Contemporary Approaches to Improving Citrus Cultivars

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Summary. Traditional genetic manipulation methods have proven ineffective or irrelevant for many citrus breeding objectives. Alternative approaches to *Citrus* genetic improvement are now available as a result of technological developments in genetics and tissue culture. For example, mapping DNA marker polymorphisms should lead to identifying markers closely linked to important loci, thereby facilitating early selection and minimizing costs associated with plant size and juvenility. Genetic transformation methods allow trait-specific modification of commercial cultivars. By selecting beneficial variants from sectorized fruit chimeras and the recovering plants via somatic embryogenesis, the problems of nucellar embryony and the hybrid nature of commercial cultivar groups can be avoided. Induced mutagenesis from mature vegetative buds may overcome these problems, as well as juvenility. Ploidy level manipulation in vitro can increase the number and diversity of tetraploid breeding parents, leading to the development of seedless *Citrus* triploids and mitigating sterility, incompatibility, and nucellar embryony.

Citrus is grown throughout the tropical and subtropical regions of the world. Its production exceeds that of *Musa* (bananas and plantain), *Vitis* (grapes), and *Malus* (apples) (Food and Agricultural Organization, 1990), thereby elevating *Citrus* to the number-one position among fruit crops. However, very few programs for improving *Citrus* cultivars have been established, despite the economic significance of this genus. Citrus breeding programs have had minimal success because of various biological factors including sterility (Soost and Cameron, 1975), self- and cross-incompatibility (Soost, 1969), widespread nucellar embryony (Frost and Soost, 1968; Soost and Cameron, 1975), and long juvenile periods resulting in large plant size at maturity. A consequence of these factors is the dearth of information on genetic control of economically important traits and rapid and effective screening procedures (Gmitter et al., 1992).

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Two of the most important *Citrus* species—*C. sinensis* (L.) Osb. (sweet orange) and *Citrus* × *paradisi* Macf. (grapefruit)—are believed to be unique interspecific hybrids, not true biological species (Barrett and Rhodes, 1976; Scora, 1975). All cultivars within these species have arisen via somatic mutation, not sexual hybridization; intraspecific hybridization results in weak or inviable hybrid progeny (indicative of inbreeding depression) that generally produce fruit unlike those of the parents. For these important cultivar groups, alternatives to sexual hybridization are essential for cultivar improvement.

Following are brief descriptions of some alternate approaches to scion and rootstock improvement for *Citrus* that have become possible in the past decade because of developments in cellular and molecular biology. Integrating these methods with classical breeding approaches has resulted in optimism and hope for future *Citrus* cultivar improvement.

Genome characterization

Very few genetic markers have been identified in *Citrus*. Genetic studies have been difficult for the same reasons as for traditional breeding, i.e., nucellar embryony and long juvenile period. However, segregating families can be developed for genetic mapping studies using monoembryonic seed parents that produce strictly zygotic offspring. Recent developments in plant molecular biology have made it possible to create saturated genetic linkage maps of plant genomes (based on isozymes), restriction fragment length polymorphisms (RFLPs), and randomly amplified polymorphic DNA (RAPD). Efforts are ongoing in Florida and California and other countries to map the *Citrus* genome with molecular markers; partial linkage maps have been developed by Durham et al. (1992) and Jarrell et al. (1992). Applying RAPD techniques has increased the number of markers available for study and the speed at which map saturation can be achieved.

Several economically important traits, such as resistance to citrus tristeza virus (CTV), citrus nematode (*Tylenchulus semipenetrans*), and *Phytophthora*, are being studied. This information is necessary to use genome maps to improve cultivars. Using molecular markers closely linked to genes of interest is a practical approach to marker-assisted selection from segregating hybrid progeny. At present, there are no rapid tests that predict mature tree performance. Screening procedures for the diseases mentioned above, for example, are complex enough to preclude their application to the large hybrid families necessary for multitrait selection. The ability to screen many plants at the seedling stage for

linked molecular markers should overcome some impediments to selecting multiple disease-resistant hybrids (i.e., costly, complex, and slow procedures). An additional benefit to the breeding program should be a decrease in the land area needed and the expense to maintain desirable hybrids in the field until maturity, because no undesirable recombinants need to be planted. This would allow greater use of zygotic seed parents in hybridization, especially for improving rootstocks. Finally, a molecular map may be used for isolating and cloning genes that can be used to transform existing cultivars or breeding parents for specific traits.

Genetic transformation

Genetic transformation is an attractive approach to improve *Citrus* cultivars, especially if cultivar integrity can be maintained while modifying only one or two traits. This method could be very useful for sweet orange and grapefruit, given the difficulties in improving these cultivar groups. Moore et al. (1992) have developed an *Agrobacterium*-mediated transformation system that relies on organogenesis from internode segments excised from young nucellar seedlings grown in vitro. Vardi et al. (1990) produced transformed *Citrus* plants through direct DNA uptake by protoplasts after polyethylene glycol treatment. Hidaka et al. (1990) reported successful *Citrus* transformation using *Agrobacterium* in cell-suspension cultures. Although transformation frequencies were lower than those reported in some other crops, *Citrus* has been transformed by several methods.

