Application of Recent Biotechnologies to *Prunus* Tree Crop Genetic Improvement[1, 2]

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Abstract

P. Martínez-Gómez, R. Sánchez-Pérez, M. Rubio, F. Dicenta, T.M. Gradziel and G.O. Sozzi. Application of Recent Biotechnologies to *Prunus* Tree Crop Genetic Improvement. Promising tools for *Prunus* breeding include germplasm introgression, molecular marker development and improved propagation and gene transfer techniques. In germplasm introgression, the introduction of genes from related *Prunus* species conferring agronomically valuable traits such as self-compatibility, improved growth habit, drought resistance, and higher kernel quality are being pursued. The analyses of twin seeds (two embryos within the same seedcoat) are facilitating genetic and cytogenetic studies. Useful propagation methods include *in-vitro* techniques for the evaluation of plant material, and *in-vivo* micrograft techniques that allow the early propagation of high-risk genotypes. In addition, plant growth under controlled environments, including the induction of an artificial rest period using cold chambers, provides a useful strategy for obtaining vigorously growing plants all the year round. Molecular markers have also become an essential tool in *Prunus* genetic improvement studies. Different types of molecular markers, including isoenzymes, RFLPs, RAPDs, AFLPs and SSRs, have been employed for the genetic characterization of germplasm, the establishment of genetic relationships between cultivars and species, and the construction of genetic maps. Methodologies for the analysis of marker-assisted selection include the use of mapping populations segregating for desired characters and bulk segregant analysis. Genetic engineering offers a resolution to problems encountered by traditional *Prunus* breeding programs including long juvenility period and large space requirements for breeding populations. A number of genetically modified *Prunus* cultivars have been obtained using different gene transfer methods. Additional research work is still required to fully develop the next generation of gene vectors and transgenic plants.

Key words: Almond, apricot, cherry, gene transfer, germplasm, molecular markers, peach, plum, propagation techniques.

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[1] This review is dedicated to Dr. Dale E. Kester, who died November 21, 2003, at Davis (California). Dr. Dale E. Kester was Professor of Pomology emeritus at the University of California, Davis. He taught courses in Plant Propagation and Pomology for four decades and authored over 100 research and technical publications. He always demonstrated great enthusiasm for the incorporation of new technologies to Prunus breeding and is remembered as a dedicated, hard-working scientist, teacher and human being whose guidance and insight are missed.

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INTRODUCTION

The *Prunoideae*, a subfamily of *Rosaceae*, includes several species producing edible drupes with economic importance. In 2002, worldwide annual production of *Prunoideae* exceeded 30 million metric tons, including almost 13.9 million tons of nectarines and peaches (*Prunus persica* (L.) Batsch), 1.8 million tons of almonds (*Prunus amygdalus* Batsch = *Prunus dulcis* (Miller) D.A. Webb), 2.7 million tons of apricots (*Prunus armeniaca* L.), 1.8 million tons of sweet cherries (*Prunus avium* L.), 0.9 million tons of sour cherries (*Prunus cerasus* L.) and 9.3 million tons of plums (*Prunus domestica* L.) (FAO, 2003). *Prunus* species are characterized by developing only one ovary in which two ovules typically form, one of which degenerates soon after anthesis. The fruit is a drupe where the mature, stony endocarp together with the seed forms a propagation unit comparable to a botanical seed surrounded by its protective testa.

Breeding practices in *Prunus* face unique challenges resulting from the narrow genetic background of commercial cultivars (Scorza *et al*., 1985), a long juvenile period combined with large plant size, and differences in trait expression between juvenile and mature trees (Baird *et al*., 1996). In the last decade, many techniques such as *in vitro* culture and the use of molecular markers have become available for *Prunus* crop breeding. These methods are now moving from laboratory evaluation to field application. Concurrently, different aspects of the emerging biotechnologies, including marker-assisted selection and genome mapping, as well as the impact of altered gene expression on *Prunus* sp., have now been extensively reviewed (Mehlenbacher, 1995; Baird *et al*., 1996; Luby and Shaw, 2001; Scorza, 2001).

This article offers an overview of the current approaches being developed to optimize breeding efficiency by improving both germplasm in general and important horticultural traits in particular. These approaches include germplasm introgression, propagation techniques, use of molecular markers, and gene transfer technologies.

GERMPLASM IMPROVEMENT

Related Prunus species. The available germplasm in *Prunus* is diverse and the origin and dissemination for several species have been extensively reviewed (Kester *et al*., 1991; Faust and Timon, 1995; Faust and Surányi, 1997, 1999; Faust *et al*., 1998). Considerable genetic variation for these species still exists, mainly in the mountainous areas of Central Asia from the Tian Shan region in China to Kurdistan, including Turkestan, Afghanistan, Iran and Iraq. However, in cultivated germplasm, a limited gene pool restricts production to specific areas and conditions (Scorza *et al*., 1985). The introduction of genes from related species through interspecific hybridization has been used in several breeding programs throughout the world primarily to develop better-adapted rootstocks.

Rootstock breeding programs using interspecific hybridization have introduced useful traits including size control, adaptation to the new environments and pest resistance. Interspecific crosses between *Prunus* species (primarily peach x almond, but also *P. webbii* x peach, and others) have been widely utilized in almond rootstock breeding in France (Bernhard, 1949), USA (Kester and Hansen, 1966), Spain (Felipe, 1975), and Yugoslavia (Vlasic, 1977).

The introgression of almond germplasm from related species, including *P. webbii* (Spach) Vieh., *P. argentea* Lam, *P. persica*, *P. bucharica* Korshinsky, *P. mira* Koehne and *P. scoparia* Batal. has allowed transfer of several useful traits including self-compatibility, fungal and pest resistance, and frost and drought tolerance (Grasselly, 1976; Kester *et al*., 1991; Kester and Gradziel, 1996; Gradziel *et al*., 2001a). *P. davidiana* (Carr.) Frans. has recently been reported to be a source of plum pox virus (PPV) resistance for peach (Pascal *et al*., 2001), while the introgression of *Prunus mandshurica* (Maxim.) Koehne genes to apricot have improved frost resistance in Eastern

and Central European programs (Paunovic, 1988).

