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BIOTECHNOLOGY AND GENE TRANSFER: THE PROMISE AND THE PRACTICE

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ABSTRACT

It is possible to regenerate whole plants from cultured cells of many plant species. This regeneration capacity is the basis of a number of novel techniques which can be collectively termed biotechnology, and which are beginning to have a growing impact on plant improvement. Applications of biotechnology will be discussed in the context of plant improvement, with particular emphasis on gene transfer systems. In the past three years, functional gene transfer systems have been developed for several species, primarily as a result of progress with vectors based on *Agrobacterium tumefaciens*. The capabilities and limitations of these vector systems will be outlined briefly, and their potential impact on crop improvement reviewed.

KEYWORDS

Plant improvement, *Agrobacterium tumefaciens,* cell culture, molecular biology.

INTRODUCTION

The application of molecular biology and biotechnology to crop improvement has been the topic of a number of review articles (e.g. Barton and Brill, 1983), as well as the subject of numerous symposia (e.g. Kosuge *et al*, 1983; Gustafson, 1984). A number of private companies support large research groups in plant biotechnology, and a growing number of research institutes and departments specialising in plant molecular biology are becoming established in the public sector. Last year the International Society for Plant Molecular Biology held its inaugural congress in Georgia, which was attended by over 1500 researchers. This burgeoning interest is unlikely to decrease, in view of the recent identification, by the National Academy of Science, of plant biology as the research area with number two priority for the U.S. in the next decade. The reason for the excitement is certainly not the contribution plant molecular biology has made to crop improvement already. That contribution has been minimal, to put a polite face on it. Rather, the excitement reflects the promise of future contributions, as perceived by science administrators and corporate executives or their investors.

Molecular biologists are by nature perhaps the most arrogant species of biologist. They have been guilty in the past of claims that their techniques would revolutionise plant breeding, and of other perhaps more insulting remarks about breeders themselves. Plant breeders tend to be a much more realistic down-to-earth breed of researcher, not easily impressed by promises and loud claims. This attitude has doubtless been fed by other disciplines who have previously proclaimed they would revolutionise plant breeding, and failed. Nevertheless, plant breeding has quietly absorbed and utilised techniques and skills from a variety of other disciplines in its quest for improved plant varieties. It is becoming increasingly clear that, notwithstanding the innate varietal differences between molecular biologists and plant breeders, some form of intercourse between them would be advantageous to both parties. The relationship would be aimed at producing a hybrid variety of biologist, trained in aspects of both disciplines. I believe that the new variety would have an unusual degree of hybrid vigour in terms of yields for a plant breeding programme. Sessions such as these are intended to foster such intercourse between the disciplines by providing information and by stimulating thought and discussion as to how each discipline can best contribute to progress in crop improvement.

In this article I will first outline areas in which biotechnology in its broadest sense might be applied to crop improvement. The remainder of the article will deal with the application of gene transfer techniques to plant breeding. This is the area of biotechnology which I believe will have the greatest impaact. I will consider the following topics: an outline of gene transfer technology, illustrated by examples of genes that confer tolerance to the herbicide glyphosate (Monsanto's "Roundup"); problems remaining with gene transfer and the consequent numbers game that must be played; factors limiting the application of gene transfer to individual crops, and how the technology might be extended to apply to cereals; traits that are most likely to be manipulated, and criteria for their identification; and finally, the likely impact of gene transfer on plant breeding, and how breeders can best take advantage of the technology.

APPLICATION OF BIOTECHNOLOGY TO PLANT BREEDING

Biotechnology is a general term that encompasses many different techniques of cell culture and molecular biology. Table 1 indicates some specific areas where these techniques have already had or may soon have an impact on plant breeding. These areas are discussed below.

Table 1. Application of biotechnology to crop improvement.