The first potentially important gene, the coat protein gene from CTV, already has been inserted into Carrizo citrange (*C. sinensis* × *Poncirus trifoliata* Raf.) and the Mexican lime (*C. aurantifolia* Christm. Swing.) on an experimental basis (G.A. Moore, personal communication). CTV is the major viral disease of *Citrus* worldwide, and all the major cultivars likely will be engineered to include the CTV coat protein gene should this control strategy be proven effective. Other candidates for transformation include genes that may induce greater cold tolerance (or dormancy) in trees, antifungal genes, or genes controlling important fruit characteristics such as pigmentation, sugar production, acidity, and bitterness.

There are several impediments to using genetic transformation to improve *Citrus* cultivars. Most research has focused on cultivars that are very responsive to in vitro manipulation, so the transformation protocols are somewhat cultivar-specific; however, not all *Citrus* cultivars are amenable to tissue culture methods. Improvements in transformation efficiency and plant regeneration

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would be very helpful. Methods to transform meristems from mature clones need to be developed, because current in vitro methods rely on juvenile tissue. Avoiding the lag time imposed by juvenility would greatly hasten field evaluation of transformants and their ultimate use by *Citrus* growers.

Identifying, isolating, and cloning significant genes is not simple. The availability of such useful sequences is limiting, but, as genetic research continues, new potentially useful genes should become available. Many important *Citrus* characteristics are polygenic, however, and manipulating these traits likely will be more problematic via the transformation approach. In addition, there may be limits to the number of foreign genes that can be inserted in a given cultivar before undesirable changes occur in other characteristics of that cultivar. Finally, despite the appeal and short-term justification for genetic transformation as a method of improving *Citrus* cultivars, relying on transformation while neglecting other genetic manipulation methods may lead to a narrower commercial cultivar germplasm base. The consequences of genetic vulnerability in monoculture production systems of perennial plants could be catastrophic.

Somatic mutation

Sexual hybridization is ineffective for genetic improvement of sweet orange or grapefruit for reasons described above. This statement is borne out by the fact that all cultivars in these two groups originated as somatic mutations—either bud-sport or nucellar-seedling variants (Hodgson, 1967). Decades ago, when growers managed small family groves or orchards, it was possible for observant individuals to identify potentially useful variation among their trees. Today’s corporate-style managers generally know their groves less intimately than the previous generations of growers, and potentially useful variation is very likely to go unnoticed. Therefore, to improve orange or grapefruit cultivars, breeders must look to either naturally occurring or induced somatic mutations. A notable exception, perhaps, is the new orange cultivar Ambersweet, which originated by hybridizing a mandarin × (grapefruit × mandarin) hybrid with sweet orange (Hearn, 1989). This cultivar is the first *Citrus* hybrid to be classified legally as a sweet orange.

One possible source of somatic mutations may be plants regenerated through various in vitro pathways, i.e., somaclonal variation; however, small populations of polyembryonic *Citrus* regenerants have not shown much variation (Kobayashi, 1987; Vardi and Spiegel-Roy, 1982). Likewise, little or no permanent variation among young seedlings

for ploidy level, isozyme genotypes, or morphology was found in more-rigorous studies (Bowman, 1990; Gmitter, 1985). Fruit characteristics were not evaluated in the latter reports on juvenile seedlings, and useful variants may possibly be identified. Therefore, the Univ. of Florida citrus breeding program has produced hundreds of regenerants of many citrus cultivars, via multiple regeneration pathways, that are being grown in field plots for evaluating mature tree and fruit characteristics.

A useful source of somatic mutations has been seeds or budwood exposed to mutagenic agents, most commonly some form of radiation. This approach has been somewhat successful; the most intensely pigmented grapefruit cultivar, Star Ruby, originated from irradiated seed of a seedy cultivar named Hudson (Hensz, 1977). ‘Star Ruby’ produces commercially seedless fruit as a result of this mutagenic treatment. Cultivar improvement programs using induced mutagenesis have focused mostly on inducing seedlessness among normally seedy cultivars such as ‘Pineapple’ sweet orange or ‘Foster’ grapefruit (Hearn, 1984, 1986). Cytogenetic aberrations (translocations, inversions) have been found to underlie fruit seedlessness among irradiated budlines of many cultivars tested (F.G. Gmitter, unpublished data).

Other objectives pursued by mutation breeding include fruit quality alterations, especially in color and acidity. Mutagenesis, especially when applied to budwood of mature clones, can circumvent nucellar embryony and inbreeding depression (no sexual crosses are required) and juvenility (mature buds are used, so offspring bear fruit in 2 years or less). However, thoroughly evaluating all irradiated progeny for major improvement objectives will be time-consuming. Further, the possibility of obtaining chimeric plants after mutagenic treatment of meristems is high, so the stability of selected budlines must be confirmed by observing their performance over several seasons. Concurrently, close attention must be paid to identifying other useful, or deleterious, phenotypic changes. Finally, well-planned field trials of several mutant budlines must be undertaken, because no new scion cultivar should be released without thoroughly evaluating its horticultural and postharvest performance.

