Biotechnology in Horticulture: 100 Years of Application

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Probably no other topic in this prospective series has such an immense scope as does biotechnology. The evolution of biotechnology has drawn on many disciplines and techniques; just defining a boundary around the subject is elusive. The importance and impact of biotechnology varies widely with the observer, some seeing it as part of the continuum in the development of our ability to manipulate the genetics of plants and others seeing it as truly revolutionary. Indeed, it is this latter aspect that contributes yet another broadening character to this topic—the controversial nature of the application of this powerful technology to our food, fiber, and ornamental crops.

Because of the space limitations, I will bias my discussion toward horticultural crops, but the discussion must involve nevertheless considerable reference to other disciplines from which applications were imported into horticulture. I will assume that the other crop-oriented treatises in this prospective series will include the relevant applications of biotechnology, thus I will not delve deeply into crop specific applications. One advantage of addressing a topic that is in a state of logarithmic growth and also has generated a good amount of controversy is that there are many preceding reviews; I will rely heavily upon such works for perspective and will not repeat that extensive literature. Finally, biotechnology is in a rapid state of growth and thus the future directions must occupy considerable attention.

COMMERCIAL ADOPTION OF BIOTECHNOLOGY

Many of the recent reviews of biotechnology allude to the potential, even the essentiality, of this technology contributing substantially to solving our growing problem of world hunger, a premise that is not without its own controversy (Evenson et al., 2002). Hunger discussions center on agronomic crops, not horticultural crops. Of the 79 major world food crops (classified as to tonnage, Khachatourians, 2002), about 50 of the 79 listed can be classified as horticultural crops. However, few horticultural plants are in the top 10 major food crops. Whether such estimates properly consider the contributions of small market and family kitchen gardens is doubtful. Nevertheless, the potential to apply biotechnology to horticultural food crops to help alleviate real nutritional and not simply mass caloric intake problems should not be ignored (Farham et al., 1999; Lindsay, 2002).

Biotechnology has impacted world agriculture, including horticulture. "Biotechnology continues to be the most rapidly adopted technology in agricultural history…" (James, 2002), a statement referring only to genetically-engineered crops. In 2002, the global area growing crops modified by genetic engineering was more than 55 million hectares (145 million acres). Genetic engineering technology has been adopted by more than 5.5 million farmers and shows acreage growth rates above 10% per year. That all of this commercial adoption of genetically engineered crops has occurred in a little more than a decade is impressive. With the exception of *Carica papaya*, the highest percentage penetration of genetically engineered selections into commercial crop production has been in agronomic crops. However, if other aspects of biotechnology are considered, the impact on commercial horticultural crops is equally impressive. For example, estimates of the number of cloned, mostly horticultural plants produced annually worldwide through micropropagation approach 500 million propagules, with the Netherlands alone producing more than 50 million annually (Thorpe and Harry, 1997).

COMPONENTS OF PLANT BIOTECHNOLOGY

Simplistically, biotechnology has evolved to include two interdependent applications important to horticulturists: cloning of elite germplasm and the genetic improvement of our existing germplasm. Each has an array of technologies associated with it (Fig. 1). In all cases, these technologies only rise to their full potential when used in close association with conventional genetic improvement programs. As powerful as it is, biotechnology has not eliminated the necessity for a sound germplasm base within which to function and the need for effective testing and evaluation programs.

Biotechnology was borne and began its evolution in the 20th century. The fundamental scientific foundations, the application tools derived from these basic concepts, and the products of their application were all generated in that century, particularly beginning in the 1950s (Fig. 1). Three applications—microculture (tissue culture), recombinant DNA technologies, and most recently bioinformatics/genomics—provided the central technologies from which applied crop biotechnology evolved. Horticulture played a particularly strong role in the evolution of microculture technologies. Not only were many of the experimental plants horticultural crops (e.g., *Nicotiana* and *Daucus*), but horticulturists such as Murashige (1974) were principal early researchers.

CLONING USING MICROCULTURE (MICROPROPAGATION)

The first widely adopted, commercial application of horticultural biotechnology was micropropagation of ornamental crops. Although a variety of approaches for the in vitro multiplication of plants have been demonstrated, by far the one most used is shoot culture where the multiplication of shoots (microcuttings) is derived from the stimulation of axillary buds on subcultured stems (Preece, this volume). Ball (1946) used *Nasturtium* and *Lupinus* to demonstrate the first shoot culture micropropagation. Commercial applications and laboratories proliferated in the 1970s and 1980s, followed by a more conservative growth.