Clonal propagation via tissue culture
rapid multiplication
disease-free propagation
cold storage of germplasm
Altered genetic constitution via culture
haploids
embryo rescue
artificial hybrids
cybrids
somaclonal variation/cell selection
gene transfer
Molecular screening procedures for breeding
disease testing
isozyme analysis
protein patterns
RFLP's

A technique central to many biotechnology applications is that of plant propagation by *in vitro* culture. The ability to rapidly and cheaply propagate large numbers of a superior individual has significantly changed breeding strategies in a number of ornamentals and other crops. In one sense, the procedure can be likened to making an F1 hybrid, in that it provides a mechanism for producing large numbers of a superior genotype. As our knowledge of propagation improves and the use of mechanisation increases, this technique is likely to be applied to more and more species.

As a consequence of propagation in culture, many plants become free of pathogens. The production of disease-free clones has become an important application of *in vitro* plant propagation, particularly for systemic viral infections in vegetatively propagated crops. A further application for *in vitro* plant propagation which may develop increasing importance is that of germplasm storage, particularly for vegetatively propagated crops where seeds or bulbs are not available. Recent successes with cryopreservation suggest that this will be a feasible procedure in the near future.

A second general area of biotechnology is the production of genetically altered varieties by means of tissue culture (Table 1b). The culture of haploid material and its application to barley breeding is considered by Dr Kasha's paper is to this symposium. Tissue culture can also increase the ability of breeders to make hybrids between somewhat distantly related species, either by embryo rescue (discussed by Ramming in this volume), or by protoplast fusion. A particular example of this latter technique is the exchange of cytoplasms without any nuclear genetic exchange, a process that has been termed the formation of cybrids. This procedure was utilised in the production of a herbicide-resistant rapeseed variety (Pelletier *et al*, 1983). Despite yield losses of 10-20% caused by the herbicide resistance gene, it has been incorporated into a cultivar which is currently grown on half a million hectares of Canada that have severe weed problems.

Under some conditions, the passage of plant cells through culture appears to be quite strongly mutagenic, giving rise to variants with a surprisingly high frequency. The application of this type of mutagenesis, for which the term somaclonal variation has been coined, is considered by several other papers at this symposium. For some plants, particularly vegetatively propagated species, this may be the most suitable type of mutagenesis, although more work is required to determine the optimal conditions and the types of mutation that arise. Cell culture also allows the possibility of direct selection for desirable variants (Conner and Meredith, 1986).

The final type of genetic change during tissue culture that I will mention is gene transfer, that is, the deliberate addition of one or a few genes to an existing variety via a tissue culture cycle. This subject will be considered in the remainder of this article. I should note that gene transfer in plants has also been accomplished using a viral vector (Brisson *et al*, 1984). Viral vectors can be applied directly to standing crops and do not require a tissue culture step. Although this is an important advantage, the use of viruses as vectors introduces its own set of problems which have not fully been overcome, and this approach will not be considered here further.

A general consideration of the application of biotechnology to plant improvement should also mention the use of molecular techniques as aids in screening plant populations (Table 1). The major techniques are protein and isozyme analysis and DNA hybridisation. Already these techniques have been utilised in screening for bread-making quality (discussed in several papers at this symposium), in testing for viroid and virus infections, and as markers linked to genes of agronomic importance. The use of hybridization probes and restriction fragment length polymorphism (RFLP) will become an increasingly important tool for genetic mapping, for testing genetic purity, and for identifying cultivars (see Tanksley 1983).

GENE TRANSFER: THE BASIC PROCEDURE

Successful gene transfer in plants was first demonstrated by three groups during 1983 and 1984. These groups made use of a naturally occurring soil bacterium, *Agrobacterium tumefaciens*, which has evolved the capacity to transfer some of its own genes to plant cells, thereby causing a disease known as crown gall. Molecular biologists have successfully subverted this gene transfer

capability and derived strains of *Agrobacterium* that will transform plant cells with any desired DNA (reviewed by Gardner and Houck, 1984). The first gene added to plants coded for resistance to kanamycin, a common antibiotic. This gene is of no agronomic importance whatsoever, but it allows the direct selection of plants that have received DNA from the *Agrobacterium*. A simple rapid procedure, called leaf disc cocultivation, has been developed for transformation of plants (Horsch *et al,* 1985). Small discs are punched out of a leaf, dipped in a solution of *Agrobacterium* containing the kanamycin gene, and then cultured on media that contains kanamycin in addition to hormones to induce shoot formation from the leaf disc. Plants regenerated from the leaf disc in this manner usually contain one or a few copies of the kanamycin gene added to the existing genome. Crosses show that the gene is inherited normally as a single dominant gene (Horsch *et al,* 1984; De Block *et al,* 1984).

