are located. The bacterial enzyme apparently ends up instead in the cell cytoplasm because its gene lacks the transport sequence needed to direct it to the chloroplast.

Calgene has patented the mutant EPSP synthase gene and plans to use it to develop additional glyphosate-resistant crop plants, including soybean, cotton, and tomato. The company hopes to begin testing the tolerance of the genetically engineered tobacco plants in the field within the next few months if granted the required approval by the U. S. Department of Agriculture.

The second group is from Monsanto Company in St. Louis and includes Robert Fraley, Dilip Shah, Robert Horsch, Harry Klee, and Stephen Rogers. Instead of introducing a mutant EPSP gene, these investigators engineered glyphosate-resistant petunia plants by inducing them to make some 20 to 40 times the usual amount of the normal petunia enzyme. They did this by hooking the petunia EPSP synthase gene to a viral regulatory sequence that is a very active promoter of gene expression and then transferring the hybrid gene into plants. Shah had already shown that cultured petunia cells can become resistant to glyphosate if they have an increased number of EPSP synthase genes and are overproducing the enzyme.

Whole plants that acquired the hyperactive hybrid gene could also withstand exposure to the herbicide. "The four plants with the gene all survived the [glyphosate] spraying and continued to grow and flower," Fraley says, "and the four control plants are all quite dead." The transferred petunia gene, unlike the bacterial EPSP synthase gene, has a chloroplast transport sequence, and the Monsanto workers find that the product enzyme does appear in that organelle.

Although the engineered petunia plants are about ten times more resistant than the controls, Fraley estimates that the resistance will have to be increased by an additional factor of 2 to 3 to obtain commercially useful plants. It might help, he suggests, to use a glyphosate-resistant mutant of the EPSP synthase gene instead of the normal one.

There are also indications that gene transfer can be used to make plants resistant to the herbicide atrazine, according to data presented by Lawrence Bogorad of Harvard University. A few years ago investigators from Bogorad's laboratory and that of Charles Arntzen at Michigan State University showed that atrazine kills plants by blocking the action of one of the chloroplast proteins needed for photosynthesis. They also found that a change of a single amino acid in the protein can make it resistant to the herbicide.

However, the gene for the protein is encoded in the chloroplast DNA, a situa-

*The congress was organized by the International Society for Plant Molecular Biology and held in Savannah, Georgia, from 27 October to 2 November.

tion which might have militated against a successful gene transfer. Newly introduced genes are usually incorporated in the nuclear genome, and chloroplast genes do not have the correct control sequences to be expressed there.

Nevertheless, Bogorad's group, in collaboration with that of Jeff Schell at the Max Planck Institute for Plant Breeding Research in Cologne and Marc van Montagu at the State University of Ghent in Belgium, has now introduced the resistant form of the gene into tobacco plants, which subsequently become able to withstand atrazine treatment, although Bogorad notes that the plants did not appear to be completely healthy. The investigators first attached the gene to a regulatory sequence that allows its expression in the nuclear genome and also to a chloroplast transport sequence.

These experiments illustrate the growing capacity of plant molecular biologists to tailor genes to achieve specific purposes when the genes are transferred into new species. Further progress depends on a better understanding of plant gene regulation, a goal which is also being advanced by gene transfer experiments. One of the more intriguing findings is that a wide variety of plant genes show nearly normal patterns of expression when they are introduced into foreign species. According to Nam-Hai Chua of Rockefeller University, a monocot gene may even be expressed normally in a dicot. The results indicate that gene control mechanisms have been well conserved during plant evolution. They also are a source of optimism for plant genetic engineers. Normal control of genes transferred into animals has proved much more difficult to achieve.

Some of the plant genes that have been transferred are under developmental control, which leads to tissue-specific patterns of expression. For example, Robert Goldberg and his colleagues at the University of California at Los Angeles have transferred two developmentally regulated soybean genes into tobacco plants. Ordinarily the protein product of one of the genes is made primarily in seeds, while the product of the other is synthesized in soybean leaves as well as in the seeds. Expression of the two genes followed the same pattern in the tobacco plants, Goldberg reports, although they made less product than in soybeans.

Other of the transferred genes respond to environmental stimuli, such as light. Chua and his colleagues have been studying the regulation of the gene that encodes the smaller of the two protein subunits of the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase. This gene, like many others involved in photosynthesis, is only turned on by light and is expressed primarily in leaves. The Rockefeller workers have transferred a cloned small subunit gene, which was originally obtained from pea plants, into petunias. They find that both the light responsiveness and tissue-specificity of the gene are maintained there.

