ADVANCES IN TRANSFORMATION TECHNOLOGY FOR VEGETABLE *BRASSICA*

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Summary

Although transformation of *Brassica* vegetables has lagged behind similar work with rapeseed, transgenic cauliflower, broccoli, cabbage, and kale plants have been obtained by several methods. The most general approach is use of *Agrobacterium tumefaciens*. A U.S. patent for *A. tumefaciens*-mediated transformation of *Brassica*, including *B. oleracea*, was recently granted to Calgene, Inc. Transformation via *A. rhizogenes* is another option, but the plants recovered may show abnormal phenotypes. Direct DNA update into protoplasts, induced either by polyethylene glycol or electroporation, has also succeeded. Biolistic approaches have not yet played an important role in vegetable *Brassica* transformation. Even the successful procedures are still not routine, and transgenic plants are usually recovered from fewer than 10% of explants transformed. Control of ethylene and moisture levels in culture plates are among the factors that can increase efficiency. Transformants are most often selected by resistance to kanamycin or hygromycin. Other genes introduced include B-glucuronidase, genes for resistance to herbicides, an S-locus gene, and insecticidal protein genes from *Bacillus thuringiensis* (Bt). When a modified CryIA(c) Bt gene was introduced into broccoli and cabbage, about 70% of ca. 250 transformants recovered were resistant to diamondback moths, a major pest of crucifers. Progeny of some of these transgenic plants are now being used in tests of insect resistance management strategies involving refuges. Additional transgenic vegetable *Brassicas* with enhanced resistances or other horticultural improvements are likely to be available soon, but regulatory issues will delay their commercial release.

Keywords: *Agrobacterium tumefaciens, Bacillus thuringiensis, Brassica oleracea, Plutella xylostella*, transgenic

1. Introduction

Genetically altered crops, into which new DNA sequences have been introduced, are moving out of the lab and into the field, at least in the U.S.A. Some have already appeared in supermarkets and kitchens; Calgene's Flavrsavr tomato, with its extended shelf life, is now available in some U.S. cities. Transgenic squash that is virus-resistant has recently been approved, and transformed potato, cotton, soybean, and cotton are likely to be next.

Brassica crops have received attention too. A patent on transformation of *Brassica* species was recently awarded to Calgene, Inc. (U.S. Patent Number 5,188,958, 23 February, 1993). Most of the work on *Brassica* transformation has dealt with *B. napus* rapeseed because of its economic importance. The 8th Crucifer Genetics Workshop, held in Saskatoon, Canada in 1993, included a tour of the field tests of herbicide-resistant rapeseed developed by several companies. Another major area of work is manipulation of oil quality. The ratios of saturated:unsaturated C_{18} fatty acids have been altered (Knutzon et al., 1992) in order to tailor products for specific markets, such as margarine that does not require hydrogenation. Rapeseed has also been engineered to produce short chain fatty acids (C_{12} laurate) normally obtained from tropical plants (Voelker et al.,

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1992). Male fertility (Mariani et al., 1990), storage proteins (Altenbach et al., 1992), and glucosinolate levels (Chavadej et al., 1994) have been other targets for manipulation. Some transgenic rapeseed materials are already in advanced breeding trials, and commercial use seems likely.

There are fewer published reports of transformation of the *B. oleracea* vegetables, for several reasons. Efforts have been less focussed because there are several quite different crops to work with. It is also less clear which traits are of high enough value to warrant modification, in view of the effort and expense of getting the required regulatory approvals. Nevertheless this area of work deserves serious consideration; these vegetables are widely grown and are increasingly popular because of their nutritional and anti-cancer value (Zhang et al., 1992). Some transgenic plants have been recovered from each of the major *B. oleracea* vegetables (broccoli, cauliflower, cabbage, and kale); however, the emphasis has usually been on development of the transformation technique rather than interest in a specific new phenotype.

2. Transformation of *Brassica* vegetables

2.1. Transfer systems used

Several types of transformation systems have been used. As with most dicot plants, procedures involving the crown gall bacterium, *Agrobacterium tumefaciens,* are the most common. This is the method for which Calgene has obtained a patent. *A. tumefaciens* has been used to obtain transgenic plants of cauliflower (Srivastava et al., 1988; De Block et al., 1989), cabbage (Bai et al., 1993; Metz et al., 1994), broccoli (Toriyama et al., 1991; Metz et al., 1994; Lee et al., 1994), Chinese kale (Toriyama et al., 1991), and rapid cycling *B. oleracea* (Millam et al., 1994).