This new gene transfer technology allows the precise addition of single genes to an existing plant genome. Once added, the new gene is inherited normally and so can be incorporated into a subsequent breeding programme. At present it is possible to add genes, but not to replace them; hence only dominant genes will affect a plant's phenotype. The procedure is simple, and takes only as long as one tissue culture cycle for the plant concerned. Recent results suggest that it may also be possible to add genes to the chloroplast genome, thus producing a gene that is maternally inherited (De Block *et al,* 1985).

A number of genes have been transferred between species using this technique. There are now several well-documented examples where such genes are expressed in their new hosts. The pattern of expression is usually similar to that of its host. For example seed-specific genes from bean and soybean remain as seed-specific when they are transferred into tobacco (Goldberg, pers. comm.; Marx, 1985). Similarly, a leaf-specific light-inducible gene from wheat is both leaf-specific and light-inducible in petunias (Lamppa *et al,* 1985). Where genes are not expressed following transfer to a new host, it has proven possible to obtain their expression by fusing them to the controlling regions of genes which are expressed in their hosts. Thus, in theory, it is now possible to obtain a desired level and pattern of expression for any gene from any organism in any host plant. In essence, the whole world becomes the available germplasm for transfer to any crop.

GENE TRANSFER: A HERBICIDE-RESISTANT EXAMPLE

To illustrate what I have described, and to give an example of a gene with some agronomic value, I would like to outline some recent research on the isolation and transfer of two genes that code for tolerance to glyphosate, a herbicide produced by Monsanto. Glyphosate is a broad spectrum contact herbicide that acts by blocking aromatic amino acid biosynthesis. The inhibition occurs at a single enzyme, EPSPS, which is an enzyme common to plants and bacteria (but is absent from animals, which lack the capacity to make aromatic amino acids).

One gene for glyphosate tolerance has been isolated by Comai and his co-workers at Calgene (Comai *et al,* 1983; Stalker *et al,* 1985; Comai *et al,* 1985). They showed that the bacterial EPSPS enzyme was also inhibited by glyphosate, and isolated a resistant variant of the enzyme by selection. Nucleotide sequencing showed that the resistant variant contains a single amino acid substitution compared to the wild-type, sensitive enzyme. Their strategy was to express this resistant enzyme in plants, so that when glyphosate is sprayed onto the plant and blocks the endogenous plant enzyme, the resistant bacterial enzyme is able to take over synthesis of the aromatic amino acids. Since the bacterial signals that control gene expression are very different from plant signals, they first engineered a gene fusion that spliced the coding region for the bacterial gene to plant signals. Once this engineering was done the chimaeric gene was transferred to *Agrobacterium* along with the gene for kanamycin resistance. Plant transformation was then achieved by selection for kanamycin resistance in leaf disc cocultivation. When transformed tobacco plants containing the chimaeric glyphosate resistance gene were sprayed with different concentrations of glyphosate, they displayed a tolerance level that was elevated two to three fold over control plants. This increased tolerance is well below the level of resistance that would be required for a field situation. However, Calgene believe that by directing their engineered protein to the chloroplast rather than the cyptoplasm of the cell, they will obtain useful resistance levels (Comai *et al* 1985).

