11 Almond

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11.1 Introduction

The almond [*P. dulcis* (Mill.) D.A. Webb; syn. *P. amygdalus* Batsch] is a species of genus *Prunus* and subgenus *Amygdalus* (Rosaceae, subfamily Prunoideae) that is commercially grown worldwide. The cultivated almond is thought to have originated in the arid mountainous regions of Central Asia (Grasselly 1976a). Several wild species are also found growing in these mountainous areas from Tian Shan mountain in western China through the mountainous areas and deserts of Kurdistan, Turkestan, Afghanistan and into Iran and Iraq (Grasselly 1976b; Kester and Gradziel 1996). The *Prunus* species *P. fenzliana* (Fritsch) Lipsky, *P. bucharica* Korschinsky and *P. kuramica* Korschinsky (of the Section Euamygdalus) from these regions are described as the wild species most closely related to almond (Grasselly 1976b; Browick and Zohary 1996), and may be the ancestral species of the modern cultivated almond (Kester et al. 1991). Ladizinsky (1999), however, identified only *P. fenzliana* as the wild ancestor of almond. *P. webbii* (Spach) Vieh, which is thought to have originated on the Balkan peninsula, is also described as closely related to almond (Grasselly 1976a, b; Browick and Zohary 1996). The evolution and distribution of almonds, both in cultivation and in the associated semi wild state, has been divided into three stages: Asiatic, Mediterranean, and Californian, corresponding to the geographical areas where is grown (Fig. 1) (Grasselly 1976a; Kester et al. 1991; Kester and Gradziel 1996).

The fruit of almond, as with other *Prunus* species, 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. The almond is the earliest deciduous fruit and nut tree to bloom in spring due to its low winter chilling requirements and quick growth response to warm temperatures. The almond growth cycle is adapted to a Mediterranean type climate (Kester et al. 1991; Kester and Gradziel 1996). Almond is a predominantly self-incompatible species. This self-incompatibility is gametophytic and it is controlled by a single *locus* with multiple codominant alleles (Socias i Company and Felipe 1988; Dicenta and García 1993a). Since self-compatible almond cultivars were reported in Puglia region in Italy, self-compatibility has become one of the main objectives for almond breeding programs in Europe and the USA (Grasselly et al. 1981; Vargas et al. 1984; Socias i Company and Felipe 1988; Dicenta and García 1993a; Gradziel and Kester 1998). Cultivated almond is among the most polymorphic of all cultivated fruit and nut species (Hauagge et al. 1987a; Byrne 1990; Kester et al. 1991; Socias i Company and Felipe 1992; Bartolozzi et al. 1998; Martínez-Gómez et al. 2003a). Sixteen ($2n = 2x = 16$) small, but distinguishable (Corredor et al. 2004), chromosomes and a small diploid genome of approximately 300 Mbp (Baird et al. 1994) also characterize this species.

Horticulturally, almonds are classified as a nut in which the edible seed (the kernel) is the commercial product. Almond kernels are concentrated energy sources because of their high lipid content. The oil is primarily unsaturated, composes mostly oleic and linoleic fatty acids (García-López et al. 1996). The kernel also contains considerable proteins, minerals, and some vitamins (Kester et al. 1991; Kester and Gradziel 1996). However, native almond species predominantly have bitter kernels because of high levels of the glucoside amygdalin (Grasselly 1976b; Kester et al. 1991).

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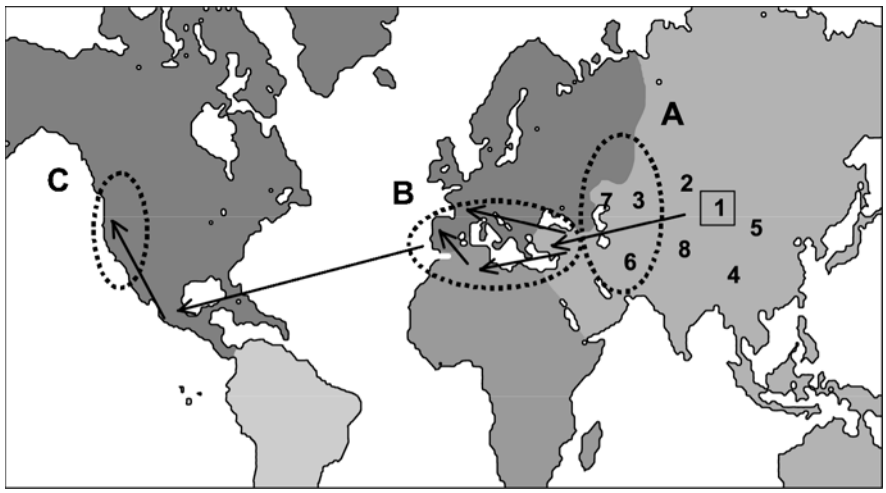

