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A molecular marker approach using intron flanking EST-PCR to map candidate genes in peach (*Prunus persica*)

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Abstract In Peach (*Prunus persica*) several physiological changes, such as woolliness, triggered by chilling injury are involved in major production losses due to cold storage of the fruits during shipping. Additionally, the low level of polymorphisms among peach varieties is an important limitation in the search for new molecular markers that could be associated with economically important traits. Therefore, a functional approach was employed to associate candidate genes with an informative marker in peach. The data was obtained from the results of an *in silico* analysis of four different cold peach treatments. Thirty two candidate genes were selected that were aligned against *Arabidopsis thaliana* genomic sequences to design intron-flanking EST-PCR markers. These markers were used to position the candidate genes on the *Prunus* genetic reference map. In the physiological response to chilling injury, cell wall integrity, carbohydrate metabolism and stress response pathways could be involved, therefore candidate genes associated by Gene Ontology annotation to these pathways were included in the analysis. The designed markers were positioned to the Texas X Earlygold (TxE) genetic reference map through selective mapping methodology (Bin mapping). 72% of these new markers showed polymorphism in the TxE Binset population and 31% of them were successfully mapped to a genetic position on the *Prunus* reference map. The bioinformatic methodology used in this work includes a first approach in search for functional molecular markers associated to differentially expressed genes under certain physiological condition which in addition to the Bin mapping approach allows addressing a genetically anchored position to these new markers.

Keywords: bin mapping, molecular markers, *Prunus*, Rosaceae

INTRODUCTION

Peach (*Prunus persica*) is one of the most important fruit exported by Chilean industry. Its production was 100,318 tons in 2011 with a 112.3 million dollars market (Bravo, 2012). Since fruit quality is critical to sustain the position of the product in the market, post harvest alterations are key issue. Due to the long term shipment, fruits are under several adverse conditions that include biotic and abiotic stress, causing considerable damages to the final product, such as woolliness (Lill et al. 1989; Lurie and Crisosto, 2005). The main problem associated with exportation of peaches is related with the woolly phenotype caused by the low temperatures in which the fruit must be storage in order to delay ripening. In this frame of time many cellular processes are triggered, therefore a fine regulation is needed (Lill et al. 1989; Lurie and Crisosto, 2005). Approaches to manage these conditions have been reported. Intermittent warming (Fernández-Trujillo and Artés, 1997; Lurie and Crisosto, 2005) and pre conditioning (Infante et al. 2009) has been used. These two approaches involved temperature changes through the fruit cold chain transport, which is directly associated with additional costs.

Alternative approaches to try to solve this problem are Molecular Assisted Selection (MAS) and transgenic plant technologies. In order to find a solution that could be used in a MAS program, identification of genes involved in this physiological problem in peach is needed. Several approaches have been taken, such as candidate genes identification (Horn et al. 2005; González-Agüero et al. 2008; Vecchietti et al. 2009; Vizoso et al. 2009) and molecular markers mapped to genetically anchored maps (Joobeur et al. 1998; Wang et al. 2002; Jung et al. 2005). To accomplish the identification of molecular markers that can be associated with higher tolerance to woolliness and to other traits in *Prunus* species, markers such as RFLP, isozymes (Joobeur et al. 1998), SSR (Yamamoto et al. 2002) and EST-SSR (Howad et al. 2005) has been anchored to a reference map. This reference map is considered key in the development of a selective mapping approach known as Bin mapping (Howad et al. 2005). This approach consist in the positioning of newly developed molecular markers within a previously described genetically anchored map, which in the case of *Prunus* species belongs to the F_2 backcross of the almond variety Texas with the peach variety Earlygold (Joobeur et al. 1998).

The development of an approach known as intron-flanking EST-PCR markers based on the alignment of EST cDNA sequences against known genomic sequences of *Arabidopsis thaliana* has been described (Wei et al. 2005). These types of markers are based on the prediction of putative splicing sites and the amplification of them. Considering the assumption that intronic regions are richer in polymorphism than exonic ones, an increment in the use of these markers as fingerprinting tool has been reported for non-model species such as *Rhododendron* (Wei et al. 2005; De Keyser et al. 2009), *Lolium*, *Festuca* (Tamura et al. 2009) and Rosaceae species (Sargent et al. 2009). Taking in consideration the functional association of these type of markers, it could be a good approach in order to search for Quantitative Trait Loci (QTL), which in *P. persica* has been described already for commercially interesting traits such as soluble sugar and organic acids content (Dirlewanger et al. 1999), fruit weight, skin colour, total soluble solids, juice acidity and juice pH (Eduardo et al. 2011).

