Biotechnology of Vegetable Crops

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It is now a matter of routine to transfer exogenous genes to many dicotyledonous plants using Ti plasmids derived from *Agrobacterium tumefaciens*. Experiments are frequently carried out first with tobacco to test the feasibility of an idea, since genetic manipulation of this species is rapid and reproducible. However, it is now relatively simple to transform potato, tomato (Fig. 1), and many other commercially important plants. Methods are rapidly being developed also for genetic transformation of monocotyledonous species, and we can expect many developments in this area in the near future.

The simplest type of genetic engineering involves the production of a transgenic plant that expresses a foreign gene in many different organs and tissues throughout the life cycle. This so-called constitutive expression is suitable for genetic engineering of single-gene resistances to, for example, insects, viruses, or herbicides. Transcription promoters, such as the CaMV 35S promoter (from cauliflower mosaic virus), are frequently used to give this type of expression pattern. However, it may sometimes be necessary to arrange for cell-specific or developmental regulation of added genes to obtain expression in, for example, the epidermis or in fruits, seeds, tubers, or flowers. In addition to adding new genes, it is also possible to reduce or even abolish the effect of undesirable existing genes using antisense RNA technology. These developments open up the prospect of being able to modify plant performance or quality by the addition or subtraction of specific characters. Eventually, it will be possible to modify complete developmental programs in plants. In this article, I review some of the successes achieved by this new technology and discuss possible future developments that may have an impact on vegetable crops.

SINGLE GENE RESISTANCES

Herbicide resistance

The criteria for an ideal herbicide are that it should 1) have no toxicity toward humans, animals, or soil organisms and 2) kill selectively weeds and not crop species. Unfortunately, many herbicides effectively kill crop plants and weeds. Genetic engineering has enabled a new approach to this area by making it possible to confer on crop plants resistance to specific herbicides that may then be used to kill competing weeds.

Herbicides generally work by inhibiting an essential metabolic reaction (Comai and Stalker, 1986). Examples of this area: 1) glyphosate, which inhibits an enzyme (EPSP synthase = 5-enolpyruvylshikimic acid-3-phosphate synthase) that catalyzes a specific step in aromatic amino acid biosynthesis, 2) sulfonylurea and imidazolinone herbicides, which inhibit an enzyme (acetolactate/acetohydroxy acid synthase) required for the synthesis of branched chain amino acids, and 3) bialaphos and phosphinothricin, which kill plants by preventing detoxification of ammonia by the enzyme glutamine synthase. The generation of transgenic plants resistant to these herbicides can be achieved either by 1) transferring a gene enabling plants to synthesize an enzyme not inhibited by the herbicide to catalyze the essential metabolic step, or 2) adding a gene that directs the synthesis of an enzyme that detoxifies the herbicide, thus preventing it from inhibiting its target (Fig. 2). The former strategy has been used to produce plants tolerant to glyphosate, sulfonylureas, and imidazolinones by identifying genes for the appropriate enzymes in bacteria or higher plants that are resistant to the herbicides, by tailoring, where necessary, the genes to be expressed in higher plants, and by using Ti plasmids to transfer the resistance genes (Stalker, 1990). Examples of the second approach

include the transfer to higher plants of the bar gene from *Streptomyces hygroscopicus*, conferring on transgenic plants the ability to acetylate and thus detoxify phosphinothricin (De Block et al., 1987), and the generation of plants expressing a bacterial nitrilase that renders them resistant to bromoxynil (see Stalker, 1990).

Virus resistance

Three approaches have been used in attempts to engineer virus resistance. The first method aims to exploit the phenomenon of cross protection, where infection of a plant with a virus strain producing mild symptoms often renders the plant resistant to a subsequent infection by a related strain of virus that normally causes severe symptoms. This is believed to be due to inhibition of the effectiveness of the virus by the coat protein derived from the mild strain. It has been shown that transgenic plants expressing virus coat protein genes alone show some resistance to infection with intact virus particles (Fig. 3) (Buck, 1990; Nelson et al., 1988; Powell et al., 1986). The virus coat protein produced by the transgenic plants appears to block a receptor in the plant cell that is required for uncoating the virus nucleic acid, or to interfere with some other function important for the replication or expression of virus genes.

A second approach to engineering virus resistance has centered on satellite RNAs. These are small RNA molecules that sometimes accompany virus particles. They are not essential for virus function but, when present in plant cells, are replicated together with the virus and encapsulated by the virus coat protein. It has been shown that expression of satellite RNAs in transgenic plants can reduce symptoms of tobacco ringspot virus and cucumber mosaic virus (Gerlach et al., 1987; Harrison et al., 1987). The molecular mechanism whereby satellite RNAs bring about this effect is unclear. Caution must be exercised in the use of this approach, since satellite RNAs are known to reduce or exacerbate symptoms, depending on their detailed structure. Thus, expression of certain satellite sequences in transgenic plants could, under some circumstances, be detrimental. Attempts to generate virus resistance by producing transgenic plants that express antisense virus RNA (see below) have not thus far proved as successful as the former methods. This may be because current strategies are unable to prevent the virus RNA

Fig. 1. Transferring genes to tomato plants using *Agrobacterium* Ti plasmids. DNA sequences located within the T-DNA of the plasmid are transferred to the nucleus of plant cells and integrated in one or more of the chromosomes. Generally, a selectable marker gene for antibiotic resistance is added with the DNA to be transferred.