Gene transfer experiments are also being used to identify the DNA sequences that regulate plant gene expression. They show that the regulatory sequences are located before the start of the protein-encoding regions of the genes, just as are those of most other genes, and may be fairly long, perhaps 1000 base pairs or more. The Chua group finds that only the nucleotides between -2 and -35 (counting backward from the start of the gene) are needed for the light response of the small subunit gene, but an additional 700 nucleotides, which extend from positions -352 to -1052, are needed for maximal expression of the gene. The regulatory region contains an enhancer element similar to those found in other genes of higher organisms.

June Simpson and Luis Herrera-Estrella of the Ghent laboratory, with Anthony Cashmore and Michael Timko of Rockefeller University, and Schell have made similar findings regarding the control regions of the small subunit gene. Other light-regulated genes may use different control sequences. For example, Hildegard Kaulen and Fritz Kreuzaler of Schell's Cologne group have been analyzing the 1200–base pair regulatory region of a gene for a pigment-synthesizing enzyme but do not see a segment comparable to the 33–base pair sequence identified by the Chua group.

One of the more complex regulatory systems discussed at the Plant Molecular Biology Congress is that of the genes coding for certain proteinase inhibitors that are part of plants' defenses against insect pests and fungal and bacterial pathogens. The inhibitors block the activities of the protein-digesting enzymes of insects, bacteria, and fungi, thereby diminishing the nutrition that the parasites can obtain by dining on the plants.

Work by Clarence Ryan and his colleagues at Washington State University and also by the Max Planck researchers, who have been studying the expression of the proteinase inhibitor genes in tomato and potato plants, shows that they are subject to a two-tier control involving both developmental and environmental regulation. Normally the genes are off in leaves but are on in potato tubers and tomato fruits. But when the leaves are wounded—as when a hungry insect takes a bite, for example—the genes are turned on there. Both groups plan experiments aimed at identifying the gene segments that participate in this complex control.

Monsanto Co.

Glyphosate resistant plants

The plants in the bottom row, which were genetically altered to produce large quantities of the enzyme inhibited by glyphosate, tolerated spraying with the herbicide. The control plants did not.

Gene transfer into dicots is so advanced primarily because investigators can use bacteria of the *Agrobacterium* genus to introduce genes into foreign species. The bacteria are pathogens that cause diseases marked by abnormal growths either of the "crown gall" or "hairy root" type, depending on the bacterial species. When the bacteria infect plants they act as natural genetic engineers. They inject a plasmid into the cells, thus transforming them with new DNA and producing the abnormal growths. Molecular biologists can insert the gene they want transferred into an *Agrobacterium* plasmid, infect plant tissue with bacteria carrying the plasmid, and then regenerate whole plants from transformed cells.

However, the *Agrobacteria* do not usually infect monocot cells and only recently have attempts to introduce new genes into these cells proved successful. At least three groups have now developed methods that do not require the bacteria for transferring genes into

monocot protoplasts, denuded cells that have been stripped of their cell walls.

In a last-minute addition to the congress program, Michael Fromm, who works with Loverine Taylor and Virginia Walbot at Stanford University, described his group's achievement in introducing an antibiotic resistance gene into maize protoplasts. The investigators used a technique called "electroporation" that is becoming generally popular for gene transfer. The method involves subjecting cells to an electric current that induces the formation of transient membrane pores through which large molecules may pass.

Simply incubating protoplasts with the DNA to be transferred can also work. Horst Lörz and his colleagues at the Max Planck Institute in Cologne have transformed protoplasts of the primitive wheat *Triticum monococcum* with an antibiotic resistance gene by this method, and Ingo Potrykus and his colleagues at the Friedrich-Miescher-Institute in Basel, Switzerland, have done the same with protoplasts of the ryegrass *Lolium multiflorum*. The efficiency of gene transfer may be better with electroporation, however.

Transformation of monocot protoplasts is thus becoming a reality. The main problem remains the difficulty in regenerating whole plants. There is a bit of a paradox here. Whole monocot plants can be regenerated from some types of cells, particularly those derived from embryonic tissue, but these cells have walls and investigators have not been able to get foreign DNA into them. They can now introduce new DNA into protoplasts, but as Edward Cocking of the University of Nottingham, England, points out, "No publication clearly states that you can go from protoplasts to whole plants in monocots."

