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Genetic Mapping of Blooming Time in 'Marcona' × 'Fragness' Population with Using Molecular Markers

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Abstract

Flowering time is an important horticultural trait in almond since it is essential to avoid the late frosts that affect production in early flowering cultivars. Evaluation of this complex trait is a long process because of the prolonged juvenile period of trees and the influence of environmental conditions affecting gene expression year by year. In this research flowering time was studied in an F1 almond progeny of 90 seedlings from the cross between the Marcona and the Fragness. In addition, a set of 63 co-dominant microsatellites or simple-sequence repeat (SSR) markers developed from peach, cherry and almond were used for the molecular characterization of the progeny. A genetic linkage map was created with 17 of these SSRs. Molecular studies at the DNA level confirmed this polygenic nature by identifying several genome regions (Quantitative Trait Loci, QTL) involved. QTL mapping detected two loci for flowering time (Ft-Q1 and Ft Q4) in Linkage groups 1 and 4 that close with BPPCT011 and UDP96-021 respectively. Finally, the development of efficient MAS strategies applied to almond and other *Prunus* breeding programs are also discussed.

Keywords: Almond, Flowering time, Microsatellites, Molecular markers, QTL.

Introduction

Studies of genetic variation and genetic relatedness assisted by molecular markers can improve the use of the various genotypes in breeding programs and the design of new crosses. In *Prunus* breeding programs, evaluation of agronomic traits in *Prunus* species is a time-consuming and laborious process because of the long juvenile period of trees, the influence of the juvenility on the expression of the trait, and the existence of climatic factors affecting this evaluation. For these reasons, marker-assisted selection (MAS) is particularly useful in these cases (Scroza, 2001). Simple sequence repeat sequences (SSRs) are becoming the markers of choice for molecular characterization and mapping in *Prunus* because of their high polymorphism, abundance, co-dominant inheritance and transportability across *Prunus* species (Dirlewanger *et al.*, 2004).

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An effective approach to developing breeding programs might be to identify and map genes that respond to stress using molecular markers, and to determine the relationship of these genes to phenotypic traits. Grain yield is a particularly complex trait, which usually has low heritability (Quarrie *et al*., 2005) and is influenced significantly by the environment (Cuthbert *et al*., 2008). Due to the importance and complex nature of yield and yield components, mapping these traits is a critical factor for most breeding programs. Most of the quantitative trait loci (QTL) for the yield of crops such as wheat and barley that have been identified account for less than 10 % of the total phenotypic variation (McCartney *et al*., 2005; Cuthbert *et al*., 2008; Xue *et al*. 2009). From a commercial point of view, flowering time is one of the most important agronomic traits in almond (*Prunus dulcis* (Miller) D. A. Webb) as it determines the vulnerability of production to late frosts, as well as the use of cultivars for cross-pollination in order to achieve successful pollination when the flowering times of two varieties must coincide (Dicenta *et al*., 2005).Marker linkage analysis was first performed in almond with isoenzyme genes (Arus *et al*. 1994). The first genomic studies performed used RAPDs (Random Amplified Polymorphic DNA) and bulk segregant analysis in an F1 progeny from "Tardy Nonpareil," corroborating the presence of the previously mentioned major gene *Lb* controlling late flowering time. Moreover, three RAPDs were found to be associated with *Lb* in linkage group 4 (G4) of the "Felisia" × "Bertina" ("Felisia" is a descendant from "Titan," that is a seedling of "Tardy Nonpareil") genetic map (Ballester *et al*., 2001). In addition, Silva *et al*. (2005) described several QTLs linked to flowering time in an interspecific F1 almond × peach progeny using a Candidate Gene (CG) approach in G1, G2, G3, G5, G6, and G7. More recently, different works using SSR markers in an F1 population between a seedling of "Tardy Nonpareil" ("R1000") × "DesmayoLargueta" (R×D), also confirmed the location of *Lb* in G4 and identified other QTLs to flowering time in G1, G6, and G7 (Sánchez-Pérez *et al*., 2007; Martínez-Gómez *et al*., 2012; Rasouli *et al., 2013*; Rasouli *et al., 2014b*) In this work, flowering time trait have been studied in Marcona× Fragness.

