SESSION 1

THE ELEVENTH BAWDEN LECTURE

CHAIRMAN MR E. LESTER

SPEAKER DR MARY-DELL CHILTON

Genetic Engineering - Prospects for Use in Crop Management

MARY-DELL CHILTON

CIBA-GEIGY Biotechnology Facility

Post Office Box 12257

Research Triangle Park, NC 27709

ABSTRACT

The Agrobacterium tumor-inducing (Ti) plasmid inserts a segment of its DNA, called T-DNA, into host plant cells, transforming them into tumor cells that grow rapidly. Although the mechanism of this gene-transfer process is not understood, it can nevertheless be exploited by plant genetic engineers. The T-DNA on the Ti plasmid can be replaced with genes of interest for crop improvement. If such genes come from very unrelated organisms (bacteria or yeast for example), they must be modified to make them function in the crop plant. Model experiments in such gene modification have been successful, and plants have been engineered with functional bacterial genes. This genetic engineering strategy has two limitations. Only one class of crop plants is susceptible to Agrobacterium infection, i.e. the dicots (soybean, potato, tomato, tobacco, and many vegetable crops). Monocots (corn, wheat, rice) seem immune. Secondly, the crop plant must be regenerable from tissue culture cells, a serious problem at present for soybean.

Supposing that means are developed for direct gene insertion into valuable crop plants, the next challenge will be choosing and isolating single genes that will improve the crop. Today it is feasible to produce plants resistant to agricultural chemicals. Improvement of nutritional quality of seeds can readily be accomplished by directed changes in seed storage protein genes. The prospects are more long range for protecting plants against pathogens, improving yield, affecting plant size and shpae or protecting the crop against environmental stress. Problems of patent protection and governmental regulation add an element of risk to all genetic engineering projects.

INTRODUCTION

A central figure in the new technology of plant genetic engineering is the plant pathogen Agrobacterium tumefaciens, agent of crown gall disease. The bacterium carries a large virulence plasmid, the Ti (tumor-inducing) plasmid, that possesses the unique ability to insert a part of its DNA into the chromosome of the host plant cell. The transferred DNA, T-DNA, is a specific part of the Ti plasmid and contains genes that function only after transfer to the plant cell. In response to these new functions, the plant cell is stimulated to divide rapidly and to synthesize novel metabolites, opines, that are specific nutritional substrates for the pathogenic bacterium. The gall is thus a factory for production of nutrient for Agrobacterium, and the pathogen is a microscopic genetic engineer. The details of the discovery of T-DNA transfer and opine biosynthesis have been summarized in several recent reviews (Nester and Kosuge 1981, Van Montagu and Schell 1982, Bevan and Chilton 1982, Hooykaas and Schilperoort 1984).

T-DNA is a specific small part of the large Ti plasmid, and luckily what marks it for transport to the plant cell is not the genes in T-DNA. There are signals at the left and right borders of T-DNA (Yadav et al. 1981, Zambryski et al. 1982), and all of the genes in T-DNA are dispensible (Garfinkel et al. 1981, Leemans et al. 1982.) Genes elsewhere on the Ti plasmid are responsible for sending T-DNA along to the plant cell. Addition of extra DNA to the middle of T-DNA did not interfere with the T-DNA transfer process, and indeed this passenger DNA was carried along to the plant cell together with the natural T-DNA (Hernalsteens et al. 1980). T-DNA could therefore be exploited as a carrier (vector) for introducing desirable genes.

INSERTION OF FOREIGN DNA INTO T-DNA

Insertion of DNA into small plasmids can be achieved by a few simple enzymatic steps (Figure 1). The small plasmid is cut with a restriction endonuclease that cleaves in one site. Novel DNA fragments that have been cut with the same enzyme are added to the plasmid. The mixture is reassembled by use of the "stitching" enzyme ligase, forming recombinant plasmids containing novel DNA inserts.