Fig. 1. Map of world showing the origin for almond [*Prunus dulcis* (*1*)] and different relative *Prunus* species [*P. bucharica* (*2*), *P. fenzliana* (*3*), *P. davidiana* (*4*), *P. persica* (*5*), *P. scoparia* (*6*), *P. webbii* (*7*), and *P. argentea* (*8*)], the dissemination routes for the cultivated almond [→], and the three main areas for diversification and cultivation of almonds [Asiatic (*A*), Mediterranean (*B*), and Californian (*C*)]

The principal almond-producing area of the world is the central valley of California with around 50% of the world production. In 2003, worldwide annual almond production exceeded 1679 thousand metric tons, including 741 thousand metric tons in California. The second major almond-producing area includes the European countries bordering the Mediterranean Sea, including Spain (the second leading country after the United States with 197 thousand metric tons in 2003), Italy (91 thousand metric tons) and Greece (40 thousand metric tons). Finally, emergent areas exist in central and southwestern Asia including Syria (139 thousand metric tons in 2003), Iran (109 thousand metric tons) and Turkey (38 thousand metric tons) (Fig. 2) (FAO 2004).

The three basic objectives of almond improvement are to increase yield (self-compatibility, late flowering, flower density, and productivity), to improve quality (maturity date, kernel bitterness), and to decrease production costs (pest and disease resistance, drought resistance) (Socias i Company 1998). The efficiency of breeding programs depends on the information available on the transmission of those traits to be improved. There has been a considerable progress in the study of inheritance of agronomic traits in almond. In this species most of the important agronomical characteristics are quantitative. These quantitatively inherited characters constitute the bulk of the variability selected during the breeding process (Kester and Asay 1975; Grasselly and Crossa-Raynaud 1980; Dicenta et al. 1993a, b; Socias i Company 1998). Late flowering allows the avoidance of the spring frosts in colder areas and has been an objective of early almond breeding programs (Kester 1965; Vargas et al. 1984; Dicenta et al. 1993a; Socias i Company et al. 1999). Genetic studies have demonstrated a positive response to selection for this trait (Kester et al. 1973; Dicenta et al. 1993a). Flowering density and productivity are also two important traits, which have been studied by Kester and Asay (1975), Grasselly and Crossa-Raynaud (1980), Vargas et al. (1984) and Dicenta et al. (1993a). Few studies have been performed regarding the time of maturity (Kester and Asay 1975; Dicenta et al. 1993b). On the other hand, other important agronomic traits in almond seem to be controlled by major genes, including kernel bitterness or self-compatibility. There are many studies regarding the transmission of the kernel traits (see Kester et al. 1977; Vargas et al. 1984; Dicenta et al. 1993b). In addition, kernel bitterness has been characterized as a monogenic trait, the bitter genotype being recessive (Heppner 1923, 1926; Dicenta and García 1993b; Vargas et al. 2001). Finally, self-compatibility was studied by different authors who have determined its monogenic nature with a multi-allelic *S* series, and identified the S_f allele as the responsible for self-compatibility (Socias i Company and Felipe 1988; Dicenta and García 1993a; Ortega and Dicenta 2003). Self-compatibility is expressed within the styles of flowers and results in the successful growth to fertilization of self-pollen tubes (Bošković et al. 1997, 2003).

The absence of extensive crossing barriers among the different *Prunus* species in the initial hybridization and the subsequent backcrosses, demonstrates a direct accessibility of this rich germplasm to almond breeding (Browicz and Zohary 1996; Gradziel et al. 2001a; Martínez-Gómez et al. 2003b). The encouraging performance of interspecific hybrids and back-

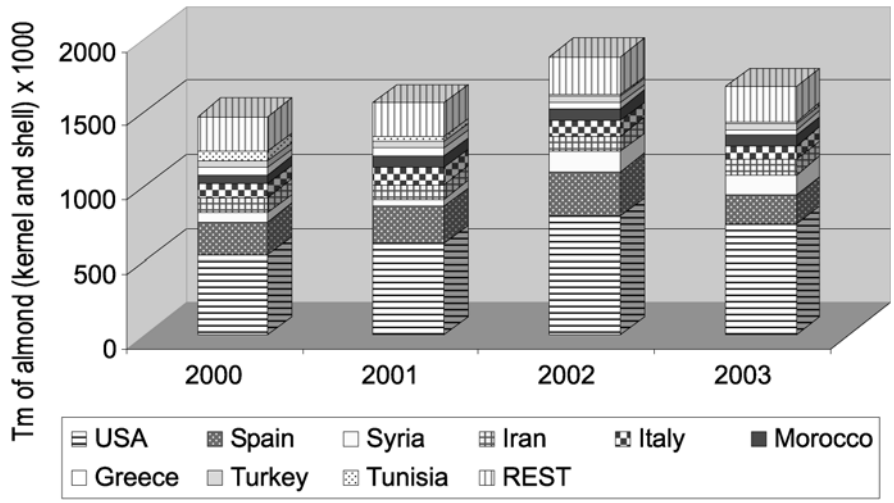

Fig. 2. World almond production according with FAO (2004)

crosses 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). The direct utilization of these related almond species as a rootstock, mainly under non-irrigated native conditions, has been reported by several authors (Grasselly 1975; Denisov 1988). Interspecific crosses have also been used as peach and plum rootstocks (Kester and Hansen 1966; Felipe 1975). Related species have also been reported as having potential in almond breeding to improve the quality of kernels and as sources of self-compatibility (Kester and Gradziel 1996; Gradziel and Kester 1998; Gradziel et al. 2001a). However, a major impediment to the full utilization of this rich germplasm is the tedious selection process emphasizing the need for accurate molecular markers allowing efficient and rapid selection tools (Martínez-Gómez et al. 2003b, c).