A second gene for glyphosate tolerance has been isolated by Monsanto (Fraley, pers. comm.; Marx, 1985). They used glyphosate selection to isolate a petunia cell line with an elevated level of EPSPS enzyme, resulting from amplification of DNA containing the EPSPS gene. The over production of enzyme requires excess glyphosate to inhibit activity, and so the cells are resistant. Using over-production as a screen, they were able to clone the wild-type (glyphosate-sensitive) petunia gene. By fusing this sensitive gene to a high-level plant viral promoter, they obtained a chimaeric gene that overproduces EPSPS enzyme in plant cells. When this chimaeric gene was introduced to *Agrobacterium* and then to plants, the overproducing gene conferred glyphosate resistance at levels up to 10 times higher than background. This level of resistance still does not represent an acceptable level of field resistance. Two options are open to Monsanto to increase the resistance level: further overproduction using a different promoter, or development of a resistant enzyme similar to the strategy used by Calgene. It is probable that either approach will rapidly lead to a gene that is agronomically useful.

GENE TRANSFER: UNANSWERED QUESTIONS

While results to date have been very encouraging, a number of critical questions regarding gene transfer have

yet to be answered. Many of these questions impinge on the numbers game that must be played in gene transfer, namely, how many independent transformants must be grown in order to be certain of obtaining one with the desired characteristics.

One important requirement of a transferred gene is that it be stable. The DNA should be present and the expression of the gene should remain constant during subsequent generations. Results with gene transfer in animals has suggested that the stability of introduced genes can sometimes be much lower than that of "endogenous" genes, depending primarily on the method used for gene transfer. While it is clear that genes introduced via *Agrobacterium* are reasonably stable, careful long-term experiments have yet to be reported.

A second question regards the quantification of gene expression. Some variability between individual transformants has been observed in the rate of expression of the introduced gene. The basis for the differences are unclear, although they may well be due to "position effects" (i.e.) the surrounding linked genes affecting expression of the inserted DNA. Position effects have been elegantly demonstrated in *Drosophila,* both in the level of expression of the inserted gene and in its pattern of expression (Levis *et al,* 1985). The extent of this phenomenon in plants needs to be determined.

A third aspect of the numbers game about which little is known concerns the effect of the inserted DNA on surrounding genes. The additional DNA would undoubtedly disrupt any gene in which it was inserted. It remains to be tested whether expression of the inserted gene would also disrupt expression of neighbouring genes to any extent (a kind of reverse position effect). Fortunately plants contain vast amounts of "non-essential" DNA, so that this is unlikely to be too serious a problem.

A final consideration is the frequency with which the tissue culture step gives rise to somaclonal variants. The aim of gene transfer is to obtain an individual that is completely unchanged except for the addition of a single new gene. It is clear that the number of variants can be minimised by regenerating plants from organised explants rather than protoplasts or callus, and by keeping plant cells in culture for as short a time as possible (Scowcroft 1984). The extent of the problem is almost certain to vary between species and between different transformation conditions. If it proved to be impossible to avoid introducing additional undesirable mutations, a backcrossing programme could be used to remove them, in species where this is feasible.

APPLICATION OF GENE TRANSFER TO INDIVIDUAL CROPS

Two factors determine whether gene transfer can be applied to a particular crop. The most important one is the ability of the crop to be regenerated from culture. A second factor is the host range of *Agrobacterium,* which appears to be limited to dicotyledons and a few monocotyledons (including asparagus).

For crops that are hosts of *Agrobacterium,* the gene transfer procedure requires regeneration of plants from a leaf disc, or from some other tissue explant. Regenerations must occur from at or near wound sites, since only these cells are tranformed by *Agrobacterium.* A number of crops fulfil these requirements. These include tobacco, rape, clover, cotton, soybean, sunflower, many vegetables (including potato, tomato, asparagus, most Brassica's, lettuce), many ornamental species, and a few trees. In some species regeneration is still difficult and restricted to a few cultivars (e.g. soybean and cotton). Nevertheless, some degree of gene transfer is currently available, or soon will be available, in these crops.