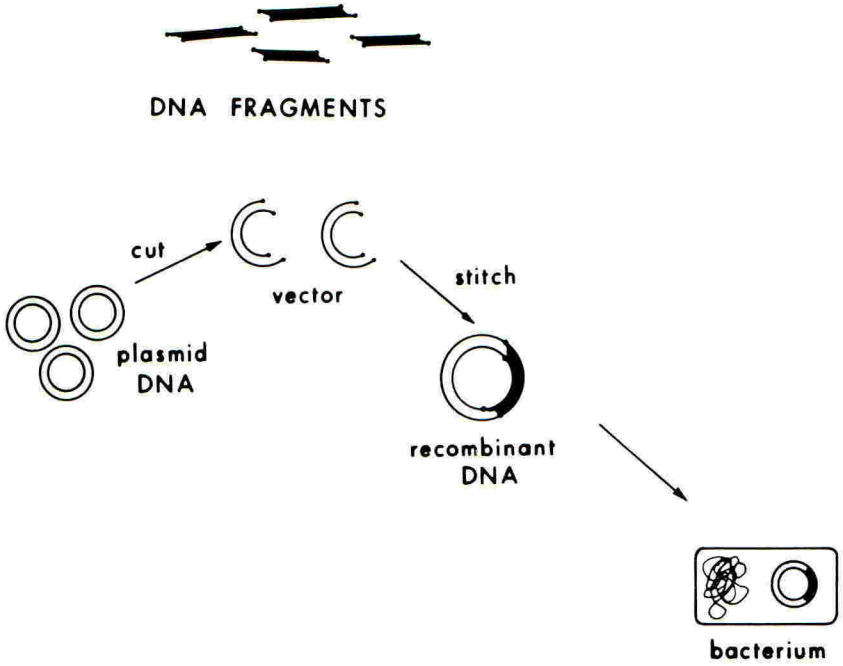

Fig. 1. Insertion of DNA fragments into a small plasmid. Small circular plasmid DNA (white) is cut once with a restriction endonuclease. DNA fragments (black), cut from plant or other DNA of interest with the same restriction endonuclease, are added. The fragments are stitched together by adding DNA ligase, to yield a mixture of recombinant DNA plasmids, each containing a different black fragment. The recombinant plasmids are introduced into a population of bacteria (one per bacterium). A pure culture derived from each bacterium contains one type of recombinant plasmid. Many such bacteria constitute a "library" of DNA fragments, among which the desired "book" (plasmid can be found by several techniques.

Novel DNA cannot be directly inserted into T-DNA of the Ti plasmid by this approach because all restriction endonucleases cut the Ti plasmid into 10-50 pieces (Sciaky et al. 1978). To circumvent this problem we use a combination of recombinant DNA manipulation and bacterial genetics to get new genes into T-DNA at any desired location (Matzke and Chilton 1981, Leemans et al. 1981). Details of one type of procedure are shown in Figure 2. Slight modification of the strategy allows us to delete small or large protions of the natural T-DNA during the insertion process. This powerful but laborious procedure makes it possible to rebuild T-DNA in any way we wish.