11.2 Variability Analysis with Molecular Markers

Traditionally, the identification and characterization of almond cultivars has been based on morphological traits. However, such traits are not always available for analysis, are affected by changing environmental conditions and may only be visible in adult materials and so requiring a long time for their analysis. Molecular markers have offered a solution to many of these problems allowing a fast, accurate, highly discriminative and environmentally stable test that has been used for variability analysis, pedigree determinations or cultivar identification (Wünsch and Hormaza 2002; Martínez-Gómez et al. 2003b; Sánchez-Pérez et al. 2004a). Moreover, some markers, such as isozymes, restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs) and other markers derived from the knowledge of specific genome sequences; allow the comparison of variability among homologous regions of the same or different species.

Isozymes were the first molecular markers used because of their environmental stability, their codominant expression, and their good reproducibility (Arulsekar et al. 1986; Hauagge et al. 1987a, b; Cerezo et al. 1989; Foolad et al. 1995; Vezvaei et al. 1995; Sathe et al. 2001). Isozyme studies have detected high levels of variability in almond and allowed the individual identification of most genotypes studied. A comparative study of isozyme variability in *Prunus* (Byrne 1990) showed that almond and Japanese plum, both with a strong self-incompatibility system were more variable than apricot and peach that have different degrees of self-compatibility. 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 at most loci. On the other hand, RFLPs are codominant and can detect a virtually unlimited number of markers, thus providing an efficient method for discovering linkages between markers and for constructing genetic maps. RFLPs also proved to be useful for variability analysis and cultivar identification in almond

(Viruel 1995). However, RFLP analysis has important limitations: it is laborious and time-consuming and it often involves the use of radioisotopes. The recent utilization of PCR-based markers has increased the opportunities for mapping and tagging a wide range of traits. RAPDs, based on the PCR amplification of random locations in the genome, typically use arbitrary primers. A single oligonucleotide is utilized for this random amplification of genomic DNA. Unlike RFLPs, RAPDs can be obtained with a simple method, but have some disadvantages when compared to isoenzymes and RFLPs: they are dominant markers and have a variable degree of repeatability which limit their utilization for cultivar identification and map construction. RAPD techniques have been used in almond for the study of germplasm variability (Bartolozzi et al. 1998; Martins et al. 2003).

SSR (or microsatellite) markers, also based on the PCR technique, are currently becoming the markers of choice for genetic fingerprinting studies for a wide range of plants. 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 closely related species (Gupta et al. 1996; Powell et al. 1996). In the case of *Prunus*, SSR markers covering the almost whole genome have been obtained in different species including peach, apricot, Japanese plum and cherry almost (Cipriani et al. 1999; Downey and Iezzoni 2000; Sosinski et al. 2000; Testolin et al. 2000; Cantini et al. 2001; Aranzana et al. 2002, 2003; Dirlewanger et al. 2002; Georgi et al. 2002; Wang et al. 2002; Yamamoto et al. 2002; Clarke and Tobutt 2003; Decroocq et al. 2003; Schueler et al. 2003; Hagen et al. 2004; Messina et al. 2004; Mnejja et al. 2004). Recently, the first set of almond SSRs has been published (Testolin et al. 2004). They have been successfully used for the molecular characterization and identification of almond cultivars (Martínez-Gómez et al. 2003a; Testolin et al. 2004) and related *Prunus* species (Martínez-Gómez et al. 2003c). Electrophoresis in polyacrilamide gels with radioactive and silver staining was the first method used in the analysis of the PCR amplified fragments of DNA obtained from the SSR markers. Electrophoresis in Metaphor® agarose gels was an alternative method to polyacrilamide gels due to its easier application (Morgante et al. 2003). More recently, new methods for PCR amplified DNA have been developed including the utilization of automated sequencers. While the use of Metaphor® agarose gels appears less useful for genotype characterization that the other two methods, this method may be the most convenient in mapping of populations involving alleles separated more than 5 bp due to its lower cost and easier routine application (R. Sánchez-Pérez et al. 2006) (Fig. 3). The comparative analysis of the variability of five *Prunus* species with 125 SSRs has detected that the most polymorphic species was almond, followed by Japanese plum, apricot, cherry and peach (M. Mnejja and P. Arús, unpublished results). This is the same order of variability that was found with isozymes by Byrne (1990), which confirms almond as a species with a very high level of polymorphism.