In almond, the absence of extensive crossing barriers in either the initial hybridization or subsequent backcrosses demonstrates a direct accessibility of this rich germplasm to breeding (Browicz and Zohary, 1996; Gradziel *et al*., 2001a). Potential barriers to successful interspecific gene introgression include male sterility, poor germplasm maintenance, and problems associated with character quality (Smith, 2002). The encouraging performance of interspecific hybrids and backcrosses to date, support continuing opportunities for transferring useful traits, including self-compatibility, resistance to important pests and diseases, improvement of seed oil quality, tolerance to aberrant environments, and modified tree architecture and bearing habit (Gradziel *et al*., 2001a) (Table 1). International collaborations have further allowed a more thorough evaluation of wild and related germplasm prior to extensive gene introgression (Esquinas-Alcazar, 1993), and have helped minimize breeding obstacles imposed by quarantine restrictions (Mora-Aguilera *et al*., 1998).

Table 1. *Prunus* species with reported value for the genetic improvement of peach and almond.
Cuadro 1. *Especies de* Prunus *descritas de gran valor para el mejoramiento genético de duraznero y almendro.*

Section	Species	Use in peach and almond breeding[1]
Amygdalus Spach.	*P. persica* (L.) Batsch.	Self-compatibility and pest and disease resistance in almond
	P. davidiana (Carr.) Franch.	Disease resistance in peach and self-compatibility in almond
	P. mira Koehne.	Disease resistance in peach and self-compatibility in almond
	P. dulcis (Mill.) D.A. Webb	Pest and disease resistance in peach
	P. argentea Lam.	Self-compatibility and frost resistance in almond
	P. bucharica Korschinsky	Self-compatibility, growth habit and frost resistance in almond
	P. kuramica Korschinsky	Self-compatibility and disease resistance in almond
	P. webbii (Spach) Vieh.	Self-compatibility and growth habit in almond
Chameamygdalus Spach.	*P. petunikowii* Lits.	Pest and disease resistance in almond
	P. tangutica Batal.	Pest and disease resistance in almond
Spartioides Spach.	*P. scoparia* Batal.	Self-compatibility and drought resistance in almond
Leptopus Spach.	*P. pedunculata* Pall.	Pest and disease resistance in almond

[1]Hesse, 1975; Grasselly, 1976; Denisov, 1988; Kester *et al*, 1991; Kester and Gradziel, 1996; Scorza and Sherman, 1996; Gradziel *et al*., 2001a, 2002.

Multiple embryos. Twin seeds (multiple embryos within the same seed coat) occur spontaneously in several *Prunus* species including peach and almond (Hesse, 1971; Kester and Gradziel, 1996). The occurrence of these multiple embryos varies greatly among years and is strongly influenced by environmental conditions. This phenomenon has been mainly studied in peach and in the almond cultivars 'Nonpareil' and 'Mission'. Seedlings from the same twin peach seed are frequently viable and show similar growth habits, though occasionally one of the seedlings show weak growth and develops poorly (Hesse, 1971; Gulcan, 1975). Some of these low-vigor plants have been shown to be haploids from which true-breeding dihaploids can be generated (Hesse, 1971) for genetic studies, hybrid rootstock production, and transformation and regeneration studies. Some of the low-vigor, twin almond seedlings were found to be aneuploids (Martínez-Gómez and Gradziel, 2003) and thus, have value for developing near isogenic lines (NIL). A collection of these haploid/aneuploid NILs are presently being developed to aid in genetic (locating genes, selective transfer of particular chromosomes) and molecular (isolation and sequencing of genes, genetic transformation, etc.) studies as demonstrated by Muehlbauer *et al*. (1988) and Young *et al*. (1988).

NEW PROPAGATION TECHNIQUES

In vitro evaluation of agronomic traits. The possibility of growing plants and even isolated plant cells in a test-tube under controlled *in vitro* conditions offers unique opportunities for improving selection efficiency. Advantages include minimizing environment influences, the potential to handle large numbers of individuals in a very small space, and accelerated growth and development (Wenzel and Foroughi-Wehr, 1993). The increasing availability of other biotechnological techniques (biochemical markers, DNA analysis, transient reporter transgene expression, genetic transformation, etc.) further complements *in vitro* culture opportunities.

Wenzel and Foroughi-Wehr (1993) reported the application to herbaceous species of *in vitro* callus culture for the selection of resistance to environmental stress (freezing tolerance in wheat, salt tolerance in rice) and diseases (*Phytophthora* sp. and *Fusarium* sp.), and herbicide tolerance in tobacco. The application of *in vitro* culture techniques for the selection of horticultural characters, however, may be more difficult. For temperate fruit species, tissue culture propagation has progressed rapidly during the last years. The application of tissue culture techniques as alternative propagation methods has been reported as early as the 1960s. Initially, tissue culture has involved micropropagation and somatic embryogenesis. Axillary shoot production (meristem culture) is the system most frequently utilized to regenerate plantlets by micropropagation techniques (Hammerschlag, 1986, Bornman, 1993; Pérez-Tornero *et al*., 1999). Research in somatic embryogenesis has recently increased in anticipation of more widespread attempts at regeneration of genetic transformants (Singh and Sansavini, 1998). Tissue culture has numerous potential applications for temperate fruit and nut tree species, including propagation of rootstocks, own-rooted scion cultivars, virus-free stock plants, and elite genotypes (Negueroles and Jones, 1979; Hutchinson, 1987; Gella and Errea, 1998). These techniques also offer unprecedented opportunities for the evaluation of horticultural traits in breeding programs. Applications have been reported in *Prunus* for the evaluation of the compatibility between cultivar and rootstock (Jonard, 1986), the resistance to abiotic stress (Datée and Branchard, 1986), and the resistance to biotic stress (Martínez-Gómez and Dicenta, 2000).

Recently, a method for measuring *in vitro* sprouting success for populations of individual vegetative buds of almond has been developed to assess their bud viability (sprouting percentage) and state of dormancy (sprouting rate) (Kester *et al*., 2003). Two periods of dormancy in the annual growth and development cycle could be distinguished. The first period of growth suppression (paradormancy) occurs during late spring and summer as the trees are subjected to increasing ambient summer temperature and decreasing soil moisture. The second period (endodormancy) occurs during the late fall and winter and is associated with winter chilling. These periods of suppressed bud growth coincide with the occurrence of adverse growth in the desert regions of central Asia where the almond originated. Similar growth conditions occur in the Mediterranean climates of southern Europe, California, Australia and other areas where commercial almond production presently occurs. Results of this research showed that the apparent somaclonal breakdown of a crucial high temperature gene (HTD) controlling paradormancy is associated with the development of noninfectious budfailure (BF), an economically important, epigenetic-like disorder in almond (Kester *et al*., 2003).