The major limitation of shoot culture-based micropropagation is the cost of subculturing and handling the individual microcuttings. Labor costs usually are more than 60% of the total production cost (Altman and Loberant, 1998) and attempts to reduce these involved automation, particularly robotics (Aitken-Christie, 1991; Altman and Loberant, 1998; Fujita and Kinase, 1991). Although manipulating shoots using visually-aided robotics has been demonstrated, commercialization has been thwarted by the cost of the robotic systems, the necessity for subjective evaluation as to tissue quality, and the nonuniformity of explants both within a shoot culture and between different crops. A complimentary approach has been to attempt to develop systems that produce a propagule more uniform and readily manipulated than a complex shoot and which are adaptable to liquid culture systems (Cantliffe, 2001; Ziv, 2000). The three leading choices have been somatic embryos (Merkle, 1997), nodular systems (McCown et al. 1988), and specialized structures (McCown and Joyce, 1991; Takayama et al., 1991). Although successful application of these technologies was demonstrated for a few crops, widespread application has been thwarted by problems associated with moving the propagules from the microculture environment to the greenhouse with a high percentage of uniformity and survival (Altman and Loberant, 1998; Cantliffe, 2001). One solution to developing a more robust and readily acclimated propagule is the use of autotrophic culture systems (Jeong et al., 1995), though these have not found wide commercial application.

Micropropagation has been accompanied by disease-removal procedures, including meristem culture and thermotherapy (Towill, 1989). The early demonstration of virus removal using microculture included work on *Dahlia*.

GERMPLASM PRESERVATION

Towill (1989) and Withers and Engelman (1998) reviewed the use of biotechnology for preserving (storing) germplasm. Microculture-based storage systems have shown some advantages over field or greenhouse collections, including disease control, year-around availability, and lower space requirements. Of great importance is the ability

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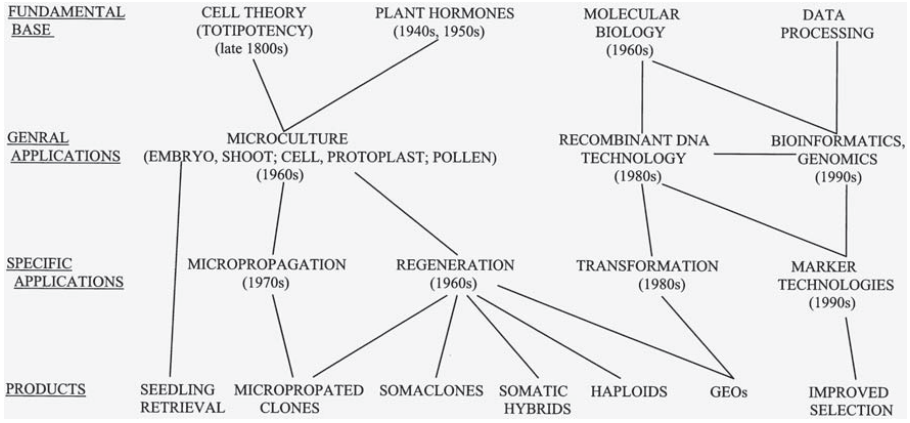

Fig. 1. A generalized progression of the development of plant biotechnology during the 20th century. Numbers in parenthesis represent the time period when that technology first became generally available. GEOs = genetically engineered organisms.

to keep the biological materials relatively disease-free. Thus meristem culture, thermotherapy, and evaluation techniques based on molecular probes logically accompanied germplasm storage programs. In order to reduce costs, extended storage (slow growth using low temperature) and long-term storage techniques (no growth or cyropreservation) have been researched and demonstrated. A variety of tissues have been used including shoot culture, embryos, and dissected meristems (Williams et al., 1987). However, extensive information on the long-term stability of such culture techniques is absent.

No germplasm collection relies entirely on microculture storage techniques. In vitro germplasm storage with such horticultural crops as *Solanum*, *Fragaria*, *Ipomoea*, *Mentha*, *Prunus*, and *Vaccinium* is exemplary.

EMBRYO, OVULE AND SEED CULTURE

Williams et al. (1987) reviewed embryo and ovule culture. Embryos of *Raphanus* were among the first plant organs to be successfully microcultured (Hannig, 1904). One of the major uses of embryo culture is the rescue of hybrid embryos, often from wide crosses. This has been applied to fruits (*Prunus*, *Carica*), vegetables (*Allium*, *Phaseolus*, *Brassica*, *Cucumis*), and ornamentals (*Ulmus*, *Quercus*, *Lilium*, *Viburnum*, *Nicotiana*, *Impatiens*).