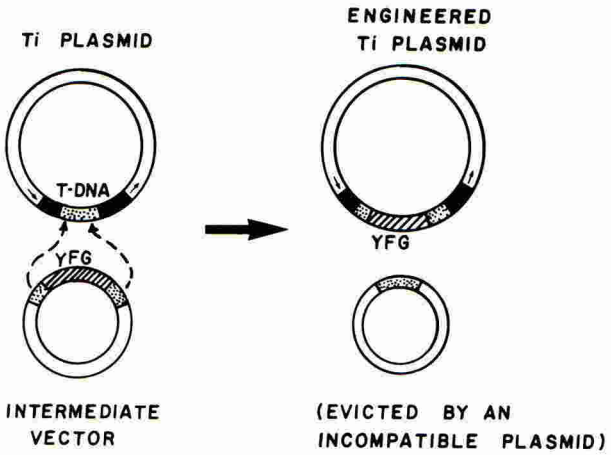

Fig. 2. Strategy for insertion of foreign DNA into T-DNA on the Ti plasmid. A subfragment (stippled region) of T-DNA (black region) is cloned into a wide host range intermediate vector. Foreign DNA, symbolized by YFG (your favorite gene) together with a selectable genetic marker (antibiotic resistance), is cloned into the center of the stippled region. The engineered intermediate vector is introduced into Agrobacterium containing a Ti plasmid. Two recombination events (dotted arrows) bring about rare exchange of the engineered trait onto the Ti plasmid. The small arrows flanking T-DNA represent the border repeats that define the region of the Ti plasmid transferred to the plant cell.

Simplification of the procedure has provided more convenient methods for producing genetically engineered Ti plasmids (Comai et al. 1983, Van Haute et al. 1983, Zambryski et al. 1983). However the ultimate simplification was the discovery that T-DNA need not be on the Ti plasmid: it can be on a separate MINI-Ti plasmid in Agrobacterium, together with a "helper" Ti plasmid lacking T-DNA (Figure 3). The helper plasmid provides all the functions needed to send T-DNA on its way into the plant genome (De Framond et al. 1983, Hoekema et al. 1983). This MINI-Ti strategy offers great promise for design of versatile, convenient vector systems into which desirable genes can be inserted directly. A MINI-Ti vector consists of a wide host range plasmid containing left and right T-DNA border signals and between the borders, one or more unique cut sites for restriction endonucleases.

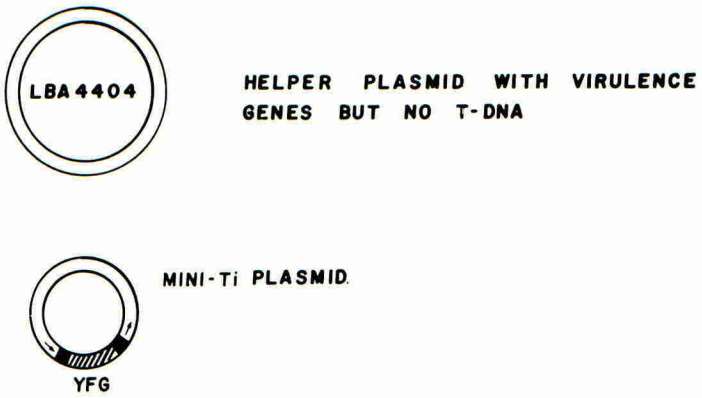

Fig. 3. MINI-Ti Vector. A MINI-Ti plasmid vector contains left and right borders on a wide host range plasmid. YFG is cloned between the borders, and the vector is introduced into Agrobacterium containing a helper plasmid. No recombination onto the helper plasmid is required: T-DNA transfer is mediated by the helper plasmid in trans.

SELECTABLE MARKERS

Tumorous plant cells do not regenerate into complete healthy plants as do their normal counterparts. Elimination of one (Barton et al. 1983) or more (Zambryski et al. 1983) genes in T-DNA solves this problem. However it creates another problem: it is difficult to find the transformed cells among large populations of untransformed cells. (Tumor cells are easy to find: they grow on hormone-free agar, while the normal cells die.) To allow easy detection or selection of transformed cells, "chimeric" genetic markers have been constructed. Bacterial genes, yeast genes and other very foreign types of genes do not function in plants because the start and stop signals in plant genes are different. Attaching plant start and stop signals to a bacterial gene produces a chimeric gene structure that does function in the plant. In most of the experiments reported thus far, the "plant" signals were in fact cannibalized from a T-DNA gene, the nopaline synthase gene. Chimeric bacterial drug resistance genes (Herrera-Estrella et al. 1983, Bevan et al. 1983, Fraley et al. 1983) and β-galactosidase (Helmer et al. 1984) have been shown to function in plant cells after insertion by Ti plasmid T-DNA.