Transgenic plants resistant to herbicides

Fig. 2. Strategies for generating herbicide-resistant transgenic plants by adding a gene encoding a detoxifying enzyme (B) or a resistant enzyme (C).

from replicating itself and overcoming the effect of antisense RNA by virtue of its higher concentration.

Insect resistance

Transgenic plants have been generated that express specific proteins that deter or kill feeding insects. The most celebrated example of this strategy is the expression in plants of genes encoding specific proteins from *Bacillus thuringiensis* (Fischhoff, 1989; Fischhoff et al., 1987). These proteins are not harmful to mammals but kill insects by interfering with ion transport in the midgut. Different types of protein are available that selectively kill specific groups of insect, raising the possibility of producing plants resistant to particular pests.

Plants also have endogenous defense mechanisms against insect attack. Several plants have been shown to synthesize systemically protease inhibitors in response to wounding (Ryan, 1978). These proteins are believed to provide some defense against feeding insects; also, recent work has shown that cowpea seeds containing a high concentration of chymotrypsin inhibitor are resistant to insect attack (Hilder et al., 1990). Transgenic tobacco plants have been generated that express constitutively different amounts of the cowpea seed chymotrypsin inhibitor. Feeding experiments have shown that there is a direct relationship between the amount of chymotrypsin inhibitor produced and the degree of protection afforded against a range of insects.

DEVELOPMENTAL REGULATION

It is now quite clear that plant development and differentiation involve the regulated expression of specific genes. There are at least three aspects to this developmental regulation. First, *cis*-acting DNA sequences in the vicinity of a gene contain information that determines its expression at a particular stage of development, in a specific organ or tissue, or in response to a particular signal. Second, regulator genes located elsewhere in the genome encode proteins (*trans*-acting factors) that interact with these *cis*-acting DNA regions to regulate expression of the neighboring genes. Third, other genes

Transgenic plants resistant to viruses

Fig. 3. Diagrammatic representation of the role of virus coat protein expressed in transgenic plants in reducing symptoms of infection by virus particles.

affect, in a broad sense, one or more aspects of the developmental program by controlling the synthesis of, or response to, hormones and other regulators. An example of the latter type of control comes from studies by Keller et al. (1989), who, by overexpressing an oat phytochrome gene in tobacco plants, caused a dramatic change in the appearance and growth of the plant.

Promoters governing differential gene expression

When entire plant genes, including introns, exons, and upstream (5') and downstream (3') sequences, are transferred to other plants, they frequently retain their normal pattern of developmental regulation in their new host. This feature indicates that the transferred gene contains information (*cis*-acting sequences) governing its own expression, and *trans*-acting factors in the host plant can recognize this information and regulate expression of the gene accordingly. By testing the role of different portions of the gene in transgenic plants, it has been shown that sequences to the 5' side of the transcription start site frequently determine the developmental pattern of expression. There are also indications that sequences within an intron or to the 3' side of a gene may occasionally modulate expression.

Promoters governing developmental regulation have been identified for storage protein genes expressed specifically in seeds (Shirsat, 1991), for the small subunit of rubisco and other photosynthetic proteins expressed in green organs and tissues in the light (Jenkins, 1991), for the potato storage protein patatin expressed predominantly in tubers (Bevan, 1991; Willmitzer et al., 1990), and for many other genes expressed in various parts of flowers (Smith et al., 1990a) in response to heat shock (Schöffl et al., 1990), wound-

ing (Stanford et al., 1989), and hormones (Huttley and Baulcombe, 1990). In addition, specific amino acid-coding regions of genes have been shown to target their protein products to different sub-cellular locations (Bennett and Osteryoung, 1990; Robinson, 1990). Thus, molecular biology is providing a gene "tool kit" of DNA sequences that can be used to cause a foreign gene to be expressed at a specific stage in the life cycle, in a particular cell type, and with the protein located in the appropriate cell compartment.

One promoter that may have an application in the genetic engineering of horticultural crops has been obtained from the tomato polygalacturonase (PG) gene. PG is an endo-acting polygalacturonic acid hydrolase that is synthesized specifically during ripening of some fruit. The enzyme functions to degrade the pectin fraction of the fruit cell walls and, as discussed below, may play a role in softening. The gene for tomato PG has been entirely sequenced, and a 1450 base pair fragment (the PG promoter), located immediately upstream from the coding region, has been shown to contain genetic information determining ripening-specific expression. This was demonstrated in an experiment where the PG promoter was fused to the bacterial chloramphenicol acetyl transferase (CAT) gene and the chimeric gene transferred to tomato plants using a Ti plasmid (Bird et al., 1988). The results showed that the PG promoter caused the CAT gene to be recognized in the transgenic plants and regulated as if it were a ripening gene (Fig. 4). This result supports the idea that promoters from the PG gene, and other developmentally regulated genes, could be used to express foreign genes in particular plant organs at specific stages in development. It should also be possible to rearrange the expression pattern of existing plant genes by changing their promoters. Thus, it is feasible to consider altering by genetic engineering the color, flavor, texture, aroma, nutritional value, or storage life of horticultural produce. The main requirement to accomplish this successfully is to understand sufficiently plant physiology and biochemistry to enable identification of the appropriate genes.