In vivo micrograft. Grafting has been widely used over the centuries for asexual propagation of fruit trees (Hartman and Kester, 1959). Micrografts, developed in the 1970s, involve the grafting of millimeter-size vegetative meristems. Initially, this technique was used for virus elimination in fruit trees. Subsequently, it has been used for the early assessment of rootstock-scion incompatibility, commercial multiplication, virus detection and phytoplasma studies (Navarro *et al*., 1982; Deogratias *et al*., 1986; Gebhardt and Goldbach, 1988; Jarausch *et al*., 2000).

Micrografts proved to be a useful technique when the early propagation of plant material was desired

and to invigorate weak material. Optimum propagation efficiency is achieved by maximizing the different parameters involved in micropropagation as well as subsequent shoot growth (Martínez-Gómez and Gradziel, 2001a). *In vivo* micrografting avoids tissue culture transplant shock when transplanting from sterile *in vitro* conditions. This technique has been employed to recover aneuploids of almond, which occur at low frequencies in sexual embryos with seeds (Martínez-Gómez and Gradziel, 2001b).

Artificial growth cycles. The growth of woody plants from temperate climates requires a periodic endodormancy, which can be artificially achieved through controlled rest periods in cold chambers (Pedryc *et al.*, 1999; Martínez-Gómez *et al.*, 2000b). Two cycles of vegetative growth per year can be carried out by employing two 4-month periods of growth in the greenhouse and two 2-month periods of rest in cold chambers. The size of the pots, the periodic renewal of the soil and the control of mites and fungi are key considerations for the successful management of almond seedlings under these controlled conditions (Martínez-Gómez *et al*., 2000b). The cold treatments can also be used to control fungi and mites. Seedlings, properly maintained under these conditions, can provide vigorously growing plant tissue (e.g., leaves, root tips) throughout the year. This approach has been used in virus resistance studies, as well as to maintain quarantine conditions (e.g. in the study of dangerous viruses such as the PPV causing sharka disease; Martínez-Gómez *et al*., 2000a). Artificial cycles of growth have also been used to invigorate weak genetic material such as the aneuploid seedlings of almond (Martínez-Gómez and Gradziel, 2003). Similarly, artificial cold treatments have been successfully utilized in studies of frost tolerance in stone fruit flowers (Pedryc *et al*., 1999).

USE OF MOLECULAR MARKERS

Characterization of genotype identity and genetic relationships. Traditionally, the identification and characterization of *Prunus* cultivars and species has been based on morphological and physiological traits. However, such traits are not always available for analysis and are affected by changing environmental conditions. Molecular marker technology offers several advantages over the sole use of conventional markers. Molecular markers developed for *Prunus* also offer a powerful tool to study the evolution of the genome, and for understanding of genome structure and determinants of genetic diversity (Wünsch and Hormaza, 2002a).

Isoenzymes: Isoenzymes were among the first genetic markers to be widely utilized. They have been used for cultivar identification in *Prunus* because of their environmental stability, their codominant expression, and their good reproducibility. Nevertheless, their utilization is limited by the small number of loci that can be analyzed with conventional enzyme staining methods, as well as a low variation in some loci. Electrophoretic surveys were particularly useful in characterizing almond (Arulsekar *et al*., 1986; Hauagge *et al*., 1987; Cerezo *et al*., 1989; Sathe *et al*., 2001) and plum (Byrne and Littleton, 1988a) cultivars, because both almond and plum are outcrossing species with high level of isoenzyme polymorphisms. In contrast, peach, a predominantly autogamous species, shows few isoenzyme polymorphisms in spite of its extensive morphological variability (Arulsekar *et al*., 1986; Durham *et al*., 1987; Mowrey *et al*., 1990; Agarwal and Nath, 2001). Apricot shows intermediate levels of variability (Arulsekar *et al*., 1986; Byrne and Littleton, 1989a), with the predominantly outcrossing non-European populations exhibiting higher isoenzyme variability than the predominantly inbreeding European populations (Byrne and Littleton, 1989a). Isoenzyme analysis has also been used to identify different interspecific hybrids (Byrne and Littleton, 1988b, 1989b; Mowrey *et al*., 1990; Bošković *et al*., 1997b) and detect phylogenetic relationships among species (Mowrey and Werner, 1990). More recently, isoenzymes in combination with DNA-based markers were employed to create the genetic maps for woody perennials (Weeden, 1994) and for the genetic characterization of multiple embryos in almond (Martínez-Gómez and Gradziel, 2003).

RFLPs: Restriction fragment length polymorphism (RFLP) markers are based on the differential hybridization of cloned DNA to bulk DNA fragments from restriction-enzyme digestion. Thus, RFLPs are defined by specific enzyme-probe combinations (Tanksley *et al*., 1989). RFLP markers are codominant. The primary sources of clones for RFLP mapping are cDNA clones and Pstl-derived genomic clones. Genomic clones that represent random sequences may be a poor choice for hybridization probes because of the large percentage of repeated sequences. RFLPs can detect a virtually unlimited number of markers, thus providing an efficient method for discovering linkages among markers and for constructing genetic maps. This is particularly important in *Prunus* because of the relatively low level of variation typically present in this genus. There are several reports of the use of RFLPs in *Prunus* for map-based selection (Rajapakse *et al*., 1995; Viruel *et al*., 1995; Dirlewanger *et al*, 1998; Wang *et al*., 2000) and for elucidating the extent of genetic variability (de Vicente *et al*., 1998). However, RFLP analysis has important limitations: it is laborious and time-consuming and it often involves the use of radioisotopes. To overcome some of the difficulties, an alternative called sequence tagged sites (STS) has been developed (Olson *et al*., 1989) which is PCR-based but not requiring radioactive probing. Different STS markers tightly linked to the resistance genes to root-knot nematodes have been successfully developed and can be utilized for introgression of new root-knot nematode resistance genes into peach rootstocks (Yamamoto and Hayashi, 2002).