In vitro seed germination has been used to decrease the generation time in breeding programs involving woody ornamental and fruit crops. Its advantages include the avoidance of dormancy (*Prunus*, *Iris*, *Vaccinium*), high seedling recovery rates (*Rubus*), and the simultaneous establishment of the germplasm in microculture to allow both germplasm storage and rapid clonal propagation to facilitate testing (*Vaccinium*, *Citrus*).

ANDROGENESIS (HAPLOID PLANT GENERATION)

Ferrie (2002) and Wenzel (1998) reviewed haploid plant generation and estimated over 2000 publications covering more than 250 species describing these techniques and their application. The main advantage of androgenesis is the production of homozygous lines in one generation (haploid production followed by chromosome doubling) which may save years of selfing to establish homozygosity. Although finding limited use in horticultural plant breeding, anther/microspore culture has been used with Solanaceous crops and *Asparagus*, as well as some woody species.

PROTOPLAST CULTURE AND SOMATIC HYBRIDIZATION

Probably one of the more technologically elegant procedures in biotechnology involves protoplast culture and subsequent protoplast fusion to form somatic hybrids. The process captivated many researchers in the 1970s and 1980s but has been eclipsed by transformation technologies. The first reported successful application was with *Nicotiana*, and subsequently the Solanaceous crops have been the prime targets because of their ease of in vitro culture. After protoplast formation using enzymatic digestion of the cell walls, the most common fusion techniques were chemical (PEG) or electric shock (electrofusion). Johnson and Veilleux (2001) and Waara et al. (1995) have reviewed somatic hybridization.

The thought of combining the genes of two parental lines by simply fusing the cells (somatic hybridization) was intriguing and has been demonstrated with a number of horticultural crops, among them being genera in the Rutaceae (*Citrus*) and Solanaceae (*Lycopersicon*, *Nicotiana*, and *Solanum*). Much of the effort was directed at combining the genomes of two species (interspecfic hybridization), although intergeneric fusions were also common. Another use of somatic hybridization was the combining of an enucleated protoplast with a complete protoplast, thus creating a cytoplasmic hybrid or cybrid. Cybrids could be useful in developing cytoplasmic male sterile systems.

Unfortunately, the complications accompanying somatic hybridization presented severe hurdles. These included complex cell manipulations, change in ploidy, and the necessity for extensive further breeding. Thus the use of somatic hybrids in breeding has been limited and the release of cultivars developed by these methods is rare.

SOMACLONAL/GAMETOCLONAL VARIANTS

Variants produced in microculture systems were termed somaclonal variants (Larkin and Snowcroft, 1981) referring to the potential genetic variation produced through somatic cell culture. Somaclonal variation is one of the many two-edged swords of plant biotechnology. For those uses where true genetic fidelity is desired (such as micropropagation or genetic transformation), somaclonal variation is a major problem (Preece this volume; Veilleux and Johnson, 1998). However, somaclonal variation has also been viewed as a new source of genetic variation (Bouman and de Klerk, 1997) that may be useful for plant improvement. Many reviews are available covering somaclonal variation (Bouman and de Klerk, 1997; Morrison and Evans, 1987; Veilleux and Johnson, 1998).

The potential causes of somaclonal variation probably vary as much as the observed changes; prominent are the expression of preexisting variation in the explant tissue, karyotype changes, chromosomal rearrangements, and activation of transposable elements. Some authors (Bouman and de Klerk, 1997) differentiated somaclonal variation from changes in gene expression (amplification, methylation), although on a practical level this differentiation is often difficult to discern. The observed rates of somaclonal variation are dependent on many factors, paramount among them being the genotype being cultured and the culture system used. Some genotypes and even species are more prone to variation. By far the most universal source of variation has been systems where callus culture is followed by plant regeneration. Thus in vitro culture systems where regeneration is direct (Merkle, 1997) have shown stability close to what is commonly observed in cutting propagation (Arene et al., 1993; Ostry et al., 1994).

A major problem with somaclonal variation is the lack of a sensitive and exact method of detecting such variation in micropropagules or transformants. Although using molecular markers and genomics is one approach to this problem, the techniques have not been sufficiently sensitive as they do not screen the total genome of the plant being cultured. Unfortunately, in micropropagation systems, some variation may not be detectable until the propagules have been put into production (Ilan and Khayat, 1997) and this has had significant economic consequences in some cases, for example with oil palms and *Rhododendron*. The end result is a strong limitation on multiplication systems that rely on adventitious regeneration, especially those using cell and callus culture.