ANTISENSE GENES

Recently, it has been shown that endogenous plant genes can be inhibited very effectively by antisense genes. This was first shown by van der Krol et al. (1988), who used a chalcone synthase antisense gene to inhibit pigment production in the flower petals of transgenic petunia plants. Shortly after, it was reported that expression of the tomato PG gene during ripening could be inhibited by as much as 90% by a single antisense gene (Sheehy et al., 1988; Smith et al., 1988). Antisense genes are presumed to inhibit expression of the target gene by directing the synthesis of a complementary RNA. Although the precise site of action is unknown (e.g., transcription, mRNA processing, transport, or translation), it has been shown that this inhibition causes a major reduction in the amount of mRNA that accumulates from the sense gene.

Fig. 4. Ripening-specific expression of the CAT gene in transgenic tomatoes directed by the 1450 base pair PG promoter. The activity of the CAT enzyme is demonstrated by the appearance of 1-acetyl and 3-acetyl chloramphenicol. Activity was measured in roots (1,10), stems (2,9), leaves (3,8), green fruit (4,7), and red fruit (5,6) of nontransformed and transformed plants. Lanes 11-13 show activity with 0, 0.01, and 0.02 units CAT activity. From Bird et al. (1988).

Physiological effects of reducing PG activity

The PG antisense gene was shown to be stably inherited, and in progeny with two antisense genes, the level of PG enzyme was 1% of normal (Fig. 5). An extensive biochemical investigation of the ripening of antisense fruit containing 1% PG compared to normal fruit established the following points (Smith et al., 1990b). First, no change in the timing or extent of the synthesis of lycopene or ethylene was observed. Second, the activity of other enzymes, such as pectin esterase and invertase, was essentially unchanged. Third, no significant change in compressibility of the fruit was detected during the ripening of control and 1% PG fruit. Fourth, the normal reduction in the average molecular weight of the soluble pectin produced during ripening was largely inhibited in the transgenic fruit. Biochemical studies on 1% PG fruit do not support the view that pectin fragments liberated by PG action play a major role in regulating other ripening processes. It will, however, be necessary to reduce PG activity further before this can be established unequivocally (Smith et al., 1990b).

Inhibition of ethylene synthesis by antisense genes

The construction of an antisense gene is possible for any gene for which a cDNA or genomic sequence is available, even if the function of the gene has not been established. We took advantage of this to attempt to identify genes involved in ethylene synthesis, which is implicated in the regulation of a number of important physiological processes, such as leaf and flower senescence, abscission, and fruit ripening.

There are two enzymes, ACC synthase and ACC oxidase (or "ethylene-forming enzyme"), that are specific to the ethylene biosynthetic pathway. Since no cDNA or genomic clones were available for these enzymes, we tried an indirect approach to the identification of ethylene biosynthesis genes by testing an antisense gene based on the sequence of a cDNA clone known as pTOM13 (Slater et al., 1985). This was considered a possible candidate for an ethylene biosynthesis gene since mRNA homologous to pTOM13 accumulates during ripening and in response to wounding, both situations involving ethylene synthesis (Holdsworth et al., 1987;

Fig. 5. Polygalacturonase activity in normal (O) and transgenic tomatoes with 0 (●), 1 (□), or 2 (■) PG antisense genes.

Smith et al., 1986). The production of ethylene is inhibited by 93% in ripening transgenic tomatoes expressing pTOM13 antisense genes (Hamilton et al., 1990). The ACC oxidase activity is reduced by a similar amount, suggesting that pTOM13 may encode this enzyme. The pTOM13 antisense fruit ripen more slowly than normal. These results indicate the general feasibility of down-regulating plant hormone synthesis. It seems likely that if the production of ethylene can be reduced further, it may be possible to delay processes such as abscission, ripening, and senescence in transgenic plants and accelerate them at will by supplying ethylene or ethylene-generating compounds.

CONCLUSION

It is clear that plant genetic engineering has advanced to a stage where many theories of how plant growth and development is regulated can now be tested at the molecular level. In some cases, there are obvious commercial applications for the knowledge that is being gained from experiments with transgenic plants. We are now entering a phase where knowledge of physiology and biochemistry will be at a premium to capitalize on this potential. Application of the new technology raises important issues concerning consumer acceptance, safety, and regulation, which are of concern to both scientists and the wider community. It is now essential to encourage a wide-ranging public discussion of the aims, objectives, and methods of plant genetic engineering, both as a means of discovering more about how plants function and of altering plant produce for the benefit of producers and consumers.

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