The outlook for crops that are outside the host range of *Agrobacterium,* which includes the cereals, recently improved somewhat with the development of alternative procedures for gene transfer. Purified DNA of a kanamycin gene can be taken up by isolated protoplasts of wheat (Lorz *et al,* 1985), corn (Fromm, pers. comm.; Marx, 1985) and ryegrass (Potrykus *et al,* 1985). The kanamycin gene become stably integrated into the chromosomes in each case. More recently, microinjection of isolated tobacco protoplasts with purified DNA has also resulted in transformation (B. Miki, A. Crossway; pers. comm.). The end result in each case is essentially the same as for *Agrobacterium* transformation, namely the addition of a single new gene. However, so far all protoplast systems for cereals give rise to callus but not to plants. Hence it is not yet possible to obtain cereal plants which are transformed and can be used in a subsequent breeding programme.

Several approaches have been suggested in order to overcome this barrier to gene transfer in the cereals:

- Further manipulation of culture conditions may result in cereal protoplast regeneration into plants. A large amount of basic research is needed and I believe should be undertaken, in order to determine the factors which affect the capacity of plant cells to divide and diffentiate in culture.
- The ability to regenerate could be bred into modern cereal cultivars from wild species that possess this capability.
- Research into the basis of the host range limitation of *Agrobacterium* may enable the host range to be extended to cereals. Regeneration from certain explants is possible for many cereal cultivars, even though protoplast regeneration has not be obtained.
- Transformation of pollen cells, for example by microinjection, would allow gene transfer to occur within the normal sexual cycle and bypass the regeneration problem altogether.

Until one or more of these pathways has been established, gene transfer in cereals will have to remain but a gleam in the eyes of the molecular biologists.

SINGLE GENES FOR CROP IMPROVEMENT

Given that the capability for transfering single genes

already exists in some species and should soon be available in others, what traits can be manipulated using this technology? It is clear that the most important traits that plant breeders deal with are affected by large numbers of genes. Nevertheless, plant breeders have identified and used a growing number of single genes in their breeding programmes. These include genes for cytoplasmic male sterility, dwarfing, earliness, disease resistance, and a number of quality characteristics. Thus there are a number of traits which are potential targets for improvement by genetic engineering of single genes.

The shortage of available genes is currently a major factor limiting the application of gene transfer to crop plants, and has two components. The first is the lack of *cloned* plant genes. Cloning the gene is a prerequisite to transferring it, and comparatively few plant genes have been cloned to date. Because of the relatively fast pace at which molecular biology moves, and because of the widespread recognition of this problem, I believe the lack will be largely overcome within a few years. The second component is perhaps more serious, and that is the lack of basic information about biochemistry, physiology and gene action in plants. There are many cases where it may be possible to define a useful trait which could be attacked via gene transfer, but it is very difficult to identify the gene which would confer the desired phenotype. I believe that this is a much more serious long-term problem. An additional aspect of the lack of information about plant biochemistry and gene action is the difficulty of predicting whether a particular gene will function in a new host. Most of the single genes manipulated by plant breeders are part of complex biochemical or developmental pathways that are not well understood. The functioning of one of these genes across species barriers would require that the new host also possess the unknown pathway. The usefulness of many of these genes may be a matter of trial and error, highly influenced by the effect of the genetic background into which it is inserted.

Despite these difficulties, a number of genes have been cloned that confer or have the potential to confer useful phenotypes. Several of these single genes confer their phenotype by the independent action of their gene product, and are thus likely to be free from the background effects considered above. The glyphosate resistance genes described above is probably closest to application. Other herbicide resistance genes have been identified, and in the case of atrazine and possibly chlorsulfuron, have been cloned. The atrazine resistance gene has been reported to confer partial tolerance when reintroduced to plants (Bogorad, pers. comm.; Marx, 1985). Another gene which has been cloned and transferred to plants, but does not yet confer a useful phenotype is the *Bacillus thuringiensis* toxin. This bacterial gene codes for a protein which is toxic to many insects. It is hoped that expression of this gene in plants will provide insect resistance. However, currently the level of expression of the toxic protein in plants is not high enough to kill insects which eat the leaves (Barnes, pers. comm.). Another suggestion for a useful gene is the idea of obtaining viral resistance by expressing in plants antibodies against the plant virus. Applied *in vitro,* the antibodies block viral infectivity, and the genes would be readily available from rabbits or goats for transfer to plants.