A novel source of potentially valuable genetic variation has been identified in naturally occurring mutations that are found as sectorized fruit chimeras (Bowman et al., 1991). Fruit sectors have been observed, with differences from the normal phenotype including more or less intense color (sometimes internal color changes coincide with external differences), thicker or thinner rinds, possible tolerance of fungal or insect agents that induce rind

blemishing, or earlier or later maturity. Many of these mutations, e.g., earlier maturity or more intense pigmentation, could be of great economic value. Thickened rind sectors are polyploid and could give rise to autotetraploid nucellar offspring of potential breeding value. *Citrus* breeders can access this externally visible variation, because *Citrus* fruit rinds originate from the L-II histogen, as do the gametes and nucellus. Therefore, nucellar embryos in seeds found beneath mutant rind sectors should be genetically identical to the rind, and new plants should be recovered that are nonchimeric and entirely of the new phenotype. When seeds have not been found beneath desirable mutant sectors, it has been possible to recover plants by culturing undeveloped ovules on standard *Citrus* tissue culture media (F.G. Gmitter, unpublished data). Based on the evidence (Bowman et al., 1991; Iwamasa et al., 1977) that rind mutations can be recovered in nonchimeric form from such seedlings, much effort has been expended in Florida to collect as many chimeric-sectored fruit exhibiting beneficial mutations as possible and to recover plants from those sectors. I have recently recovered >300 plants from chimeras of various grapefruit, sweet orange, and mandarin cultivars. However, none of these plants has fruited; clearly, juvenility, selection, and the need for field trials are not avoided by this approach. It is likely that breeding problems associated with the hybrid nature of the grapefruit and sweet orange can be overcome and improved cultivars developed from plants recovered from chimeric fruit.

Manipulating ploidy level

Nearly all *Citrus* scion and rootstock cultivars are diploid ($2n = 2x = 18$). Polyploids have been found among nucellar seedling families (Cameron and Frost, 1968) and have been induced by colchicine treatment of axillary meristems in vivo (Barrett, 1974), treatment of undeveloped ovules and embryogenic callus (Gmitter and Ling, 1991; Gmitter et al., 1991), or a combination of colchicine treatment and micrografting (Oiyama and Okudai, 1986). *Citrus* somatic hybridization has resulted in the creation of several tetraploids composed of complementary diploid parents, and intergeneric somatic hybrids combining *Citrus* with sexually incompatible relatives have been produced (Grosser and Gmitter, 1990).

Citrus autotetraploids have been less vigorous and productive and fruit quality has been less desirable than diploid progenitors (Soost and Cameron, 1969), but some triploid hybrids have been developed that are reasonably vigorous and productive (Soost and Cameron, 1980, 1985). The interest in triploids arises from their potential to pro-

duce seedless fruit (Soost and Cameron, 1969), a commercially significant attribute. Triploid citrus plants have been recovered after endosperm culture in vitro (Gmitter et al., 1990); however, the frequency of plant recovery via this route and the vigor of recovered plants have been unsatisfactory. A more direct method for producing triploid hybrids has been hybridizing diploid seed parents (preferably clones that produce strictly zygotic progeny) with tetraploid pollen parents and the subsequent rescue by in vitro culture of triploid embryos from abortive seeds (Starrantino and Reforgiato-Recupero, 1981). Using interploid hybridization to develop seedless cultivars is not a new idea; it has been pursued in California and, more recently, in Italy. Success has been limited by the availability of suitable breeding lines. New interest in this breeding approach has come with an increase in the number of useful monoembryonic diploids and the development of new tetraploid clones.

Conclusion

Advances in molecular genetics techniques and tissue culture-based genetic manipulation of plants have yielded new opportunities for developing novel *Citrus* cultivars. Some of the technology can overcome the limitations of sexual hybridization. For example, somatic hybridization can create new combinations that were previously impossible because of sterility or sexual incompatibility. Selecting somatic mutations or genetic transformation may allow the modification of very few traits while retaining the essential characteristics that typify specific cultivars or cultivar groups.

Other new methods may allow greater exploitation of the opportunities for genetic advance provided by sexual hybridization. Interploid hybridization for developing seedless triploid clones can be facilitated by embryo rescue in vitro and applying flow cytometry to determine hybrid ploidy levels. Improved endosperm culture methods may be useful for producing triploids and also can circumvent nucellar embryony, because endosperm tissue is of hybrid origin. Multitrait, marker-assisted selection, based on genetic links of molecular markers with economically important genes, can improve the *Citrus* breeder's ability for selecting, and allow greater exploitation of, monoembryonic seed parents and their segregating progeny.

It is important to recognize, however, that these new approaches simply facilitate the genetic manipulation aspects of cultivar development—they do not diminish the critical need for final selection based on the results of properly designed field trials. Such field trials are costly to establish and conduct, but

“Technology can overcome the limitations of sexual hybridization.”

their importance to the process of *Citrus* cultivar improvement should not be overlooked. *Citrus* breeding programs that recognize that the value of contemporary methods comes from their integration into genetically sound, broad-based cultivar development programs will be the most successful in the coming century.

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