The objective of the present work is to design polymorphic markers, using an intron flanking EST-PCR approach. These markers are representative of previously undescribed genes associated to metabolic pathways that could be involved in the triggering of the woolly phenotype. This will allow assigning a position in common regions of the genetically anchored *Prunus* reference map, which are projected to be useful for future MAS programs.

MATERIALS AND METHODS

Genomic DNA material

The genomic DNA used for the screening through the Bin mapping (Howad et al. 2005) approach belongs to the F_2 population of Peach cv. Earlygold X Almond cv. Texas described for the *Prunus* reference map (TxE) in Joobeur et al. (1998) kindly donated by Dr. Peré Arús and Dr. Werner Howad (IRTA, Spain).

Candidate gene selection

The information describing differentially expressed genes reported in a previous work (Vizoso et al. 2009) was obtained from our database and managed using the JUICE software (Latorre et al. 2006). 32 unigenes were selected for Bin mapping (Table 1). These selection of unigenes previously shown a differential expression when fruits were stored in cold conditions for a prolonged period of time (Vizoso et al. 2009). Based on their ontology, these genes may be related to cell wall metabolism and stress response. From a group of 249 unigenes differentially expressed between harvest and post harvest cold treatment, we select 2 subsets of unigenes according to its Gene Ontology categorization (Harris et al. 2004) for cell wall integrity and stress response. For cell wall integrity a total of 18 genes were selected. The second subset considered 13 genes related with stress response, which in addition to the Hsp70 control makes a total of 32 unigenes (Table 1) used to identify intron flanking EST-PCR molecular markers and position them on the *Prunus* reference map.

Table 1. Selected candidate genes. Selected unigenes according to its Gene Ontology categorization, contigs identity numbers and description of the annotation for each gene are showed according to Vizoso et al. (2009) and their positioning in the available physical map prunus v1.0.