Somaclonal variants have been observed in vegetable, fruit, and ornamental crops. Since the mutations generated in somaclonal variation are generally not unique, that is they can be generated by mutagenesis, the process is not readily controlled but rather random and may include multiple variations. Since most of the variants have no economic value as in mutation breeding, somaclonal variation has not made many important contributions to horticultural crop improvement.

REGENERATION OF PLANTS

Probably no plant biotechnology technique is more fundamental than the regeneration of plants from cells and tissues. Without reliable and predictable regeneration, all processes based on adventitious meristem development, including most transformation protocols, would have been failures. However, there is probably no other aspect of biotechnology that is more dependent on the specific genotype being manipulated than regeneration, an observation that hints at a strong host genetic control of the process. Regeneration has been reviewed extensively (de Klerk et al., 1997; Ritchie and Hodges, 1993; Su, 2002)

De Klerk et al. (1997) delineated three phases commonly observed in any regeneration process: 1) dedifferentiation or the acquisition of competency, 2) induction leading to meristem organization under appropriate stimuli, and 3) realization leading to the outgrowth of the meristems into functional structures. This overview highlights the complexity of the process and thus it is not surprising that the basis of competency and totipotency are not well understood. Work on this aspect has intensified in the last decade, especially with somatic embryogenesis systems. Horticultural plants (*Daucus*, *Lycopersicon*) often have been the research organisms. Many cellular changes have been observed and correlated with regeneration and prominent among these are DNA methylation, chromatin structure, and enzyme dynamics. However none of these markers have been sensitive enough or universal enough to be utilized as a general indicator of regenerative competency.

It is clear that the regeneration process as viewed at the macro level can take a number of avenues. Organogenesis involves the de novo formation of isolated shoot or root meristems. Embryogenesis in contrast involves the formation of a true bipolar structure containing both root and shoot meristems. A much less studied route involves the formation of differentiated nodular masses that have the capability of self replication and eventual adventitious organ regeneration (McCown et al., 1988). Indirect regeneration systems having an intervening callus stage have shown higher rates of somaclonal variation and losses of competency.

For woody and long-lived horticultural crops, a thorough understanding of phase change is critical for regeneration. One general theme that permeates the work of the last 100 years is that juvenile or rejuvenated tissue is much more competent in most all microculture systems, from micropropagation to transformation. The partial rejuvenation that accompanies adventitious meristem development and stabilization of shoot cultures has been used to advantage in providing tissues from adult phase plant sources for biotechnological manipulations (McCown, 1985, 2000).

TRANSFORMATION

Many have equated biotechnology with genetic transformation—the insertion of DNA foreign to the host cell. The volume of work on transformation of horticultural crops is immense and continues to increase at an impressive pace (Altman, 1998; Barz and Oksman-Caldentey, 2002; Geneve et al., 1997; Khachatourians et al., 2002; Singh and Sansavini, 1998). Persley et al. (2002) described the history of genetically-engineered crops, beginning with the first experimental transgenic plants in the mid 1980s and progressing through the first field trials in late 1980s (*Lycopersicon*), the first commercial cultivation in mid 1990s, and the present commercial plantings of millions of hectares in 13 countries. Present commercial applications involve primarily production traits (insect and herbicide tolerances) in agronomic field crops, but will be followed by a wave of other introductions featuring quality and nutritional traits where horticultural crops may predominate.

Two general modes of moving genes into plant cells have been utilized: vector-mediated gene transfer and direct gene transfer. Vector-mediated transfer employs *Agrobacterium*, usually *A. tumefaciens* but also *A. rhizogenes*. Two classes of *A. tumefaciens* vectors have been developed, co-integration and binary vectors (Walkerpeach and Velten, 1994). Vector-mediated transfer has been used more commonly than direct-gene transfer.

An ingenious variety of direct DNA delivery systems has been developed. Deroles et al. (1997) list 14 such systems, though three predominate. Electroporation was one of the earliest systems devised (patented in 1986) and relied on inducing the uptake of DNA into cells (usually protoplasts) suspended in the DNA preparation. A second system employed a liquid mixture of fine silicon-carbide whiskers or microfibers, plasmid DNA, and cell suspensions; uptake of the DNA is induced by vortexing the mixture. The technique is simple, quick and inexpensive and is not patented. Microprojectile bombardment (patented in 1990) uses carriers, usually gold pellets, accelerated to a high speed to move the DNA into cells. This system has proven the most versatile and also has been used for plastid transformation as well as a complement to vector systems.