TRANSFORMATION OF PROTOPLASTS BY A NON-T-DNA VECTOR

Recently Paszkowsky et al. (1984) have shown that plant protoplasts can be transformed by naked plasmid DNA containing a selectable marker but no T-DNA border sequences. A chimeric kanamycin resistance gene with signals from a plant viral gene (cauliflower mosaic virus gene VI) was introduced into tobacco protoplasts. After selection of the transformed cells on kanamycin-containing medium, plants were regenerated and shown to transmit kanamycin resistance to their progeny as a dominant Mendelian trait. This finding points the way to a transformation method for plants outside the host range of Agrobacterium, which is restricted to dicots.

REGULATION OF FOREIGN GENES INTRODUCED INTO PLANTS

Chimeric genes formed from the nopaline synthase signals express in all types of plant cells: they seem not to be developmentally regulated. If the signals are taken from a light-regulated plant gene, the resulting chimeric gene is also light-regulated (Herrera-Estrella et al. 1984). It is plausible to suppose that signals from a plant gene that expresses only in roots would provide chimeric genes that are root-specific, etc. This will be important for some kinds of genetic engineering projects: engineering a nematode resistance gene to express in leaves would be uselss when the roots are the point of attack. Herbicide resistance, on the other hand, might be needed only in leaves.

TECHNICAL PROBLEMS REMAINING

The major problems in vector design are solved for plants within the host range of the Agrobacterium Ti plasmid. So far as we now know, this is restricted to dicotyledonous plants. More detailed knowledge of why Agrobacterium fails to produce tumors on monocots may reveal a means of adapting the Ti plasmid to this group of plants. Alternatively, it may be necessary to develop completely new strategies. Transformation of plant protoplasts by naked DNA (Paszkowski et al. 1984) is an attractive approach; however important cereal crops fail to regenerate from protoplasts to complete plants. Transformation of pollen with DNA would provide an ideal solution, yielding genetically engineered plants with minimal in vitro manipulation.

Soybean, an important dicot crop that is susceptible to the Ti vector strategies outlined above, is not readily regenerable from tissue culture. Thus genetically engineered soybean plants have not yet been produced. Several groups are currently reporting hopeful results in soybean regeneration, and it appears likely that this challenge will be met within a year.

A general problem for the genetic engineer wishing to produce plants from engineered plant cells is the occurrence of high genetic variability in regenerated plants of clonal origin. This "somaclonal" variation, while it may prove useful in its own right as a source of new kinds of genetic variation for plant breeders, is unwanted in engineering new genes into existing desirable genotypes. An understanding of the molecular basis of this variation might aid in eliminating it during genetic engineering projects.

The most challenging technical problem is the choice of single-gene-encoded traits that can be used to improve the quality of a crop plant. The most obvious choices are genes that confer resistance to herbicides or other useful agricultural chemicals. Nicotiana plumbaginifolia and N. tabacum plants resistant to the antibiotic kanamycin have been engineered by introduction of chimeric genes that detoxify kanamycin (Horsch et al. 1984, Paszkowski et al. 1984). Neither of these plants is a major food crop, nor is kanamycin an important agricultural chemical, but the success of these model experiments augurs well for projects of this type. It is less clear how to find or construct single genes that will solve other problems for the farmer: resistance to fungal or bacterial pathogens, tolerance to environmental stress, susceptibility to insects or nematodes, or improvement of yield. Improvement of the amino acid balance of seed storage protein can be attempted by manipulation of single genes that are readily isolable from plants. This objective however, is not high on the farmer's wish list. Many approaches are clear for combatting viral diseases, but further basic research is needed to determine the best method.

Foreign proteins introduced by genetic engineers may find themselves in a hostile environment for which they have not been prepared by natural selection. Plant proteases may cleave them, or low pH environment in the plant cell may make them enzymatically inactive. We have had too little experience to predict how frequently we may encounter problems of this kind. It may prove important to direct the engineered protein to a particular compartment of the plant cell in order to allow it to function.