RAPDs: Random amplified polymorphic DNA (RAPD) markers are based on the PCR amplification of random locations in the genome (Welsh and McClelland, 1990). RAPDs are characterized by using arbitrary primers and permit the quick construction of genetic maps and the saturation of specific genomic regions with molecular markers. A single oligonucleotide is utilized for the amplification of genomic DNA. In contrast to isoenzymes and RFLPs, RAPDs are dominant markers. This feature, as well as their variable degree of repeatability and problems in transferring across populations, limits their utilization primarily to map construction. RAPD techniques have been successfully used in *Prunus* for identifying cultivars (Lu *et al*., 1996), estimating genetic diversity and assessing possible origins for selected genotypes (Warburton and Bliss, 1996; Bartolozzi *et al*., 1998; Martins *et al*., 2003; MirAli and Nabulsi, 2003), and construction of maps. Problems with DNA quality and a general sensitivity to changes in the reaction conditions can hamper the routine utilization of RAPD markers. These difficulties can be overcome by converting RAPDs to sequence-characterized amplified regions or SCARs (Paran and Michelmore, 1993). In contrast to RAPD and AFLP methods, SCAR is a PCR-based method that employs specific primers. These primers amplify single bands corresponding to genetically defined loci. SCARs can potentially be converted into codominant markers and are less sensitive to reaction conditions. Different SCAR markers are being evaluated for marker-assisted selection in *Prunus*, including identification of the *Ma1* root-knot nematode resistance gene in Myrobalan plum (Lecouls *et al*., 1999) and the identification of the *Ff* (flesh adhesion) gene in peach (Jun *et al*., 2002).

AFLPs: Amplified restriction fragment length polymorphism (AFLP) technology is a powerful DNA fingerprinting technology based on the selective amplification of a subset of genomic restriction fragments using PCR (Vos *et al*., 1995). DNA is digested with restriction endonucleases and double-stranded specific adapters are ligated to the ends of the DNA fragments to obtain template DNA for subsequent amplification by PCR. The subset of amplified fragments is then analyzed by denaturing PAGE to generate the fingerprint. AFLP has a number of advantages over the RAPD technique: more loci analyzed per experiment and better reproducibility of banding patters resulting from the higher specificity of primer annealing to complementary adapters. Powell *et al*. (1996) found that AFLPs had a much higher multiplex ratio (number of polymorphic products per "reaction") than other molecular marker systems. Consequently, AFLPs also shows a higher marker index. These

markers have been mainly used in *Prunus* for genetic mapping and molecular characterization such as the estimation of genetic diversity among apricot cultivars (Hurtado *et al*., 2002a).

SSRs: PCR-based, simple sequence repeat (SSR) markers (microsatellites) are becoming the marker of choice for fingerprinting and genetic diversity studies for a wide range of plants (Gupta *et al*., 1996). Because of their high polymorphism, abundance, and codominant inheritance, they are well suited for the assessment of genetic variability within crop species, and of the genetic relationships among species (Powell *et al*., 1996). In the case of *Prunus* species, primer pairs flanking SSRs have been cloned and sequenced in peach (Cipriani *et al*., 1999; Downey and Iezzoni, 2000; Sosinski *et al*., 2000; Testolin *et al*., 2000; Aranzana *et al*., 2002, 2003a; Dirlewanger *et al*., 2002; Georgi *et al*., 2002; Wang *et al*., 2002; Yamamoto *et al*., 2002), apricot (Decroocq *et al*., 2003), and cherry (Downey and Iezzoni, 2000; Cantini *et al*., 2001; Schueler *et al*., 2003). These SSR markers were used for the molecular characterization and identification of cultivars in different species including peach, almond, apricot, cherry and *Prunus* rootstocks using different methods for the analysis of the DNA (Table 2). In addition, SSR markers were used also for genetic mapping in peach (Sosinski *et al*., 2000; Dettori *et al*., 2001; Etienne *et al*., 2002; Aranzana *et al*., 2003b), almond (Joobeur *et al*., 2000; Bliss *et al*., 2002), and apricot (Hurtado *et al*., 2002b; Vilanova *et al*., 2003). Electrophoresis in polyacrilamide with radioactive and silver staining was the first method used in the analysis of the PCR amplified fragment of DNA obtained from the SSR markers. Electrophoresis in Metaphor® agarose was a method used as alternative to the polyacrilamide due to its easier application. More recently, new methods for the PCR amplified DNA have been developed including the utilization of ABI® sequencer (Table 2).

Table 2. Reports of molecular characterization of *Prunus* species by microsatellite markers (SSR) using different methods to analyze amplified DNA.
Cuadro 2. *Reportes de autores que han efectuado caracterización molecular de especies de* Prunus *mediante marcadores microsatélites (SSR) utilizando diferentes métodos de análisis del ADN amplificado.*

	Methods for the DNA analysis			
Species	Polyacrylamide Radioactive	Polyacrylamide Silver staining	Metaphor® agarose	ABI® sequencer
Peach	Aranzana *et al*., 2002; Georgi *et al*., 2002; Sosinski *et al*., 2000; Testolin *et al*., 2000	Cipriani *et al*., 1999; Dirlewanger *et al*., 2002; Bliss *et al*., 2002; Martínez-Gómez *et al*., 2003a	Cipriani *et al*., 1999; Dettori *et al*., 2001; Martínez-Gómez *et al*., 2003a	Aranzana *et al*., 2003a; Wang *et al*., 2002; Yamamoto *et al*., 2002; Etienne *et al*., 2002
Almond		Joobeur *et al*., 2000; Bliss *et al*., 2002; Martínez-Gómez *et al*., 2003a	Martínez-Gómez *et al*., 2003a	
Apricot	Zhebentyayeva *et al*., 2003	Hurtado *et al*., 2002a; Vilanova *et al*., 2003; Decroocq *et al*., 2003	Hormaza, 2002; Sánchez-Pérez *et al*., 2005	Decroocq *et al*., 2003
Cherry	Downey and Lezzoni, 2000; Aranzana *et al*., 2002; Schueler *et al*., 2003	Cantini *et al*., 2001; Dirlewanger *et al*., 2002; Struss *et al*., 2003	Wünsch and Hormaza, 2002b	
Other Prunus	Cipriani *et al*., 1999	Decroocq *et al*., 2003; Martínez-Gómez *et al*., 2003b	Serrano *et al*., 2002; Martínez-Gómez *et al*., 2003b	