Materials and Methods

Plant material and DNA isolation

The mapping population assayed was an F1 progeny of almond [*Prunus dulcis* (Miller) D.A. Webb] of 90 seedlings from the cross made in 2004 between the Iranian selection Marcona × Fragness. The Marcona was near to Shahrood 1 local cultivar and the Fragness was too near toShahrood 12 local cultivar. Therefore the name of local hybrid in this research was Shahrood1× Shahrood12. Total genomic DNA was isolated using the procedure described by Doyle and Doyle (1987) with the modifications of Sonneveld and *et al.* (2001). Evaluation of flowering time trait: The following flowering time was evaluated in the Marcona× Fragness population during years 2012 and 2013. This trait was evaluated in Julian days (natural days from 1 January) until 50% of the flowers were open.

The extracted almond genomic DNA was PCR-amplified for identification. PCRs were performed with the reaction mixtures containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1 unit of Taq DNA polymerase and 90 ng of genomic DNA. The cycling parameters were: one cycle of 95 $^{\circ}$C for 3 min, 35 cycles of 94 $^{\circ}$C for 1 min, 57 $^{\circ}$C for 1 min and 72 $^{\circ}$C for 2 min, followed by a 10 min final extension. Amplified PCR products were separated by electrophoresis on 1.5% agarose gels (1x TAE buffer), stained with ethidium bromide (0.5 µg/ml) and visualized under UV light using a 1 kb Plus DNA Ladder (Invitrogen TM Life Technologies, Carlsabad, CA, USA) as a molecular size standard.

SSR analysis

Genomic DNA of a small set of seedlings was PCR amplified using 63 published primer pairs flanking SSR sequences from peach (20 SSRs) and almond (43 SSRs) that the table 1 showed the type SSRs for Marcona × Fragness hybirid population. The SSRs subsequently used in the full progeny were those found to segregate in SSRs and have a good coverage of the *Prunus* reference map (Aranzana *et al.*, 2003, Dirlewanger *et al.*, 2004).

Table 1. The study type SSRs for hybrid population Marcona× Fragness

SSR Marker	Species	Reference
BPPCT	Almond	Dirlewanger*et al*., 2002
CPPCT	Almond	Aranzana*et al*., 2003
M1A	Almond	Yamamoto *et al*., 2002
pchcms	Peach	Sosinski*et al*., 2000
pchgms	Peach	Sosinski*et al*., 2000
UDP	Peach	Ciprianiet *al*., 1999
UDP	Peach	Testolin*et al*., 2000

PCRs were performed in a total volume of 12.5 µL containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 2 mMMgCl_2, 0.2 µMof each primer, 0.1 mM of each dNTP, 1 unit of Taq DNA polymerase (Ecogen S.R.L.) and 90 ng of genomic DNA based on OD readings between 260 nm to 280 nm. The cycling parameters were: 1 min at 94 °C; 35 cycles of 15 s at 94 °C, 15 s at the appropriate annealing temperatures and 30 s at 72 °C, followed by a 5 min extension at 72 °C. PCRs were carried out in a 96-well block Eppendorf Mastercycler Gradient. Amplified PCR products were separated depending on the differences in the sizes of the segregating alleles. If the difference was more than 5 bp, 3% Metaphor Agarose gel electrophoresis (Biowittaker, Rockland, ME, USA) was used according to the manufacturer's instructions, and the gels were stained with ethidium bromide (0.5 µg/ml) and the bands visualized under UV light. If the difference was <5 bp polyacrylamide gel electrophoresis (PAGE) was used. For PAGE, the PCR products were denatured by adding 2.5 µL of the 95% formamide/bromophenol blue loading buffer. Samples were loaded on to sequencing gels (6% polyacrylamide, 7.5 M urea) and electrophoresis conducted in 1x TBE buffer at a constant current of 120 W and gel temperature of 50 °C. Results were visualized using a silver staining kit from Promega (Promega Inc., Madison, WI, USA) following the manufacturer's instructions. A 1-kb Plus DNA ladder (InvitrogenTM Life Technologies) was used as the molecular size standard for Metaphor agarose gels and a 10-bp Plus DNA Ladder (Invitrogen TM Life Technologies) for PAGE.