11.3 Construction of Genetic Linkage Maps

Linkage analysis was first performed in almond using isozyme genes (Arús et al. 1994a; Vezvaei et al. 1995), but the low number of isozymes that can be analyzed with conventional enzyme staining methods in a given population precluded the use of these markers for the construction of genetic maps. The development of RFLPs at the beginning of the 1980s provided a virtually unlimited source of high quality markers located all over the genome, making map construction with markers a feasible endeavor for most animal and plant species. The first map for almond was constructed by Viruel et al. (1995) based almost entirely on these markers (120 RFLPs and 7 isoenzymes) for the F_1 progeny between 'Ferragnès' and 'Tuono' (the F×T map). This map detected the eight expected linkage groups and spanned approximately 400 cM. Another map constructed by Foolad et al (1995) with an F_2 population of the interspecific cross between a peach selection (54P455) and the almond cultivar 'Padre' (P×5), had a similar marker composition (101 RFLPs and 6 isozymes). This map was longer than that of Viruel et al. (1995), with a total length of about 800 cM.

As a result of a European project (see Arús et al. 1994b), a saturated linkage map of for *Prunus* was obtained in an almond (cv. 'Texas', syn. 'Mission') × peach (cv. 'Earlygold') F_2 progeny (Joobeur et al. 1998) including 246 markers (235 RFLPs and 11 isozymes). All markers studied mapped in the eight linkage groups found, with a total distance of 491 cM. Given that this map (the T×E map), considered as the

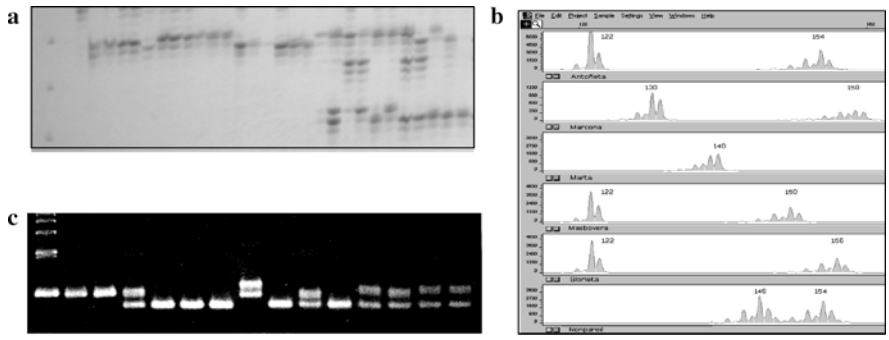

Fig. 3. Analysis of DNA polymorphisms of SSR markers in several almond cultivars using polyacrylamide electrophoresis gels (*a*), Metaphor® agarose electrophoresis gels (*b*) and automated capillary sequencers (*c*)

Prunus reference map, had many markers in common (67) with the 'Ferragnès' × 'Tuono' (F×T) map, it was possible to compare them, having the same distribution of makers among linkage groups and a complete colinearity of markers within each linkage group. For that reason the terminology for linkage groups coined for the F×T almond map was adopted for the T×E reference *Prunus* map. The T×E map has been progressively improved (Aranzana et al. 2003) with the addition of more markers of good quality, such as additional RFLPs and simple-sequence repeats (SSRs). The current version (Dirlewanger et al. 2004a) includes 562 markers (361 RFLPs, 185 SSRs, 11 isozymes and 5 STSs), which cover a total distance of 519 cM with high density (average density 0.92 cM/marker and largest gap of 7 cM).

The development of markers that could be obtained with simpler methods than RFLPs, such as RAPDs and SSRs (particularly given the latter's high quality) fostered the improvement of other maps, and more saturated versions were produced such as the F×T map (Joobeur et al. 2000) with 174 markers and the P×5 ['Padre' (almond) × 54P455 (peach)] map (Bliss et al. 2002) with 161 markers including six morphological genes and eight resistance-gene analog sequences.

Two more maps were constructed after T×E and used the information of this map to elaborate framework maps with a low number of markers selected from it that covered the whole genome at distances of 10–25 cM. The first of these maps was obtained in the F_1 progeny of the cross between two almond cultivars 'Felisia' (syn. D-3-5) and 'Bertina' which allowed a study of the map position of genes involved in self-incompatibility (Ballester et al. 1998, 2001), shell hardness (Arús et al. 1999) and blooming time (Ballester et al. 2001). The second map was based on an F_2 progeny between 'Garfi' almond and 'Nemared' peach (Jáuregui et al. 2001), which located genes involved in nematode resistance, and flower color (Jáuregui 1998).