Studies of genetic diversity and genetic relatedness utilizing molecular markers thus offer unprecedented opportunities for improving *Prunus* breeding efficiency when using either established cultivars or interspecific germplasm. Similar opportunities have also been demonstrated for the analysis of chloroplast DNA (Badenes and Parfitt, 1995). SSRs are currently being employed for the molecular characterization and estimation of genetic diversity and the genetic relationships among peach and almond cultivars and related *Prunus* species (Serrano *et al*., 2002; Martínez-Gómez *et al*., 2003b). In addition, recent studies have shown promise for analyzing variation of internal transcribed spacers (ITS) in nuclear ribosomal DNA (Lee and Wen, 2001) and chloroplast DNA (Bortiri *et al*., 2001).

Genetic mapping. Several intraspecific and interspecific *Prunus* maps have been developed using different types of molecular markers. The utilization of PCR-based markers has made mapping and tagging of a wide range of traits possible (Arús *et al*., 1994; Baird *et al*., 1996). The analysis of cosegregation among markers greatly facilitates linkage analysis between markers and major or quantitative loci controlling horticulturally important traits.

Different research teams have released linkage maps using morphological traits, isoenzymes, RFLPs and RAPDs in peach (Chaparro *et al*., 1994; Dirlewanger and Bodo, 1994; Rajapakse *et al*., 1995; Warburton *et al*., 1996; Abbott *et al*., 1998; Dirlewanger *et al*., 1998, 1999; Lu *et al*., 1998; Sosinski *et al*., 1998; Dettori *et al*., 2001; Yamamoto *et al*., 2001), almond (Viruel *et al*., 1995; Joobeur *et al*., 2000; Jauregui *et al*., 2001; Bliss *et al*., 2002), sweet cherry (Stockinger *et al*., 1996), sour cherry (Wang *et al*., 2000), apricot (Hurtado *et al*., 2002b; Vilanova *et al*., 2003), and peach x almond hybrids (Foolad *et al*., 1995; Joobeur *et al*., 1998). Similarly, AFLPs allow detection of a higher level of polymorphism in peach than isoenzymes, RFLPs or RAPDs (Dirlewanger *et al*., 1998, 2003). SSR has also been used for mapping in peach (Dettori *et al*., 2001; Etienne *et al*., 2002; Dirlewanger *et al*., 2003) and almond (Joobeur *et al*., 2000; Aranzana *et al*., 2003b). The first genetic linkage map for a *Prunus* rootstock population was constructed using AFLPs (Lu *et al*., 1998).

The similar order of markers observed in different *Prunus* maps suggests a high level of synteny within the genus (Aranzana *et al*., 2003b; Dirlewanger *et al*., 2003). This homology among *Prunus* species partly explains the low level of breeding barriers to interspecific gene introgression and supports the opportunity for successful gene transfer between closely related species.

Another strategy to locate new markers in a known genetic linkage map is the "selective" or "bin" mapping approach. This technique uses a subset of plants that belongs to a population from which a map is already available. This subset of plants is selected to maximize the information on linkage, so that their joint genotype for any marker identifies a unique genome fragment (a bin) as small as possible. This strategy reduces the time and cost of mapping and is adequate to simplify the construction of high density maps or to add large amounts of interesting markers (such as SSRs or EST-derived markers) to a preexistent map. Recently, Howad and Arús (2004) have incorporated 151 SSRs to the *Prunus* reference map using only 6 individuals from the TxE ('Texas' x 'Earlygold') *Prunus* reference population. The use of this set of 6 individuals promises to be a useful resource for *Prunus* geneticists in future.

Marker-assisted selection. Marker-assisted selection (MAS) is emerging as a very promising strategy for increasing selection gains (Knapp, 1998). If sufficient mapping information is known, MAS can dramatically shorten the number of generations required to "eliminate" the undesired genes of the donor in backcrossing programs (Arús and Moreno-González, 1993). Marker loci linked to major genes can be used for selection, which is sometimes more efficient than direct selection for the target gene (Arús and Moreno-González, 1993; Baird *et al*., 1996). Selection by molecular markers is particularly useful in fruit, nut, and other tree crops with a long juvenile period, and when the

expression of the gene is recessive or the evaluation of the character is otherwise difficult, as with resistance to biotic or abiotic stress (Luby and Shaw, 2001; Scorza, 2001; Testolin, 2003).

The principal approach for the analysis of marker-trait association in *Prunus* is the use of mapping populations segregating for the characters of interest. The different linkage maps developed in *Prunus* (peach, almond, cherry, and apricot) include markers associated with several traits of horticultural value. Mapping quantitative characters by identifying quantitative trait loci (QTL) is also becoming an important tool in tree breeding. QTLs are generally recognized by comparing the degree of covariation for polymorphic molecular marker with phenotypic trait measurements. Important characters and QTLs that are presently being mapped in stone fruits include the control of flower (bloom time, self-incompatibility, pollen-sterility, double-flowers), fruit (shape, pubescence, flesh color, acidity and sweetness), leaf (red vs. green color) and tree (pillar or weeping architectures) traits, and resistance to various pests and diseases including root-knot nematodes, powdery mildew, leaf curl, and Plum pox potyvirus (Asins *et al*., 1994; Dirlewanger *et al*., 1996, 1998, 1999; Abbott *et al*., 1998; Ballester *et al*., 1998, 2001; Bliss *et al*., 2002; Etienne *et al*., 2002; Hurtado *et al*., 2002b) (Tables 3 and 4). The high degree of genome synteny observed among *Prunus* species (Aranzana *et al*., 2003b; Dirlewanger *et al*., 2003) should also facilitate the successful transfer of sets of markers and coding sequence among species.