For some kinds of genetic engineering objectives, it will be important to direct the expression of the foreign gene precisely. Although results available thus far are encouraging (Broglie et al. 1984, Herrera-Estrella et al. 1984), the chimeric gene approach may not solve all problems in this area. We may find that the position at which the foreign gene is inserted in the host plant genome affects the outcome. There are already isolated reports of changes in gene expression after the new gene is transmitted by seed.

This li t of challenges clearly shows that the genetic engineer still has important work ahead. There are few agriculturally significant objectives that can be approached with complete confidence today.

STRATEGIC PROBLEMS

In addition to the scientific problems outlined above, companies working in genetic engineering face strategic problems in evaluating project alternatives. It is not clear how to protect a product that is a genetically engineered plant (Williams 1984). It is not clear whether genetically engineered plants will, like novel chemicals, come under governmental regulation. The criteria for acceptance, if regulation comes into being, are completely unknown. These two important problems introduce an element of risk into all genetic engineering projects.

An additional strategic problem in evaluation of many genetic engineering projects is the estimation of their marketability. Genetic engineers could, in principle, produce plant genotypes so novel that

their marketability is completely unkown. For example, who will buy high-protein potato tubers? Will they be susceptible to attack by new kinds of pests? Will they require storage at temperatures inconsistent with maintenance of the starch content? Will they have acceptable taste and texture? Will the yield suffer?

CONCLUSIONS

Impressive progress in plant genetic engineering over the last five years seems to predict a bright outcome to the significant scientific challenges remaining. The problems of protectability and regulation add an element of risk to all genetic engineering projects. The most novel products to emerge from this new technology will carry the added business risk of unpredictable marketability. It is clear that the technology of plant genetic engineering is well-suited to certain kinds of crop improvements. For other problems, it may never be useful. Genetic engineering will provide plant breeders with a unique new source of genetic traits that will make significant contributions to traditional breeding programs by the turn of the century.

REFERENCES

Barton, K.; Binns, A.N.; Matzke, A.J.M. and Chilton, M.-D. (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32, 1033-1043.

Bevan, M.W. and Chilton, M.-D. (1982) T-DNA of the Agrobacterium Ti and Ri plasmids. Annual Review of Genetics 16, 357-384.

Bevan, M.W.; Flavell, R.B. and Chilton, M.-D. (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304, 184-187.

Broglie, R.; Coruzzi, G.; Fraley, R.; Rogers, S.; Horsch, R.; Niedermeyer, J.; Fink, C.; Flick, J. and Chua, N.-H. (1984) Light-regulated expression of a pea ribulose-1, 5-bisphosphate carboxylase small subunit gene in transformed plant cells. Science 224, 838-843.

Comai, L.; Schilling-Cordaro, C.; Merigia, A. and Houck, C.M. (1983) A new technique for genetic engineering of Agrobacterium Ti plasmid. Plasmid 10, 21-30.

De Framond, A.; Barton, K. and Chilton, M.-D. (1983) MINI-Ti: a new vector strategy for plant genetic engineering. Bio/Technology 1, 266-275.

Fraley, R.T.; Rogers, S.G.; Horsch, R.B., et al. (1983) Expression of bacterial genes in plant cells. Proceedings of the National Academy of Sciences 80, 4803-4807.

Garfinkel, D.J.; Simpson, R.B.; Ream, L.W.; White, F.F.; Gordon, M.P. and Nester, E.W. (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell 27, 143-153.

Helmer, G.; Casadaban, M.; Bevan, M.; Kayes, L. and Chilton, M.-D. (1984) A new chimeric gene as a marker for plant transformation: the expression of Escherichia coli β-galactosidase in sunflower and tobacco cells. Bio/Technology 2, 520-527.