The similar order of molecular markers observed in different *Prunus* maps when compared to the *Prunus* reference map, suggests a high level of synteny within the genus (Aranzana et al. 2003; Dirlewanger et al. 2004a, b; Lambert et al. 2004). This homology among the genomes of *Prunus* species is in agreement with the low level of breeding barriers to interspecific gene introgression and supports the opportunity for successful gene transfer between closely related species (Gradziel et al. 2001a; Martínez-Gómez et al. 2003b). In addition, the synteny among *Prunus* genomes offers important opportunities to transfer and compare genetic information from linkage maps generated in different species of this genus.

The *Prunus* reference map has been compared with the *Arabidopsis* sequence, finding 23 syntenic blocks between them, which covered 23% of the *Prunus* map distance and 16% of the *Arabidopsis* genome (Dominguez et al. 2003). Microsynteny studies have found also a fractional conservation between these two distant taxons (Georgi et al. 2003) and indicate that the sequence of *Arabidopsis* can be employed to a limited extent for gene or marker search in *Prunus*.

11.4 Major Gene and QTL Mapping, and Gene Cloning

The usual approach for the analysis of marker-trait association is the use of mapping populations segregating for the agronomic characters of interest. The analysis of cosegregation among markers and characters allows establishment of the map position of major genes and QTLs responsible for their expres-

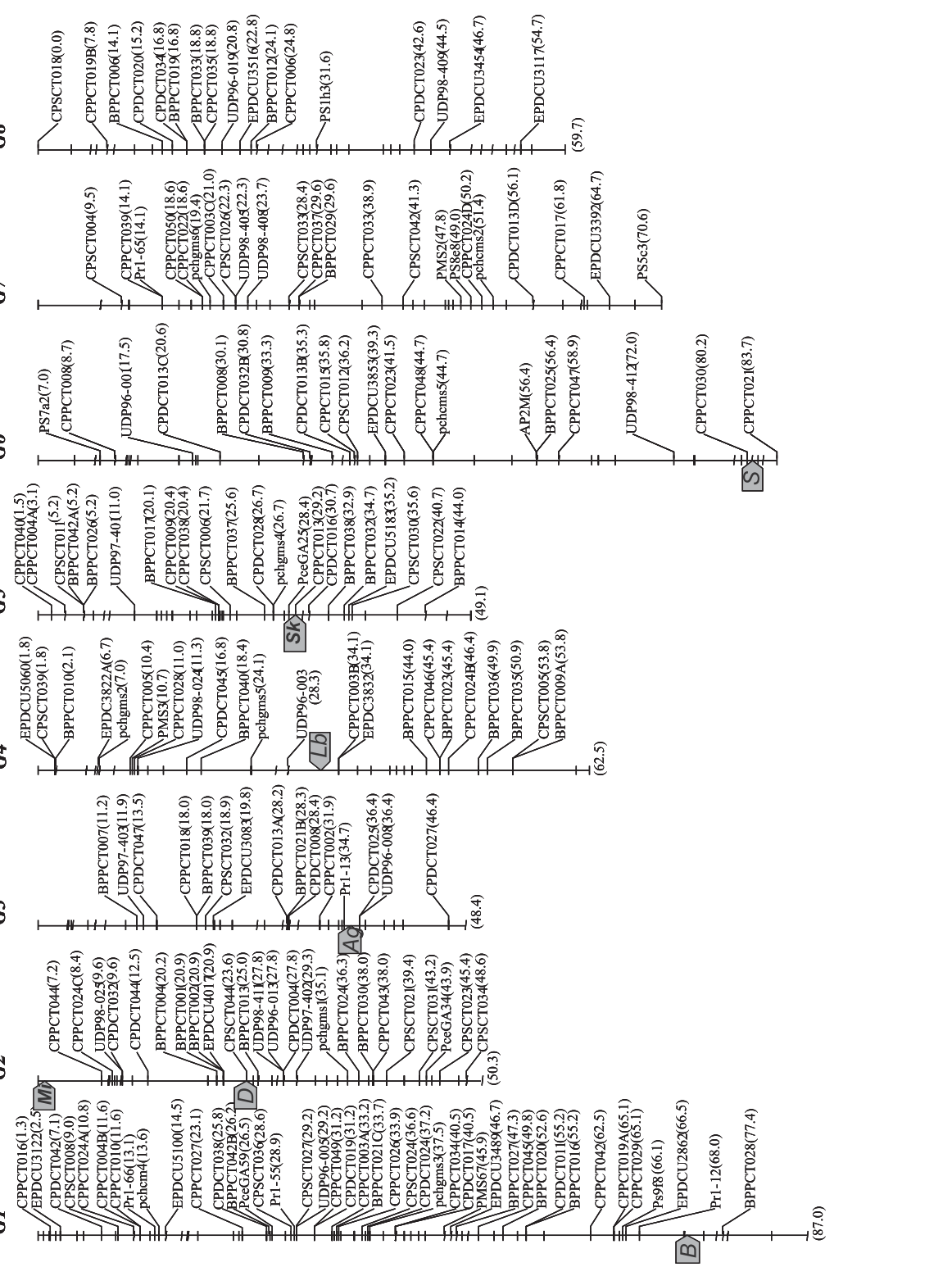

Fig. 4. Map of the 'Texas' (almond) × 'Earlygold' (peach) F2 population obtained only with the SSR markers of the map of Dirlewanger et al. (2004a) and with the approximate location of flower color (*B*), nematode resistance (*Mi*), shell hardness (*D*), anther color (*Ag*), blooming time (*Lb*), kernel taste (*Sk*), and self-incompatibility (*S*) genes