Bulk segregant analysis (BSA), where two pooled DNA samples are formed from plant sources that have similar genetic backgrounds but differ in one particular trait, is another promising approach for the analysis of molecular marker-horticultural trait association. This method also makes possible the identification of markers linked to the trait of interest (Michelmore *et al*., 1991). A strategy combining different markers with bulk segregant analysis was used to identify markers linked to loci of specific characters in peach and peach x almond crosses (Warburton *et al*., 1996), RAPD markers flanking the red-leaf (Gr) and malate dehydrogenase loci in the NC174RL x Pillar and Marsun x White Glory F2 peach families (Chaparro *et al*., 1994), and three RAPD markers associated with a gene conferring delayed bloom in almond (Ballester *et al*., 2001). BSA has facilitated the study of self-incompatibility and male sterility in almond (Badenes *et al*., 2000). BSA and RAPD analysis were recently utilized to distinguish markers linked to the *Ma1* gene, a major dominant gene that controls a wide-spectrum resistance to root-knot nematodes in Myrobalan plum (Lecouls *et al*., 1999), as well as markers linked to resistance to PPV in apricot (Salava *et al*., 2001) and to ring nematode in peach (Blenda *et al*., 2002) (Table 3).

The first genetic linkage map for a *Prunus* rootstock population was constructed using AFLP technology (Lu *et al*., 1998) and, simultaneously, two genes that control resistance to root-knot nematodes, *Mi* and *Mij*, were mapped and tagged. The conversion of the AFLP marker linked to the *Mij* (a gene required for resistance to *Meloidogyne incognita* and *javanica*) locus to STS proved to have practical application for germplasm screening and for breeding peach rootstocks for resistance to root-knot nematodes (Lu *et al*., 1999, Yamamoto and Hayashi, 2002) (Table 3).

Apart from isoenzymes, RFLP, RAPD, AFLP, and SSR, other markers being used in the development of marker associated traits, are those based on single point mutations (SNPs) and those obtained from either cDNA sequences (ESTs) or databases (Cloned Gene Analogs, CGAs) (Van Nocker *et al*., 2002; Testolin, 2003). The large-scale single-pass sequencing of ESTs can give a global picture of the genes involved in the development and function of organs and tissues. A recent collection of ESTs from peach and almond based on cDNA libraries has been released to public databases, and more than 3,800 putative unigenes have been detected (www.genome.clemson.edu/gdr/). This work is complementary to others regarding EST development in *Prunus*. A collection of 6,817 ESTs was prepared using four cDNA libraries obtained from peach mesocarp (Lazzari et al., 2004) in relation

to the program of the Italian National Consortium for Peach Genomics (www.itb.cnr.it/ESTree). Also, the release of *Prunus* microarrays using unigene sets as probes has started. A group of nearly 4,600 unique ESTs derived from peach mesocarp and developing almond seeds have been sequenced to analyze the expression profile of the unigene set during fruit development and the identification of additional genes involved in this process (McCord *et al*., 2004).

Table 3. Markers associated to main monogenic or oligogenic traits in *Prunus*.
Cuadro 3. *Marcadores asociados a los principales caracteres monogénicos u oligogénicos en* Prunus.

Specie	Trait	Symbol	Marker[1]	Reference
Peach	Leaf color	Gr	RAPD	Chaparro *et al*., 1994
	Leaf color	Gr	SSR	Yamamoto *et al*., 2001
	Leaf glands	E	RFLP	Dettori *et al*., 2001
	Leaf glands	E	RFLP	Quarta *et al*., 2000
	Double flower	Dl	AFLP	Sosinski *et al*., 2000
	Male sterility	Ps	AFLP	Dirlewanger *et al*., 1999
	Skin hairiness	G	AFLP	Dirlewanger *et al*., 1999
	Skin hairiness	G	RFLP	Bliss *et al*., 2002
	Skin color	Sc	SSR	Yamamoto *et al*., 2001
	Flat fruit	S	RFLP	Dirlewanger *et al*., 1999
	Flesh color	Y	RAPD	Warburton *et al*., 1996
	Flesh color	Y	AFLP	Abbott *et al*., 1998
	Flesh color	Y	RFLP	Bliss *et al*., 2002
	Flesh adhesion	F	RFLP	Abbott *et al*., 1998
	Flesh adhesion	F	RFLP	Dettori *et al*., 2001
	Flesh adhesion	F	RFLP	Quarta *et al*., 2000
	Flesh adhesion	F	AFLP	Yamamoto *et al*., 2001
	Non acid fruit	D	RAPD	Dirlewanger *et al*., 1999
	Non acid fruit	D	RFLP	Bliss *et al*., 2002
	Nematode resistance	Mij	AFLP	Abbott *et al*., 1998
	Nematode resistance	Mij	AFLP	Lu *et al*., 1998
	Nematode resistance	Mij	STS	Lu *et al*., 1999
	Nematode resistance	Mia	AFLP	Yamamoto and Hayashi, 2002
	Nematode resistance	Mja	STS	Yamamoto and Hayashi, 2002
	Nematode resistance	Mja	AFLP	Blenda *et al*., 2002
Almond	Self-incompatibility	SI	RFLP	Joobeur *et al*., 1998
	Self-compatibility	Sf	RFLP	Arús *et al*., 1999
	Kernel taste	Sw	RFLP	Bliss *et al*., 2002
	Shell hardiness	D	RFLP	Arús *et al*., 1999
	Late blooming	Lb	RAPD	Ballester *et al*., 2001
Apricot	PPV resistance	Ppv	SSR	Hurtado *et al*., 2002b
	PPV resistance	Ppv	AFLP	Salava *et al*., 2001
	PPV resistance	Ppv	SSR	Vilanova *et al*., 2003
	Self-incompatibility	SI	RAPD	Badenes *et al*., 2000
	Male sterility	Ps	RAPD	Badenes *et al*., 2000
Cherry	Self-incompatibility	SI	EST	Tao *et al*., 1997
	Self-compatibility	SC	EST	Sonneveld *et al*., 2001
	Dwarf habit	Dw	RFLP	Arús *et al*., 1999
Plum	Nematode resistance	Ma1	RAPD	Salesses *et al*., 1998
	Nematode resistance	Ma1	SCAR	Lecouls *et al*., 1999

[1]RAPD: Random amplified polymorphic DNA, SSR: Simple sequence repeat, RFLP: Restriction fragment length polymorphism, AFLP: Amplified restriction fragment length polymorphism, EST: Expressed sequence tags, STS: Sequence tagged site, SCAR: Sequence characterized amplified region.