Hernalsteens, J.-P.; Van Vliet, F.; De Beuckeleer, M.; Depicker, A.; Engler, G.; Lemmers, M.; Holsters, M.; Van Montagu, M. and Schell, J. (1980) The Agrobacterium tumefaciens Ti plasmid as a host vector system for introducing foreign DNA in plant cells. Nature 287, 654-656.

Herrera-Estrella, L.; Depicker, A.; Van Montagu, M. and Schell, J. (1983) Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303, 209-213.

Herrera-Estrella, L.; Van den Broeck, G.; Maenhaut, R.; Van Montagu, M.; Schell, J.; Timko, M. and Cashmore, A. (1984) Light inducible and chloroplast-associated expression of a chimeric gene introduced into Nicotiana tabacum using a Ti plasmid vector. Nature 310, 115-120.

Hoekema, A.; Hirsch, P.R.; Hooykaas, P.J.J. and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature 303, 179-180.

Hooykaas, P.J.J. and Schilperoort, R.A. (1984) The molecular genetics of crown gall tumorigenesis, in Molecular Genetics in Plants, Ed. J. Scandalios, Academic Press, Inc., Orlando, FL, pp. 209-283.

Horsch, R.B.; Fraley, R.T.; Rogers, S.G.; Sanders, P.R.; Lloyd, A. and Hoffmann, N. (1984) Inheritance of functional foreign genes in plants. Science 223, 496-498.

Leemans, J.; Deblaere, R.; Willmitzer, L.; De Greve, H.; Hernalsteens, J.P.; Van Montagu, M. and Schell, J. (1982) Genetic identification of functions of T_L DNA transcripts in octopine crown galls. EMBO Journal 1, 147-152.

Leemans, J.; Shaw, C.; Deblaere, R.; De Greve, H.; Hernalsteens, J.P.; Maes, M.; Van Montagu, M. and Schell, J. (1981) Site-specific mutagenesis of Agrobacterium Ti Plasmids and transfer of genes to plant cells. Journal of Molecular and Applied Genetics 1, 149-164.

Matzke, A.J.M. and Chilton, M.-D. (1981) Site specific insertion of genes into T-DNA of the Agrobacterium tumor-inducing plasmid: an approach to genetic engineering of higher plant cells. Journal of Molecular and Applied Genetics 1, 39-49.

Nester, E.W. and Kosuge, T. (1981) Plasmids specifying plant hyperplasias. Annual Review of Microbiology 35, 531-565.

Paszkowski, J.; Shillito, R.D.; Saul, M.; Mandak, V.; Hohn, T.; Hohn, B. and Potrykus, I. (1984) Direct gene transfer to plants. EMBO Journal, in press.

Sciaky, D.; Montoya, A.L. and Chilton, M.-D. (1978) Fingerprints of Agrobacterium Ti plasmids. Plasmid 1, 238-253.

Van Haute, E.; Joos, H.; Maes, M.; Warren, G.; Van Montagu, M. and Schell, J. (1983) Integeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of the Ti plasmids of Agrobacterium tumefaciens. EMBO Journal 2, 411-417.

Van Montagu, M. and Schell, J. (1982) The Ti plasmids of Agrobacterium. Current Topics in Microbiology and Immunology 96, 237-254.

Williams, S.B. (1984) Protection of plant varieties and parts as intellectual property. Science 225, 18-23.

Yadav, N.S.; Vanderleyden, J.; Bennet, D.; Barnes, W.M. and Chilton, M.-D. (1982) Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proceedings of the National Academy of Sciences 79, 6322-6326.

Zambryski, P.; Depicker, A.; Kruger, K. and Goodman, H. (1982) Tumor induction by Agrobacterium tumefaciens: Analysis of the boundaries of T-DNA. Journal of Molecular and Applied Genetics 1, 361-370.

Zambryski, P.; Joos, H.; Genetello, C.; Leemans, J.; Van Montagu, M. and Schell, J. (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO Journal 2, 2143-2150.