All of the transformation technologies depend upon the ability to regenerate plants from single cells as the DNA is delivered independently into each cell, whether it is in a tissue or singulated in a suspension. Whisker and particle bombardment are particularly dependent on competent cells being at the surface of the target tissue as the DNA delivery does not penetrate deeply into tissues. Meristematic tissues may be particularly receptive using direct transfer techniques and thus pretreatment of target tissues to generate adventitious meristems prior to exposure has proven useful where effective selection is applied to avoid chimeras (Serres, et al., 1992).

A second requirement of most transformation systems is an effective selection strategy to give preferential advantage to those cells that have incorporated the foreign DNA. By far the most common selection techniques are antibiotic tolerance or herbicide resistance. However, these traits have generated considerable criticism in that the resultant crop plants carry these resistance genes (FAO/WHO, 2001; Rissler and Mellon, 1996). Strategies to avoid this problem have included post transformation removal of the selection genes (Hare and Chua, 2002) and selection based upon less environmentally sensitive traits (Zuo et al., 2002).

A third requirement of a transformation system is the generation of large numbers of transformation events (independent transformants). Genetic improvement using transformation has not eliminated the need for extensive selection and testing of the progeny. Not only are somaclonal and other variants common in some systems, but expression of the inserted genes will vary markedly between individual transformants derived from the same experiment.

Although of high potential usefulness for most crops, genetic transformation of heterozygous and long-lived horticultural crops has particular appeal. The typical problems encountered with breeding woody perennials, such as long juvenile periods, self-incompatibilities, seedlessness and sterility, apomixis, and the high cost of maintaining evaluation plots, make the thought of transferring a specific trait into an already high-valued genotype particularly inviting.

MARKER TECHNOLOGIES AND GENOMICS

The end of the 20th century was marked by the emergence of DNA marker technologies as a new era in genome analysis. DNA polymorphisms are more abundant and discrete than phenotypic or biochemical markers and thus can be used to develop more saturated genetic maps. This increases precision and efficiency in the manipulation of traits by facilitating the tagging of genes. DNA marker technologies may be useful in indirect selection schemes (MAS, marker-assisted selection), particularly for qualitative traits. These and other advantages have been reviewed (Abbott, 2002; Whetten, 1997). Although most of these technologies have been developed with non-horticultural plants, their broad applicability has been demonstrated on horticultural crops.

Two principle technologies have evolved: 1) molecular hybridization where specific probes based on restriction enzyme fragments are used to detect polymorphisms, and 2) DNA amplification fingerprinting where oligonucleotide primers are used to amplify polymorphic DNA fragments. Early molecular hybridization techniques used RFLPs to map chromosomes in many horticulturally-important families including Fabaceae, Poaceae, Pinaceae, Solanaceae, and Liliaceae. With *Lactuca* and *Lycopersicon*, genes of economic importance were tagged. As PCR systems developed, RAPD analytical techniques soon followed and were used for mapping in such genera as *Lycopersicon*, *Prunus* and *Lactuca*, and for cultivar identification.

PCR-based methods grew into DNA amplification fingerprinting where the use of shorter primers increased the number of detectable randomly amplified fragments of a genome. Two approaches, single sequence repeats (SSR) and AFLPs evolved. SSRs or microsatellites ap-

pear to possess high variability at a given locus, are highly transportable between crops, and are relatively abundant. SSRs have been researched in Rosaceous horticulture crops and may have application in fingerprinting cultivars. AFLPs have been used in linkage maps of some horticulture crops including *Solanum*, *Lycopersicon*, and *Prunus*.

Abbott (2002) suggested that the use of a combination of the three technologies may offer the most flexibility. AFLPs may give the largest number of markers while RFLP and SSRs can be used to tie-together the loci into a single map as well as to allow integration between maps. Tagging important traits (MAS) may be particularly useful for woody perennial crops where long generation times limit other selection strategies and where the existence of a marker in a seedling may infer the existence of an allele that is only expressed in an adult plant.