Map construction and QTL analysis

Band scoring for Metaphor agarose gels and PAGE was analysed using Gene Tools gel analysis software of SYNGENE (Beacon House, Nuffield Road, Cambridge, UK). The segregation data (1: 2: 1, 1: 1 or 1: 1: 1: 1) for all loci were subjected to a chi-square test for goodness-of-fit to expected genotypic ratios. Depending on the genotype of the parents, markers were scored as backcross 1 : 1 segregations when just one of the parents was heterozygous, co-dominant 1 : 1 : 1 : 1 segregations when both parents were heterozygous with no or one allele in common, and co-dominant 1 : 2 : 1 segregations when both parents were heterozygous for the same alleles. For definition of linkage group, an integrated genetic map was constructed using the 18 anchor loci on both maps with JOINMAP v.3.0 software (van

Ooijenand Voorrips, 2001) by using the CP population type. Linkage groups were established with an LOD ≥ 3.0 (Kosambi, 1944). Major gene position and interval QTL mapping was carried out using the software, QTL Cartographer v. 4.0 (Azadi *et al.*, 2014) (Table 2).

Table2. The SSRs study in PCR for linkage group and mapping preparation (Rasouli *et al., 2014a*).

NO	Marker	Sequencing	Motif	Annealingtemprature	Reference
1	BPPCT 011	F:AAT TCC CAA AGG ATG TGT ATG R:CAG GTG AAT GAG CCA AAG C	(GA)27	57	Dirlewanger*and et al.* 2002
2	BPPCT 024	F:GGGCGTGAAGGTGTTACTGT R:GGTGACACAGAAGAGAGCAGAA	(GA)?	57	http://www.bioinfo.wsu.edu/gdr/
3	UDP96025	F:TTGCTCAAAAGTGTCGTTGC R:ACACGTAGTGCAACACTGGC	(CT)11(CA)28	57	Cipriani *et al.*, 1999
4	UDP96005	F:GTAACGCTCGCTACCACAAA R:CCTGCATATCACCACCCAG	(AC)19	57	Cipriani *et al.*, 1999
5	UDP98408	F:ACAGGCTTGTTGAGCATGTG R:CCCTCGTGGGAAAATTTGA	(AG)29	57	Cipriani *et al.*, 1999
6	UDP98409	F:GCTGATGGGTTTTATGGTTTTC R:CGGACTCTTATCCTCTATCAACA	(AG)19	57	Cipriani *et al.*, 1999
7	UDP98411	F: AATTTACCTATCAGCCTCAAA R:TTTATCCAGTTTACAGACCG	(AG)23	50	Testolin *et al*., 2000
8	UDP98021	F: AAGCAGCAATGGGCAGAATC R:GAATATGAGACGGTCCAGAAGC	(AG)22	57	Testolin *et al.*, 2000
9	UDP98024	F: CCTTGATGCATAATCAAACAGC R: GGACACACTGGCATGTGAAG	(AG)8	57	Testolin *et al.*, 2000
10	CPPCT006	F:ATGGTTGCTTAATTCAATGG R: TGACATGCATGCACTAAACAA	(AG)14	57	Howad *et al.*, 2005
11	CPPCT016	F: TGACATGCATGCACTAAACAA R: TGCAAATGCAATTTCATAAAGG	(AG)15	57	Howad *et al.*, 2005
12	UDP96-018	ATTCTTCACTACACGTGCACG CCCCAGACATACTGTGGCTT	(AG)17	57	Testolin *et al.*, 2000
13	UDP97-402	F: CTGGCTTACAACTCGCAAGC R: CGTCGACCAACTGAGACTCA	(AG)12	57	Testolin*et al*., 2000
14	UDP98-407	F:AAAAGGCACGACGTTGAAGA R: TTCAGATTGGGAATTTGCAG	(AG)9	57	Testolin *et al*., 2000
15	BPPCT 010	F:AAT TCC CAA AGG ATG TGT ATG R:CAG GTG AAT GAG CCA AAG C	(GA)17	57	Dirlewanger *et al.*, 2002
16	UDP96-018	F:ATTCTTCACTACACGTGCACG R:CCCCAGACATACTGTGGCTT	(AG)27	57	Testolin *et al.*, 2000
17	UDP96005	F:GTAACGCTCGCTACCACAAA R:CCTGCATATCACCACCCAG	(AC)19	57	Testolin *et al.*, 2000