Table 1. Markers associated to main agronomic traits in almond

Trait	Symbol	Linkage group	Populations	Marker	Reference
Flower color	*B*	G1	'Garfi' (almond) × 'Nemared' (peach)	RFLP	Jáuregui 1998
Shell hardness	*D*	G2	'Ferragnés' (almond) × 'Tuono' (almond)	RFLP	Arús et al. 1999
Nematode resistance	*Mi*	G2	'Garfi' (almond) × 'Nemared' (peach)	RFLP	Jáuregui 1998
Nematode resistance	*Mi*	G2	'Padre' (almond) × '54P455' (peach)	RFLP	Bliss et al. 2002
Anther color	*Ag*	G3	'Texas' (almond) × 'Earlygold' (peach)	RFLP	Joobeur 1998
Blooming time	*Lb*	G4	'D.3.5' (almond) × 'Bertina' (almond)	RAPD	Ballester et al. 2001
Kernel taste	*Sk*	G5	'Padre' (almond) × '54P455' (peach)	RFLP	Bliss et al. 2002
Kernel taste	*Sk*	G5	'Texas' (almond) × 'Earlygold' (peach)	RFLP	Joobeur 1998
Self-incompatibility	*S*	G6	'D.3.5' (almond) × 'Bertina' (almond)	RAPD	Ballester et al. 2001
Self-compatibility	*S*	G6	'Ferragnés' (almond) × 'Tuono' (almond)	RFLP	Ballester et al. 1998
Self-compatibility	*S*	G6	'Ferragnés' (almond) × 'Tuono' (almond)	RFLP	Arús et al. 1999
Self-compatibility	*S*	G6	'Padre' (almond) × '54P455' (peach)	RFLP	Bliss et al. 2002

sion (Arús and Moreno-González 1993). Some of the linkage maps developed in almond include markers associated with several traits of horticultural value. With the previously reported high level of synteny between the genome of *Prunus* crops, and the existence of a reference map, a considerable number of genes studied in different populations of almond have been integrated in a single map. The approximate position of these genes is providing in Fig. 4 and their description in Table 1. The important characters and QTLs that are presently being mapped in almond include flower color (*B*) in the linkage group 1 (G1) (Jáuregui 1998), nematode resistance (*Mi*) (G2) (Jáuregui 1998; Bliss et al. 2002), shell hardness (*D*) (G2) (Arús et al. 1999), anther color (*Ag*) (G3) (Joobeur 1998), blooming time (*Lb*) (G4) (Ballester et al. 2001), kernel taste (*Sk*) (G5) (Joobeur 1998; Bliss et al. 2002), and self-incompatibility (*S*) (G6) (Ballester et al. 1998, 2001; Arús et al. 1999; Bliss et al. 2002). Although in some cases the location of these genes has been established in low-density maps, their position can be further defined by using the information provided by the network of maps available for *Prunus* (Dirlewanger et al. 2004a). *Prunus* genome synteny should also facilitate the successful transfer of sets of markers and coding sequence among species (Aranzana et al. 2003; Decrocq et al. 2003; Dirlewanger et al. 2004a, b). Candidate gene approaches have also proven to be useful for finding associations between genes involved in relevant metabolic pathways and the major genes or QTLs as have been reported in peach (Etienne et al. 2002).

Bulked 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 powerful approach for the analysis of molecular marker-horticultural trait association. A strategy combining different markers with bulked segregant analysis was used to identify markers linked to loci of specific fruit characters in peach × almond crosses (Warburton et al. 1996). In addition, Ballester et al. (2001) using this methodology identified three RAPD markers associated with self-incompatibility and a gene conferring delayed blooming in almond.

Although gene cloning studies in almond are very scarce, the first gene sequence reported in *Prunus* was that of extensin obtained from almond developing seeds (García-Mas et al. 1992), to which followed some other genes abundantly expressed during seed development (García-Mas et al. 1995, 1996). The genes involved in the self-incompatibility trait have also been characterized. Ushijima et al. (1998) cloned the cDNAs encoding *S*-RNases from almond after studying the primary structure and the sequence diversity of the *S*-RNases in other related *Rosaceae* species. These studies have been completed by other research groups studying other *S* alleles (Channuntapipat et al. 2001; Ma and Oliveira 2001; Certal et al. 2002). In addition, Ushijima et al. (2001) cloned and characterized the cDNAs encoding *S*-RNases in an almond cultivar 'Jeffries' which is a somaclonal mutant of 'Nonpareil' (*Sc* and *Sd* self-incompatibility alleles) and has a dysfunctional *S* allele haplotype