Table 4. Markers associated to main polygenic traits (QTLs) in *Prunus*.
Cuadro 4. *Marcadores asociados a los principales caracteres poligénicos (QTLs) en* Prunus.

Specie	Trait	Marker[1]	Reference
Peach	Leaf curl resistance	RAPD	Viruel *et al*., 1998
	Internode length	RFLP	Verde *et al*., 2002
	Powdery mildew resistance	RFLP	Quarta *et al*., 2000
	Flowering time	RFLP	Dirlewanger *et al*., 1999
	Flowering time	RFLP	Quarta *et al*., 2000
	Flowering time	RFLP	Verde *et al*., 2002
	Ripening time	RFLP	Quarta *et al*., 2000
	Ripening time	SSR	Verde *et al*., 2002
	Ripening time	RFLP	Dirlewanger *et al*., 1999
	Maturity time	SSR	Etienne *et al*., 2002
	Fruit develop cycle	RFLP	Abbott *et al*., 1998
	Fruit develop cycle	SSR	Etienne *et al*., 2002
	Productivity	RFLP	Dirlewanger *et al*., 1999
	Fruit diameter	AFLP	Abbott *et al*., 1998
	Fruit weight	RFLP	Abbott *et al*., 1998
	Fruit weight	RFLP	Etienne *et al*., 2002
	Fruit skin color	RFLP	Quarta *et al*., 2000
	Fruit skin color	SSR	Verde *et al*., 2002
	pH	RFLP	Abbott *et al*., 1998
	pH	RFLP	Etienne *et al*., 2002
	Titratable acidity	RFLP	Dirlewanger *et al*., 1999
	Titratable acidity	RFLP	Etienne *et al*., 2002
	Malic acid content	RFLP	Dirlewanger *et al*., 1999
	Malic acid content	RFLP	Etienne *et al*., 2002
	Citric acid content	RFLP	Dirlewanger *et al*., 1999
	Citric acid content	RFLP	Etienne *et al*., 2002
	Soluble solids	RFLP	Abbott *et al*., 1998
	Soluble solids	RFLP	Quarta *et al*., 2000
	Soluble solids	SSR	Etienne *et al*., 2002
	Soluble solids	SSR	Verde *et al*., 2002
	Fructose content	AFLP	Abbott *et al*., 1998
	Fructose content	RFLP	Etienne *et al*., 2002
	Glucose content	RFLP	Abbott *et al*., 1998
	Glucose content	RFLP	Dirlewanger *et al*., 1999
	Glucose content	RFLP	Etienne *et al*., 2002
Almond	Shell hardiness	RFLP	Arús *et al*., 1999
Cherry	Blooming time	RFLP	Wang *et al*., 2000
	Ripening time	RFLP	Wang *et al*., 2000
	Fruit weight	RFLP	Wang *et al*., 2000
	Soluble solids	RFLP	Wang *et al*., 2000

[1]RAPD: Random amplified polymorphic DNA, SSR: Simple sequence repeat, RFLP: Restriction fragment length polymorphism, AFLP: Amplified restriction fragment length polymorphism.

A very promising application of molecular marker assisted selection is for the manipulation of self-compatibility in *Prunus*. Most species are predominantly self-incompatibles. Self-incompatibility is of the gametophytic type and acts to prevent self-fertilization. This character is controlled by a single *locus* with multiple codominant alleles (Dicenta and García, 1993; Burgos *et al*., 1997), and is expressed within the styles of flowers as *S*-RNases glycoproteins

(Bošković *et al*., 1997a, 2003; Tao *et al*., 1997; Yaegaki *et al*., 2001) that are responsible of the subsequent inactivation of self-pollen tube growth. Almond self-incompatibility alleles (*S*-alleles) were initially identified in the field through controlled crosses with a series of known *S*-genotypes (Kester and Gradziel, 1996). More recently, molecular methods have been developed in two areas: identification of stylar *S*-RNases by electrophoresis in vertical polyacrilamide gels (Bošković *et al*., 1997a, 1999, 2003; Burgos *et al*., 1998), and the amplification of specific *S*-alleles using appropriately designed primers for PCR and electrophoresis in horizontal agarose gels (Tamura *et al*., 2000; Tao *et al*., 2000; Channuntapipat *et al*., 2001; Sonneveld *et al*., 2001, 2003). This technique is being routinely used for the identification of cross-incompatibility groupings for current almond cultivars and for efficiently breeding self-compatibility into new cultivars (Gradziel *et al*., 2001b; Ortega and Dicenta, 2003; Sánchez-Pérez *et al*., 2004).

GENE-TRANSFER TECHNOLOGIES

Genetic engineering can reduce the time and space required to improve fruit and tree characteristics compared with traditional plant breeding methodologies. A specific genetic change, with no addition of associated detrimental genes, can be accomplished rapidly, whereas traditional breeding programs require more generations and extensive acreage. Genetic engineering can also increase the diversity of genes and germplasm available by allowing the stable integration of foreign DNA into the plant genome. In the case of fruit trees, genetic engineering represents an alternative to overcome handicaps of traditional *Prunus* breeding programs (i.e. long juvenility period, self-incompatibility, evaluation of agronomic traits in the field, etc.) (Singh and Sansavini, 1998; Singh, 2000).

Even discrete genetic changes to a seemingly beneficial genotype can have unanticipated and undesirable consequences. Both the 'Tardy-Nonpareil' and the "Jeffries' mutation of the commercially important almond cultivar 'Nonpareil', while conferring the very desirable traits of late flowering and unilateral cross-compatibility with 'Nonpareil', respectively, failed commercially due to losses in tree productivity associated with the genetic change (Kester and Gradziel, 1996). In addition, the presence of genetic mosaics in regenerated transgenic plantlets could lead to later problems with losses of cultivar trueness-to-type in vegetatively propagated crops (Marcotrigiano and Gradziel, 1997).