Results

From the total of 63SSR markers evaluated, 17 were selected as markers and other markers were excluded because some of them were monomorphic. Also some of them (10 %) showed significant ($P<0.05$) segregation distortion in this study. By using distorted markers in the present study, no changes were observed in most of the linkage groups. Most of highly skewed markers with $P\leq0.0001$ could not be included and remained unlinked in the final map. A few of these could be included in the map but they altered the order of loci or were at the same positions as other markers, so they did not fill the gaps (data not shown). In order to avoid a biased estimate of marker-trait association (Gupta, 2002) or spurious linkage (Kammholz *et al*., 2001), distorted markers were excluded from the analysis as reported by previous studies (Xue *et al*., 2009; Genc *et al*., 2010; Heidari *et al*., 2011). Some researchers have used distorted markers for linkage map construction (Quarrie *et al*., 2005; Elangovan *et al*., 2008; Khedikar *et al*., 2010). A single genetic marker was placed in a group. According to for creation of a linkage group there should be least two markers; therefore they were eliminated. On the other hand some markers in this study failed to comply with Mendelianratios (1:1, 1:2:1 and 1:1:1:1) they also were removed.

All the SSR primer pairs amplified a single locus and produced a maximum of two bands (alleles) per genotype, in accordance with the diploid constitution of almond. The Mendelian segregation population of 17SSR markers was as follows: 1: 1: 1: 1 (33%), 1: 2: 1 (45%) and 1: 1 (22%). The heterozygosity of each parent for reading primers was 0.9 and 0.8 for Marcona and Fragness repectively.

This map detected 6 linkage groups (LG) fromtotal of 8 groups, covering 210cM. Distribution of SSR markers was relatively uniform in the different linkage groups: four were located in G1, five in G2, three in G4, three in G6, three in G8. The length of each linkage group ranged from 22.6 (G4) to 60.1 cM (G2).

QTL analysis

In total, two QTLs have been identified by interval mapping. Two major QTLs (G1 and G4) were detected for flowering time (Ft-Q1 and Ft-Q2). The phenotypic variance explained by Lf-Q1 (42.9%) was smaller than that explained by Ft-Q2 (45.4%). In G1 the QTL Ft-Q1 peak was located close to locus BPPCT011 in 34.5 CM, while in G4 the QTL Lf-Q2 peak was located close to UDP98-021 in 17.5 CM (Figs. 1 and 2).

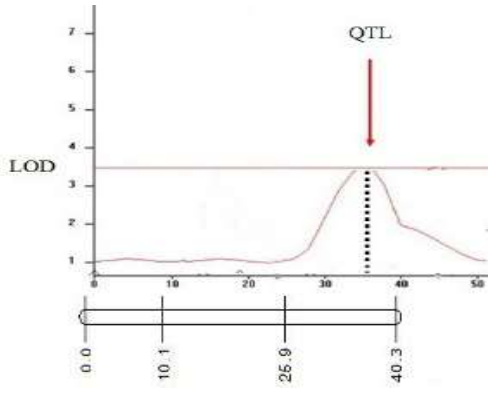

Fig.1. Position, distanceandLODquantitativetraitlocicontrollingflowering timeinMarcona × FragnessF1 progeny