both in pistil and pollen. Results indicated that at least two mutations had occurred to generate this mutant, the deletion of the *Sc* allele haplotype and the duplication of the *Sd* allele haplotype. On the other hand, Suelves and Puigdomenech (1998) identified and sequenced a gene highly expressed in the floral organs of almond and coding for the cyanogenic enzyme (R)-(+)-mandelonitrile lyase. However, the study of the mRNA levels during seed maturation and floral development in fruit and floral samples indicated a lack of correlation between these characteristics and levels of mandelonitrile lyase mRNA and the level of kernel bitterness of almond cultivars classified as homozygous or heterozygous for the sweet trait or homozygous for the bitter trait. In addition, Vezvaei et al. (2004) developed a strategy for the discovery the glucosyltransferase gene responsible for producing bitter kernel in almond using degenerate primers based on consensus regions of glucosyl-transferase genes for other plants.

11.5 Marker-Assisted Breeding

Developing new cultivars is a long and tedious process in almond, involving the generation of large population of seedlings from which the best genotypes are selected. Whereas the capacity of breeders to generate big populations from crosses is less limited, the management, study and selection of these seedlings remain the main limiting factors in the generation of new releases (Kester et al. 1991; Kester and Gradziel 1996; Socias i Company 1998). Marker-assisted selection (MAS) is emerging as a very promising strategy for increasing selection gains (Arús and Moreno-González 1993; Luby and Shaw 2001). Knowledge provided by advances in molecular genetics promise faster and more efficient approaches to cultivar improvement. Early selection utilizing molecular markers allows accurate screening of seedlings several years before the mature plant traits can be evaluated in the field, makes possible the accumulation of different genes/QTLs for horticultural traits of interest, and shortens the number of generations to recover the desired genotype particularly after a cross with an exotic genotype or wild species (Arús and Moreno-González 1993; Baird et al. 1996; Dirlewanger et al. 2004a). Selection by molecular markers is particularly useful in fruit, nut, and other tree crops with a long juvenile period, when the expression of the gene is recessive or the evaluation of the character is difficult, as with resistance to biotic or abiotic stresses (Luby and Shaw 2001; Scorza 2001; Testolin 2003). 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. Selection of marker loci linked to major genes can be sometimes more efficient than direct selection for the target gene (Arús and Moreno-González 1993; Baird et al. 1996).

A very promising application of MAS is the manipulation of self-incompatibility in almond. 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; Certal et al. 2002). Molecular methods have been developed in two areas: identification of stylar *S*-RNases by electrophoresis in vertical polyacrilamide gels (Bošković et al. 1997, 2003), and the amplification of specific *S*-alleles using appropriately designed primers for PCR and electrophoresis in horizontal agarose gels (Tamura et al. 2000; Channuntapipat et al. 2003; López et al. 2004). This latter 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) allowing earlier and more accurate selection of the most common self-incompatibility or self-compatibility alleles. More recently, a multiplex-PCR strategy has been developed for the unequivocal identification of self-incompatibility and self-compatibility alleles. This multiplex PCR opens the possibility to identify new *S*-alleles using different sets of primers (Sánchez-Pérez et al. 2004b) (Fig. 5).

In a recent study to determine the genetic basis of mechanisms involved in almond drought tolerance, several genes that were strongly expressed in response to dehydration of almond have been identified. A differential expression technique based on cDNA-AFLP (amplified fragment length polymorphism derived technique for RNA fingerprinting) has been used to identify transcripts that accumulated in mature embryos and in in-vitro-cultured plantlets subjected to desiccation or abscisic acid treatment. This study showed that the levels of expression of the identified genes in leaves of young trees of eight almond cultivars differing in drought

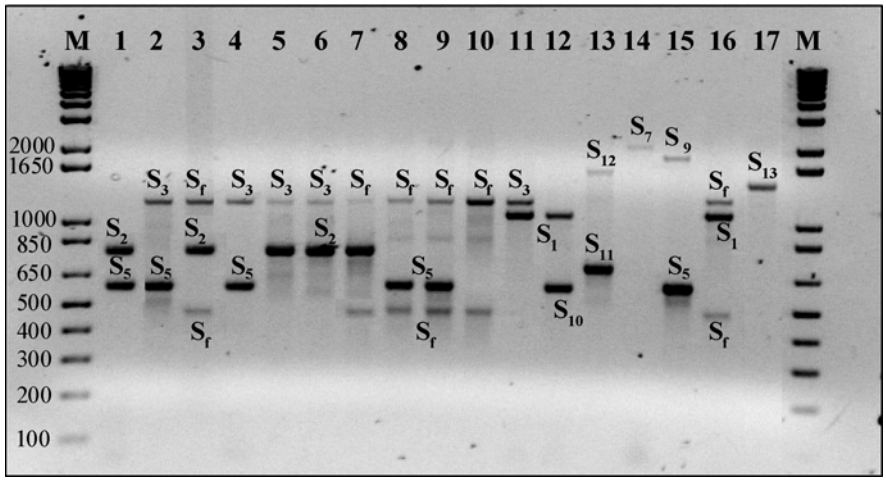

Fig. 5. Agarose 1.5% gel showing amplified *S*-alleles (self-compatibility and self-incompatibility) in 17 almond cultivars and breeding lines using PCR specific primers AS1II (Tamura et al. 2000), CEBASf (Sánchez-Pérez et al. 2004b) and AmyC5R (Tamura et al. 2000)

tolerance provided valuable information for breeding drought resistance in almond (Campalans et al. 2001).