Several groups have chosen *A. rhizogenes* (the "hairy root" bacterium) rather than *A. tumefaciens* as a vector and have used it to produce transgenic plants of cauliflower (David and Tempé, 1988), kale (Hosoki et al., 1989; Christy and Sinclair, 1992), and rapid cycling cabbage (Bethomieu and Jouanin, 1992). The disadvantage of this method is that the plants recovered often show abnormalities related to transfer of the *rol* gene from the bacteria. This has been overcome in some cases, but is a concern. Small field tests of the transgenic kale have been conducted in New Zealand (Christey and Sinclair, 1993).

Introduction of isolated DNA into protoplasts via polyethylene glycol (PEG) treatment or electroporation is an alternative to *Agrobacterium*-mediated transformation, provided an efficient system for regeneration from protoplasts is available. In this way, Mukhopadhyay et al. (1991) recovered 159 transgenic plants from cauliflower hypocotyl protoplasts.

Particle gun bombardment of explants or cell cultures is particularly useful for transient assays aimed at study promoter activity, but recovery of transgenic *Brassica* vegetables by this method has not yet been reported. Introduction of DNA into seeds has been a successful transformation method for another crucifer, *Arabidopsis thaliana* (e.g., Castle et al., 1993), but whether it would be effective with the larger seeds of *Brassica* materials is not clear. Various "novel" systems, such electroporation of DNA into shoot tips of intact plants (Chowrira et al., 1994), have been effective for some crops and may eventually have applications in *B. oleracea* as well.

2.2. Variables in *A. tumefaciens*-mediated transformation

In the *A. tumefaciens* system, many plant-related variables have been examined, including tests of different genotypes, different types of explants, and different nutrient media for regeneration. Although seedling parts, such as hypocotyls, cotyledons, and cotyledonary petioles, are the most common explants, pieces of flowering stalks also regenerate well (Christey and Earle, 1991). Flowering stalk explants are less convenient

to obtain and more subject to contamination, but do have some advantages, particularly when the supply of seeds of a particular genotype is limited. They also yield a higher percentage of diploid regenerated plants than seedling explants (Metz, 1994).

Other relevant variables are whether to preculture explants prior to exposure to the bacteria and how to control the ethylene and humidity levels in culture plates. Some groups have reported that it is essential to add silver nitrate to the medium to control ethylene (De Block et al., 1989; Mukhopadhyay et al., 1991), but we have not found it very helpful. However we have noted that sealing plates with a porous tape rather than Parafilm gives better results by reducing humidity and probably also by preventing build-up of ethylene. Both regeneration and transformation are enhanced through use of a porous seal (Metz, 1994).

The *Agrobacterium* part of the system also has many possible variables. These include the choice of *Agrobacterium* strain and the specific constructs (plasmids, promoters, genes, etc.), the induction of T-DNA transfer (through use of feeder layers, addition of acetosyringone, etc.), and the interaction of the explants with the bacteria (concentrations of bacteria applied, length of exposure to *Agrobacteria* before and after transfer to culture medium, etc.). In our experiments, transformation of broccoli and cabbage was improved when explants were cultured on filter paper over a feeder layer of tobacco cells after inoculation. Regeneration of seedling and flower stalk explants was inhibited by the feeder layer, but transformation frequency was increased (Metz, 1994).

Additional experimental options involve the selective agent (type, level, and timing). Kanamycin has been mostly widely used, generally at concentrations of about 25 mg/l, substantially less than the level used with Solanaceous plants such as tobacco and tomato. Hygromycin has also worked well (Mukhopadhyay et al., 1991; Millam et al., 1994). A initial period of co-culture in the absence of selection can increase recovery of transformants once selective pressure is applied (Toriyama et al., 1991)

Transformation of *Brassica* vegetables is not yet routine. Published values for percentage of explants giving rise to at least one transformed plant range from <5% to 30%. Because of the many variables (especially those related to plant materials and *Agrobacterium* strains or gene constructs with limited access), it is difficult to reproduce published protocols exactly. Some groups have had little success in spite of serious efforts, but failures are rarely reported. Thus further identification of the key variables will be an important contribution to transformation of these crops.