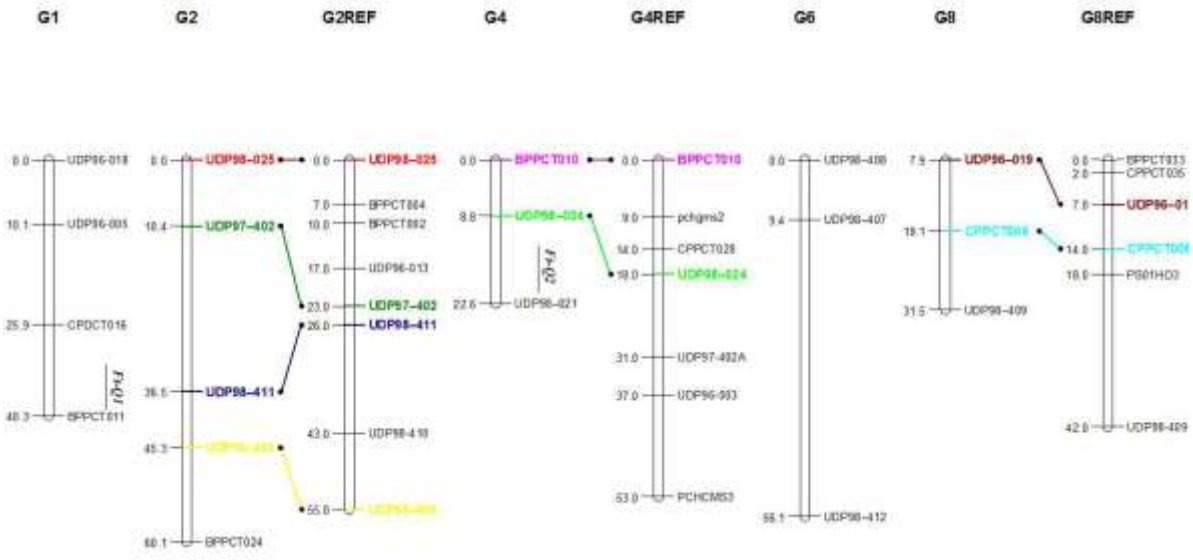

Fig. 2. Molecular linkage map constructed with the JOINMAP software of Marcona × FragnessF1 progeny obtained with 17 SSR Markers.

Discussion

Results showed a high degree of transportability for the SSR loci among *Prunus* species. Most peach and almond SSRs amplified and were polymorphic in the almond F1 progeny Marcona × Fragness. These results agree with previous reports by Cipriani *et al*., 1999, Sosinski *et al*., 2000, Cantini *et al*., 2001, Martınez-Gomez *et al.*, 2012 and Rasouli *et al.*, *2014a* on the successful utilization of these markers in different *Prunus* species.

Five of the 17 SSRs used had previously been mapped, and these new results agree with their previous location and locus order in the reference *Prunus* map (Aranzana *et al*., 2003; Dirlewanger *et al*., 2004) with minor changes. These differences were always due to permutations of two adjacent loci, indicating that they are more likely to be attributable to errors in the mapping process than to actual chromosome rearrangements. The nearly identical order of SSRs observed in different *Prunus* maps confirms the high level of synteny previously found in this genus (Dirlewanger *et al*., 2004, Arus *et al*., 2005). This synteny among *Prunus* species is in agreement with the low level of breeding barriers to interspecific gene introgression in this genus and highlights the opportunity for successful gene transfer between closely related species (Gradziel *et al*., 2001).

More recently, different works using SSR markers in a F1 population between a seedling of "Tardy Nonpareil" ("R1000") × "DesmayoLargueta" (R×D), also confirmed the location of *Lb* in G4 and identified other QTLs to flowering time in G1, G6, and G7 (Sánchez-Pérez and et al., 2007; Martínez-Gómez *et al*., 2012). Also other recent findings in almond confirm the results for late-flowering trait loci on linkage group 1 and 4(Sánchez-Pérez et al., 2007; Martínez-Gómez *et al*., 2012 and Rasouli *et al*., 2013; Rasouli *et al*., 2014b). In other hand the results in other *Prunus* species (cherry, apricot and peach) have similarity with our findings (Fan *et al*. 2010; Wang *et al*. 2000; Dirlewanger *et al*. 2012, Castede *et al*., 2014).

In conclusion, a linkage map in almond has been developed with 17 SSR markers. In addition, it was possible to place in this map 2 QTLs that will be useful in breeding programs. However, further studies with appropriate crosses between parents, which segregate

for these traits, will be necessary to apply efficient MAS strategies in the breeding programs.

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