Another area of immediate interest for providing useful genes comes from experiments in bacteria and animals. Expression of a backwards copy of a gene produces an antisense RNA, which can specifically block expression of the wild-type (forwards) copy of the same gene (Weintraub *et al,* 1985). The procedure has not been tested yet in plants. However, an obvious application would be to use a backwards copy of an essential plant viral gene, many of which are cloned and available in order to specifically shut off replication of the virus. The procedure would also be useful to block certain biochemical pathways, once the genes have been cloned.

A recent paper (Nasrallah *et al,* 1985) reported the isolation of an S incompatibility gene from *Brassica.* These genes have been used in brassicas as a means of producing hybrid seed. The use of gene transfer to manipulate this cloned gene may provide new approaches to hybrid seed production, both in brassicas and perhaps in other species.

IMPACT OF GENE TRANSFER ON CROP IMPROVEMENT

The recent development of techniques for gene transfer is likely to result in some changes in plant breeding. The principal strength of plant breeding is the ability to analyse thousands of combinations of genes and select superior combinations to get elite cultivars. Gene transfer clearly will not replace this technology, since it deals primarily with single genes. In my view it represents a kind of genetic fine tuning tool which can be used to improve existing cultivars. Its most important attribute is the greatly enlarged gene pool it opens up, since genes from any source can be utilized, and manipulated very precisely. I believe it will prove superior to traditional backcrossing methods for the introgression of a new gene from a wild relative or for moving genes between two existing cultivars, since it precisely moves one single gene without any other linked genetic material. It also offers a method for making new gene combinations. For example, it would be possible to "load up" a cultivar with many alleles of a disease resistance loci.

In the longer term, the most significant development of gene transfer is likely to be in our understanding of genes and gene action. Most plants contain in the order of 100,000 genes. Many of these are "housekeeping" genes, with a relatively small proportion having a drastic impact on phenotypes that we are interested in. I believe that in ten or twenty years, genes for many of the traits controlled by multiple genes will have been cloned, and their mode of action understood in molecular and biochemical terms. In contrast to most physiological studies of plant breeding, which have so far succeeded only in producing after-the-fact descriptions of how plant breeders have produced a new cultivar, I believe that an increased knowledge of gene

action will have great predictive value for plant breeders.

Thus far, the sole impact of gene transfer has probably been to force plant breeders to listen to numerous seminars expounding the virtues of molecular biology. However, I would be surprised if the next year or two did not see the incorporation of genetically engineered herbicide resistance genes into the breeding programmes of some easily regenerated dicotyledon crops. These herbicide resistance genes will likely be the first of an increasing number of genes that will become available, probably under license, for incorporation into crops.

How might plant breeders be best advised to make best use of these advances? The key initial step would be to develop, or to develop access to, a tissue culture system that allows regeneration of plants for their best cultivars or breeding lines. They should also give some thought to what genes or traits might be useful in their particular crop, without in any way limiting themselves to their presently accessible germplasm. They should then keep in contact with the scientific and business community to become aware of these new genes as they are made available.

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SYMPOSIUM DISCUSSION

Mr G. Pringle, Division of Horticulture & Processing, DSIR

How do you select the gene for transfer from one plant to another?

Gardner

There are a number of ways. The protein can be purified to the extent that you can raise antibodies or sequence the protein and then can go after the gene. If there is good physiological evidence about when a gene is induced or not induced, where it is or is not turned

on, then it may be possible to use differential hybridisation to get the gene. It is becoming possible, particularly in corn, to get genes through simple genetics — if you have a phenotype and a mutant, although you need a transposon.

Dr R.J. Brawn, Seed Consultant, N.Z.

Would you care to comment on the transfer of naked DNA to the pollen tube? Does that have any prospect in cereals?

Gardner

I do not believe that the evidence in the literature establishes that naked DNA can be transferred into pollen. One option for cereals may be micro injection of pollen. A needle can be put in and out of pollen and the pollen can still germinate, although it is not yet clear that the nucleus can be hit and DNA extracted. If that would work it would avoid the whole tissue culture problem.