Whether all the potentials of genetic markers and associated genomics materialize will be intriguing to observe. For example, the mapping of quantitative trait loci (QTLs,) has been used to attempt to identify locations on chromosomes of loci having major effects on crop performance (e.g., fruit quality, plant height). However, recent analyses have indicated that accuracy and precision of QTL estimates are often lacking which leads to highly inflated overestimation of the percentage of genetic and phenotypic variation explained by QTLs. (Melchinger et al., 1998; Utz et al., 2002). Major QTLs, that is, those explaining greater than 25% of the variation, detected in experiments with large population sizes (200+) of highly controlled biparental crosses are the most dependable. The identification of QTLs in highly heterozygous horticultural crops may be difficult. Presently, these and other requirements limit the direct application of QTLs in most horticultural crop genetic improvement programs.

Gene tagging and cloning will have immense importance in horticulture if the inference of function from homology to known genes proves to be broadly applicable. Functionality of tagged segments can now only be known with certainty when they are mapped to a gene of known function. Ability to predict interactions between parts of a genome or with the environment is lacking. Comparative efficiency of MAS will vary with the specific use (Dreher et al, 2002).

SOCIAL/ECONOMIC RAMIFICATIONS

As is evident from the above discussion, biotechnology has a spectrum of components. However, genetic engineering of crops, particularly foods, by far has had the highest visibility among the public and interested groups. There are a host of items that have led to citizen and consumer concerns (Evenson et al., 2002; FAO/WHO, 2001; Gressel and Rotteveel, 2000; National Research Council, 2002; Rissler and Mellon, 1996; Welsh et al., 2002). Biotechnology is an excellent example of the difficulty in evaluating and estimating risk. Not only are the methodologies of risk assessment evolving, but also the intersection of social, economic, ethical, and scientific forces makes the process bewildering.

FUTURE CONSIDERATIONS

It is difficult to see a slowing in the growth of plant biotechnology. Indicative is the increase in agricultural biotechnology patents issued to universities where we appear to be on the logarithmic increase side of the growth curve (Foltz, personal communication). The potential influence of this patent production on the structure of our public research institutions is itself quite revealing of the overarching dynamic influence of biotechnology (Foltz et al., 2003). However, on a more long-term scope, a number of scientific achievements appear to be well within our reach:

- An in depth understanding of the gene insertion process and subsequent expression of foreign genes in plants.
- A practical and widely applicable approach to selection for transformed cells without incurring the addition of controversial traits in the transformed plants.
- The availability of entire genomes of plants like *Arabidopsis* promises to lead to major advances in our understanding of the genetic components of major traits in horticultural corps (Sommerville and Sommerville, 1999). High capacity gene expression and analytical systems such as DNA chips (Lemieux et al., 1998), coupled with proteomics, metabolomics, and bioinformatics (Persley et al., 2002) will continue to dramatically increase the amount of information available for use by plant breeders (Parkin and Lydiate, 2002).
- An understanding of the genetic basis and thus the eventual control of phase change in plants has important implications for the manipulation of woody perennials.
- The control of apomixis through transgenic technologies will have strong economic application to seed crops and promises to simplify breeding schemes (Chaudhury et al., 1998; Fobert, 2002).
- The uncovering of useful markers for the acquisition of regeneration competency seems assured.
- Widely applicable plastid transformation technology promises to provide unprecedented levels of expression, and lessen environmental gene flow problems (Daniell, 1993, 2002).
- The fusion of engineering technologies such as systems engineering and sensing with improved plant microculture biologies (embryogenesis, nodule/meristemoid culture) may make clonal cultivars more economical and more widely applicable.

It is difficult not to envision a future where the acceptance of the products of this powerful technology will become commonplace. A greater focus on quality traits (Lindsay, 2002), including ornamental features (Geneve et al., 1997), that are obviously consumer friendly will find wider and quicker acceptance, an aspect of particular importance to horticulture. Probably of even greater importance, biotechnology will have to become more tightly integrated with other approaches for solving plant problems. For example, the tight interweaving of genetically engineered traits with IPM management strategies can be readily envisioned and will go a long way in preventing biotype evolution and other negative ramifications of the over-reliance on genetically engineered traits (Dewar et al., 2003; Welsh et al., 2002). In any case, plant biotechnology must be viewed as a tool—a powerful one, but just one of many tools available for plant improvement. In their text, Simmonds and Smartt (1999, p. 35) put the potential in perspective: "The potential is enormous but any rewards from its application are likely to be very hard won indeed. The cost in time, effort and resources will be vast while the products will still have to be utilized through traditional breeding programmes; they will supplement, not supplant nor supersede them." The next historical review on horticultural biotechnology should be awesome reading.

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