11.6 Advanced Works and Future Scope

Apart from the molecular markers described (isoenzymes, RFLPs, RAPDs, and SSRs), other markers being used in the development of marker associated traits in almond and other *Prunus*, are those based on single point mutations and those obtained from either cDNA sequences (expressed sequences tags, ESTs) or databases (cloned gene analogs, CGAs) (Van Nocker et al. 2002; Testolin 2003, Jung et al. 2004). The large-scale single-pass sequencing of ESTs can give a more 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 (http://www.mainlab.clemson.edu/gdr/) (Main et al. 2004). This work is complementary to the other works regarding EST development in *Prunus* performed by different research groups in other European countries (Grimplet et al. 2004; Pozzi et al. 2004). Lazzari et al. (2004) also presented a collection of 6,817 ESTs prepared from four cDNA libraries obtained from mesocarps of peach as part of the work of the Italian National Consortium for Peach Genomics (http://www.itb.cnr.it/ESTree). In almond, a study of expressed transcripts during pistil development has selected and partially sequenced over 1,000 clones from a cDNA library. Analysis of these ESTs using the National Center for Biotechnology Information (NCBI) databases indicated significant similarity to protein coding sequences in the database. The EST analysis has provided a preliminary picture of the numerous almond genes potentially involved in pistil development and provides an extensive reservoir for future gene cloning and genetic mapping in almond (Jiang and Ma 2003). As part of a worldwide collaboration effort to increase and enrich the genomics resources in different *Prunus* species, the fabrication of different *Prunus* microarray using unigene sets as probes is being initiated. 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). The development of microarrays has also been described in peach for the study of fruit quality by Trainotti et al. (2003, 2004) including the development of markers associated to these important horticultural characteristics.

A recent strategy for the location of new markers in an established genetic linkage map is the "selective" or "bin" mapping approach. This technique allows mapping with the use of a subset of plants of a population from which a map is already available (Vision et al. 2000). The plants of this subset are selected to maximize the information on linkage, so that their joint genotype for any marker identifies a small as possible unique genome fragment (a bin). The advantage of this strategy is that it allows mapping with less time and cost and is adequate for simplifying the construction of high-density maps or for the addi-

tion of large numbers of markers (such as SSRs or EST-derived markers) to a previous map. Recently, Howad et al. (2005) have incorporated 151 SSRs to the *Prunus* reference map using only six individuals from the T × E ('Texas' × 'Earlygold') *Prunus* reference population. The use of this set of six individuals, promises to be a useful resource for *Prunus* geneticists in the future.

Twin seeds (multiple embryos within the same seed coat) occur spontaneously in several almond cultivars including the Californian 'Nonpareil' and 'Mission' (Kester and Gradziel 1996). 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. Some of these low-vigor plants have been shown to be haploids from which true-breeding dihaploids can be generated (Gulcan 1975) for genetic studies, hybrid rootstock production, and transformation and regeneration studies. In addition, 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 has been presented as an interesting germplasm to aid in genetic (locating genes, selective transfer of particular chromosomes) and molecular (isolation and sequencing of genes, genetic transformation, etc.) studies for the development of new strategies of markers linkage to agronomic traits in almond (Sánchez-Pérez et al. 2004c).

In conclusion, the typical long generation time, along with the extensive space requirements and other limitations to generating the required large segregating almond progeny populations, have frustrated the development and testing of new almond cultivars. These same limitations, however, make molecular-based strategies that improve breeding efficiency particularly valuable to tree crops. Because they are vegetatively propagated, most *Prunus* tree crops such as almond have a unique advantage over other agronomic crops since desirable, unique gene/genomic combinations can be 'captured' and disseminated by clonal propagation. Future research needs include the comparative mapping between the most important genera of fruit crops and the numerous wild species. Almond species include a large number of intercompatible species which provide an enormous gene pool available for breeding. Little use has been made of this variability because the slowness of classical breeding methods. However, genomic methodologies, including the development of quick gene sequencing and cloning tools, may make it possible to rapidly discover and incorporate genes of interest from this exotic material. Additional advantages encouraging the utilization of new technologies to almond tree crop improvement include a small genome size, high levels of synteny between genomes, and a well-established international network of cooperation among researchers.

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