Most transgenic *Prunus* plants have been obtained via *Agrobacterium*-mediated transformation because of its efficacy (Mante *et al*., 1990, 1991; Scorza *et al*., 1990; Smigocki and Hammerschlag, 1991; Cámara Machado *et al*., 1994; Archilletti *et al*., 1995, Miguel and Oliveira, 1999; Petri and Burgos, 2005). Much of this research involved the introduction of reporter genes in seedling tissues. The *gus* gene, which codes for β-glucuronidase, has been successfully utilized as a marker gene in genetic transformation of almond (Archilletti *et al*., 1995), peach (Xiaojian *et al*., 1994) and plum (Mante *et al*., 1990, 1991; Cámara Machado *et al*., 1994; Scorza *et al*., 1994). In addition, *npt*II (neomycin phosphotranspherase) gene, which confers kanamycin resistance, has been used in these species (Mante *et al*., 1990, 1991; Scorza *et al*., 1994; Archilletti *et al*., 1995). Peach has been also transformed using microprojectile bombardment-based protocols with integration of *gus* and *npt*II genes (Xiaojian *et al*., 1994). The recovery of transgenic forms of established cultivars, while being most desirable owing to their commercial acceptance, has had only limited success since these older cultivars have lost most juvenile-growth characteristics including the capacity for efficient regeneration from single cells (Marcotrigiano and Gradziel, 1997).

To manipulate tree architecture, transgenic peach seedlings expressing the *ipt* gene (which increases endogenous cytokinin levels and reduces apical dominance) were obtained (Hammerschlag and Smigocki, 1998). These plants showed reduced height and increased branching. In addition,

transgenic apricots and plums bearing the coat protein gene of the *Plum pox virus* (PPV) were successfully developed (Cámara Machado *et al*., 1992, 1994; Scorza *et al*., 1994). Transgenic plants, as well as the hybrids generated from them, showed a high level of resistance to PPV (Scorza *et al*., 1994, 1998; Ravelonandro *et al*., 1997). The transgenic plum clone C5 exhibited high level of resistance to graft inoculation of PPV and an apparent immunity to aphid-vectored infection in field tests. These results demonstrate the impact that can be achieved in the *Prunus* resistance to virus by gene-silencing (Scorza and Ravelonandro, 2002).

Currently, complex traits such as yield and flavor are not likely candidates for improvement by biotechnology. Moreover, there is a need for genes from *Prunus* to be cloned, since public concern about transgene technology makes a distinction between native and non-native genes. There is also a need for fruit- or leaf-specific promoters, so that gene expression may be targeted only to the parts of the plant necessary for the desired effect (Petri and Burgos, 2005). Finally, transgenic testing should ensure that there are no non-target effects and that transgenic lines are stable and non-chimeric.

CONCLUSIONS

The typical long generation time, along with the extensive space requirements and other limitations to generating the required large segregating progeny populations, have frustrated the development and testing of new, often molecular-based, breeding strategies. These same limitations, however, make new strategies that improve breeding efficiency particularly valuable to tree crops. In addition, most *Prunus* tree crops, because they are vegetatively propagated, have a unique advantage over other agronomic crops since desirable, unique gene combinations can be 'captured' by clonal propagation. A number of technologies will enable researchers to learn not only which *Prunus* genes are expressed but also their level of expression in a given cell type under specified conditions. Quantitative technologies for evaluating differential gene expression include not only quantitative PCR (qPCR), but also serial analysis of gene expression (SAGE), microarrays, differential display, and massively parallel signature sequencing (MPSS). Results from their use will help to answer long-standing questions, such as how cells respond to changes in their environment. Drought tolerance, for example, may be determined by many genetic factors. The "one-gene-at-a-time" approach for analyzing gene function is inadequate. It is now possible to locate multiple genes of tree crops in responding to environmental stresses. There are several major new molecular tools used for gene functional analysis, such as EST and microarray technology. Genetic engineering offers an alternative approach to accelerate traditional *Prunus* breeding programs. A number of genetically modified *Prunus* species have been obtained using different gene transfer methods though additional research is needed for developing new transgenes of value to tree crops as well as methods for the efficient regeneration of commercially established cultivars. Additional advantages encouraging the utilization of these new technologies to *Prunus* tree crop improvement include a small genome size, high levels of synteny between genomes, and a well-established international network of cooperation among researchers.

RESUMEN

La utilización de nuevo germoplasma, el desarrollo de marcadores moleculares, la utilización de técnicas alternativas de propagación y la transferencia de genes, se cuentan entre las novedosas herramientas aplicables al mejoramiento de *Prunus*. En la utilización de nuevo germoplasma, se persigue la introducción de genes de especies de *Prunus* silvestres que confieren caracteres agronómicamente valiosos tales como la autocompatibilidad, el hábito de crecimiento mejorado, la resistencia a la sequía, y la mejora de la calidad del fruto o semilla. Por otro lado, los estudios con semillas poliembriónicas (dos embriones dentro de una misma cubierta seminal) pueden facilitar los estudios genéticos y

citogenéticos de estas especies. Entre los métodos de propagación alternativos se encuentran las técnicas *in vitro* para la evaluación del material vegetal, y las técnicas de microinjerto *in vivo* que permiten la propagación temprana de genotipos de alto riesgo. Además, el cultivo de los *Prunus* bajo condiciones controladas en invernadero, incluyendo la inducción de un período de reposo artificial mediante el uso de tratamientos en cámara fría, provee una estrategia útil para obtener plantas de crecimiento vigoroso durante todo el año. Los marcadores moleculares también se han constituido en una herramienta esencial para los estudios de mejoramiento genético en *Prunus*. Se han utilizado distintas clases de marcadores moleculares, incluyendo isoenzimas, RFLPs, RAPDs, AFLPs y SSRs, para la caracterización genética del germoplasma, el establecimiento de relaciones génicas entre cultivares y especies, y la construcción de mapas genéticos. Las metodologías para el análisis de la selección asistida por marcadores incluye el uso del mapeo de poblaciones segregantes para caracteres deseables y el análisis de grupos segregantes. La ingeniería genética ofrece una resolución a problemas que enfrentan los programas de mejoramiento tradicionales de *Prunus*, incluyendo un período juvenil prolongado y requerimientos de grandes espacios para las poblaciones en cruzamiento. Se ha obtenido un número apreciable de cultivares de *Prunus* genéticamente modificados utilizando diferentes métodos de transferencia de genes. Sin embargo, se requiere aún trabajo de investigación adicional para desarrollar completamente la próxima generación de vectores de genes y plantas transgénicas.

Palabras clave: Almendro, cerezo, ciruelo, damasco, duraznero, germoplasma, marcadores moleculares, técnicas de propagación, transferencia de genes.

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