Indra Vasil of the University of Florida in Gainesville described the apparent regeneration of sugarcane plants from protoplasts, but during the discussion period was closely questioned about whether he had eliminated the possibility that the plants might have been derived not from transformable protoplasts but from small clumps of cells that had retained their walls. Vasil maintained that the experiment had indeed been carefully done to exclude that possibility.

The new demonstrations of transformation of monocot protoplasts should provide additional impetus for investigators to solve the long-standing regeneration problem. If that can be done, it would put genetic engineering of monocots on the same firm footing as that of the dicots.—**JEAN L. MARX**

Neptune's Ring Arcs Confirmed

No one claims to know what it is exactly, but for the time being astronomers are calling it an arc. Its reality seems certain, although its exact location about Neptune, its extent, and its very nature remain unclear. There may even be more than one of this new sort of partial or broken planetary ring. Astronomers have again detected the arc that was reported earlier this year and possibly a nearby arc, while clearly demonstrating how abrupt the breaks are in Neptunian broken rings.

At the recent annual meeting of the Division for Planetary Sciences of the American Astronomical Society in Baltimore, four groups of observers* reported on the latest efforts to catch Neptune's mysterious arc as it passes in front of a star. Three such stellar occultations by an arc have now been detected in addition to the discovery event of July 1984†. No one has yet succeeded in recording a conventional set of ring occultations at Neptune, when a star blinks once as it passes inside a ring as viewed from Earth and again when it passes to the outside of the ring. With Neptune, it has always been one event or the other, if anything happens at all. Perhaps one out of seven observations of a star passing close to or behind the planet will detect any arc occultation at all.

Most of the Neptunian ring or rings may be missing, but when caught in front of a star their effect is a normal one and fairly consistent. An arc blocks 15 to 40 percent of the light of the star. The durations of the dimming require an arc width of 8 to 23 kilometers. Such opacities and widths are similar to those of most of the nine complete rings around Uranus. How many arcs Neptune has is not certain, but it would appear that at least two are required if they are in the equatorial plane—one at a distance of about 54,000 kilometers and another at roughly 66,000 kilometers.

What is particularly striking about the arcs, aside from their existence, is how abruptly they come and go. Corbin Covault of the MIT group reported that the star of the 7 June 1985 occultation turned out to be a double star, only one of which passed behind a detectable arc. Presumably, by the time the second star passed behind the orbit of the arc about 5000 kilometers farther along its 400,000-kilometer circumference, the particles constituting the arc had thinned out to the point of undetectability. Likewise, Lawrence Wasserman of Lowell Observatory at Flagstaff failed to detect an occultation on 20 August when the Cornell/Caltech and European groups recorded the same unmistakable event through separate telescopes farther south in Hawaii, implying a disappearance of the arc over a space of no more than 4000 kilometers.

Relatively frequent reports of the detection of the ends of arcs suggest that there is more than one arc per orbit, in effect clumps of particles separated by empty space or an undetectably tenuous ring. Mark Showalter of Cornell University and his colleagues reported at the meeting that the clumpy ringlet in the Encke gap of Saturn, a possible analog of the Neptune arcs, must have a single moonlet embedded in it. The moonlet revealed its presence by gravitationally creating a wake in the ring outside the gap. The moonlet would plow back ring particles to keep the gap open, as shepherding moonlets are thought to confine the Uranian rings, and possibly share its orbit with ringlet particles, as proposed early on for the Uranian rings by Stanley Dermott and Thomas Gold of Cornell University.

Other possibilities for the form and confining mechanism of the Neptune arcs are numerous—a recently disrupted satellite, shepherded complete rings of varying width and thus opacity, or an inclined ring. In the first formal proposal, Jack Lissauer of the University of California at Santa Barbara has pointed out that an arc might be confined ahead or behind an unseen satellite (at its L4 or L5 Lagrangian points) by a single shepherd inside or outside the satellite's orbit‡.—**RICHARD A. KERR**

*MIT group, led by J. Elliot; Cornell/Caltech group, led by P. Nicholson and K. Matthews; European group, led by A. Brahic, Observatory of Paris, Meudon; F. Vilas and W. Hubbard, University of Arizona.
†A. Brahic *et al.*, *Nature (London)*, in press.
‡J. J. Lissauer, *ibid.*, in press.