2.3. Types of genes transferred

Often only the gene for the selectable marker and/or the B-glucuronidase reporter gene were transferred. *Bar*, *pat*, or *als* genes encoding resistance to herbicides have sometimes been used (De Block et al., 1989; Mukhopadhyay et al., 1991; Christey and Sinclair, 1992; Lee et al., 1994), as in rapeseed. Transfer of an S-locus gene related to self-incompatibility has altered this phenotype (Toriyama et al., 1991). Genes encoding *Bacillus thuringiensis* (Bt) insecticidal crystal proteins have conferred insect resistance (Bai et al. 1993; Metz et al., 1994).

Various additional types of genes are being used in current experiments at Cornell and elsewhere. They are aimed at goals such as increasing resistance to various pests, improving storage characteristics through control of ethylene or hormone metabolism, or controlling male fertility (by altering pollen-stigma interactions or by inducing pollen breakdown via genes related to cytoplasmic male-sterility).

There is no shortage about other attractive ideas. It should be possible to make new types of vegetables and to enhance their nutritional qualities or resistance to stress. Moreover, antibodies or other valuable pharmaceuticals can be produced by "molecular farming" in some transgenic crops, including rapeseed (Pen et al., 1993). Cabbage might be attractive material for chemicals to be produced in leaves, rather than seeds.

The many current projects on isolation of agriculturally important genes from the small genome of *Arabidopsis thaliana* may have particular benefits for *Brassica*

materials. For example, *Arabidopsis* mutants resistant to crucifer pathogens and pests can be selected as starting material for isolation of resistance genes. Once such genes are available and have been confirmed in *Arabidopsis*, they could be transferred into the *Brassica* vegetables.

3. Transformation of *Brassica* vegetables with a Bt gene

Our current work on introduction of a Bt gene into *B.. oleracea.* offers a specific example of transformation of *Brassica* vegetables. The goal of this work is to develop and utilise a model system for study of strategies to delay insect resistance to the Bt insecticidal proteins. This issue must be addressed before Bt-transgenic plants are widely deployed because several insect species have already evolved such resistance (McGaughey and Whalon, 1992). The target insect in our work is *Plutella xylostella* (diamondback moth), the only insect in which highly resistant forms have already been recovered from fields exposed to Bt sprays (Tabashnik et al., 1990; Shelton et al. 1993). The diamondback moth is also a major pest of crucifers worldwide.

3.1. Recovery and analysis of Bt-transgenic plants

For this work, Monsanto kindly provided us with a construct carrying a CryIA(c) gene extensively modified for good expression in plants (Perlak et al., 1991) under control of a constitutive promoter (similar to 35SCaMV), as well as the *npt*II gene for resistance to kanamycin. This construct was used in *A. tumefaciens*-mediated transformation of five broccoli lines (including Green Comet hybrid), King Cole cabbage, and rapid cycling *B. oleracea* . The initial experiments used flowering stalk explants and a modification of the method of Toriyama (1992), but seedling hypocotyls and cotyledons also worked well after some alterations of the procedure (Metz, 1994). Rooted transgenic plantlets could be obtained in as little as 3 months.

Materials were confirmed as transgenic in several ways. Green shoots that formed in plates of regeneration medium containing 25 mg/l kanamycin were transferred to fresh selective medium in baby food jars. Transformants grew vigorously and formed roots, while most escapes did not. For further distinction between transformants and rooted escapes, small leaf pieces were placed on medium containing 50 mg/l kanamycin (Fry et al., 1987). Leaves from transformants remained green, enlarged, and formed roots; leaves from non-transformed controls and escapes bleached and failed to grow. Integration of the *npt*II gene and the Bt gene was demonstrated with Southern hybridization, and lines with single or multiple insertions of the Bt gene were identified. More than 250 transgenic plants were obtained (Metz, 1994; Metz et al., 1994).

A question of great interest was what would happen when larvae of Bt-susceptible diamondback moths were placed on the transgenic plants. Leaves of non-transgenic control plants were severely damaged by 1st instar larvae within a few days. In contrast, about 70% of the kanamycin-resistant transgenic plants showed virtually no insect damage. Larvae on these plants failed to develop and died.

The obvious next question was how the plants would interact with Bt-resistant diamondback moths or F_1 hybrids from crosses between resistant and susceptible moths. To test that, 30 transgenic broccoli plants were assayed with all three types of insects. Both the susceptible and the F_1 insects died on the plants, but the field-selected resistant ones survived. So expression of Bt in the transgenic plants was not high enough to control the resistant insects. The ability of the transgenic plants to kill the F_1s was as expected since insect resistance to Bt is a recessive trait (Tabashnik et al., 1992). Because our transgenic plants give a differential response to the resistant and susceptible insects, they provide an excellent model system for studies of some types of resistance management schemes.

3.2. Recovery of progeny

Insect-resistant broccoli plants with a single insertion of the Bt gene were grown to flowering in order to obtain progeny for replicated tests of resistance management strategies. The plants used came from explants taken from cytoplasmic male sterile lines, so they were pollinated with fertile non-transformed controls. To identify the progeny that were resistant, we sprayed the seedlings with kanamycin to select the bleached non-transgenic ones (Weide et al., 1989) and also assayed with diamondback larvae. The correlation between resistance to kanamycin and resistance to insects was very high. The progeny recovered segregated 1:1 for resistance, as expected. From these efforts, large numbers of resistant progeny heterozygous for the Bt gene were obtained for further use.

3.3. Resistance management studies

The resistance management strategies currently being tested in greenhouse experiments at Cornell involve planting transgenic resistant plants together with some susceptible plants that can provide refuges for susceptible insects. If susceptible insects are available to mate with any resistant ones, the F_1s will be controlled by the transgenic plants, because insect resistance to Bt is recessive. Keeping resistance alleles in the heterozygous condition should slow development of populations of resistant insects. (In our experiments, plants were initially exposed to insect populations containing a small percentage of resistance alleles).

The specific questions we are addressing include the optimal percentage and location of the susceptible plants. One approach is to plant the susceptible plants outside the crop. Alternatively, seeds of the resistant and susceptible plants could be mixed, so that the two types of plants will be close to each other. Computer models have indicated that a pure stand of resistant plants would lead to very rapid development of resistance while plantings with refuges of susceptible plants would greatly delay resistance (Mallet and Porter, 1992). Such models have also suggested that there may also be differences between outside refuges and seed mixtures, but no experimental data on these issues are available. The greenhouse tests in progress are providing such data on changes in the level of resistance in the diamondback moth population under various arrangements of transgenic and susceptible plants.

Refuges are not the only possible strategy for management of insect resistance to Bt. Other concepts include use of multiple resistance genes, either within one plant or in separate plants in the population, and Bt genes that are expressed only in specific plant parts or developmental stages or only when insect pressure is high (McGaughey and Whalon, 1992). We are now working to obtain these types of transgenic broccoli plants. A further area of interest is the extent to which environmental and developmental factors affect expression of Bt genes under control of "constitutive" promoters.

4. Concluding remarks

Our model system of transgenic broccoli and diamondback moth populations is providing information relevant to appropriate deployment of Bt-transgenic crops, including non-*Brassica* ones that industry plans to market soon (e.g., cotton). It thus makes use of transgenic *Brassica* vegetables to help maintain the important resource of insect susceptibility to Bt. Similar types of work with other mechanisms of insect resistance (e.g., transfer of genes for proteinase inhibitors toxic to insects) can also be envisioned. Ability to insert "foreign" genes or anti-sense versions of *Brassica* genes will undoubtedly lead to better understanding and control of a broad range of other developmental and biochemical characters as well.

Although transformation can produce materials for basic studies of many types, the prospects for commercial use of transgenic *Brassica* vegetables are less clear. Substantial funds are required not only for research and development, but also for

obtaining the needed regulatory approvals, dealing with legal and environmental issues, and convincing the public that these materials are safe and desirable. Which improvements would give a good return for the high costs involved? Participants at the 9th Crucifer Genetics Workshop had few suggestions to offer, other than "make broccoli taste more like a cheeseburger". This hesitation to volunteer ideas may reflect caution about revealing work in progress. It may, however, indicate that conventional breeding methods or transfer of desirable genes from crucifer relatives are still strong competitors to transgenic approaches. The 10th Crucifer Genetics Workshop will provide a good opportunity for further evaluation of the role of transgenic plants in *Brassica* vegetable improvement.

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