Group	Contig ID	GO Biological process	Annotation	best hit prunus genome sequence ID	Scaffold location prunus v1.0
Cell Wall and Carbohydrate Metabolism	1471	GO:0006098, GO:0005975	AT1G13700.1 glucosamine/galactosamine-6-phosphate isomerase family protein, [*Arabidopsis thaliana*] contains InterProScan IPR001093: IMP	ppa010073m	1: 27869871 - 27873321
	1879	GO:0005975	*Prunus persica* weakly similar to 4-alpha-glucanotransferase precursor [*Solanum tuberosum*]	ppa003327m	6: 15946189 - 15951327
	2768	GO:0005975	*Prunus persica* fructose-1,6-bisphosphatase homolog [*Pisum sativum*] contains IPR000146: Inositol phosphatase/fructose-1,6-bisphosphatase	ppa008222m	8: 2567207 - 2570516
	2825	GO:0005975	Endoxyloglucan transferase [*Daucus carota*] contains InterProScan IPR000757: Glycoside hydrolase, family 16 IPR008985: Concanavalin A-like lectin/glucanase	ppa008275m	1: 30806773 - 30809293
	2828	GO:0006073, GO:0005975	Endoxyloglucan transferase, putative [*Arabidopsis thaliana*] contains InterProScan IPR000757: Glycoside hydrolase, family 16 IPR008985: Concanavalin A-like lectin/glucanase IPR010713: Xyloglucan endo-transglycosylase, C	ppa007685m	5: 3239020 - 3242053
	2957	GO:0005975	AT5G49360.1 glycosyl hydrolase family 3 protein contains InterProScan IPR001093: IMP dehydrogenase/GMP reductase IPR001764 : Glycoside hydrolase, family 3, N-terminal IPR002772 : Glycoside hydrolase, family 3, C-terminal	ppa001718m	1: 9201981 - 9206434
	457	GO:0005975	*Prunus persica* similar to AT4G03210.1 xyloglucan:xyloglucosyl transferase, putative/xyloglucan endotransglycosylase, putative/endo-xyloglucan transferase, putative, contains similarity to xyloglucan endo-transglycosylase-like protein (XET-1)	ppa009350m	3: 16991024 - 16993725
	745	GO:0005975, GO:0000272	Identical to *Prunus armeniaca* beta-amylase (AMYB) mRNA contains InterProScan IPR001554: Glycoside hydrolase, family 14	ppa004116m	1: 10744696 - 10747638
	803	GO:0008152, GO:0006073, GO:0005975	*Prunus persica* similar to AT5G13870.1 xyloglucan:xyloglucosyl transferase/xyloglucan endotransglycosylase/endo-xyloglucan transferase (EXGT-A4), identical to endoxyloglucan transferase EXGT-A4 GI:5533315 from (*Arabidopsis thaliana*) contains InterProScan IPR000757: Glycoside hydrolase, family 16 IPR000873: AMP-dependent synthetase and ligase IPR008263: Glycoside hydrolase, family 16, active site IPR008985: Concanavalin A-like lectin/glucanase IPR010713: Xyloglucan endo-transglycosylase, C-terminal	ppa009387m	1: 21457296 - 21459443
	910	GO:0005975	AT5G24090.1 acidic endochitinase (CHIB1), identical to SP:P19172 Acidic endochitinase precursor (EC 3.2.1.14) (*Arabidopsis thaliana*) contains InterproScan IPR001223: Glycoside hydrolase, family 18 IPR001579: Glycoside hydrolase	ppa009328m	2: 25508937 - 25510028
	1590	GO:0005985	Sucrose synthase (EC 2.4.1.13) (Sucrose-UDP glucosyltransferase) contains InterProScan IPR000368: Sucrose synthase	ppa001535m	7: 18751831 - 18757844
	474	GO:0006094	Prunus persica phosphoenolpyruvate carboxykinase homolog [*Solanum lycopersicon*] contains IPR001093: IMP dehydrogenase/GMP reductase IPR001272: Phosphoenolpyruvate carboxykinase (ATP) IPR007087: Zinc finger, C2H2-type	ppa003487m	6: 20082226 - 20085411
	1322	GO:0006096	AT1G02270.1 endonuclease/exonuclease/phosphatase family protein / calcium-binding EF hand family protein [*Arabidopsis thaliana*] contains InterProScan IPR000173: Glyceraldehyde 3-phosphate dehydrogenase IPR002048: Calcium	ppa005459m	2: 20332919 - 20336529

1686	GO:0006096, GO:0006094	*Prunus persica* identical to cDNA clone PP_YEb0033G12 and similar to putative glucose-6-phosphate isomerase [*Arabidopsis thaliana*] contains IPR001093: IMP dehydrogenase/GMP reductase IPR001672: Phosphoglucose isomerase (PGI)	ppa002932m	1: 46573995 - 46579710
1737	GO:0006096	Prunus persica similar to hexokinase-related protein 1 [*Solanum tuberosum*]; IPR001312:Hexokinase, complete CDS	ppa004715m	1: 32589942 - 32593010
2575	GO:0006096	Glyceraldehyde-3-phosphate dehydrogenase [*Capsicum annuum*] contains InterProScan IPR000173: Glyceraldehyde 3-phosphate dehydrogenase IPR001093: IMP dehydrogenase/GMP reductase IPR011596: Glyceraldehyde 3-phosphate dehydrogenase	ppa023763m	4: 3754796 - 3757806
3328	GO:0006096	AT3G04120.1 glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC)/NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, identical to SP:P25858 Glyceraldehyde 3-phosphate dehydrogenase, cytosolic (EC 1.2.1.12)	ppa008250m	5: 13923035 - 13926433
3749	GO:0006096	Pyruvate kinase [*Glycine max*] contains InterProScan IPR001697: Pyruvate kinase IPR011037: Pyruvate kinase, beta-barrel-like	ppa008250m	5: 13923035 - 13926433
1190	GO:0050832, GO:0042742, GO:0010204, GO:0009870	*Prunus persica* identical to cDNA clone PP_LEa0007J18f and weakly similar to gb|AAM14351.1| unknown protein [*Arabidopsis thaliana*] contains IPR008409: Breast carcinoma amplified sequence 2	ppa010316m	5: 17577235 - 17578955
1263	GO:0006979	*Prunus persica* AT2G43350.1 glutathione peroxidase, putative weakly similar to contains InterproScan IPR000889: Glutathione peroxidase IPR012336: Thioredoxin-like fold	ppa011682m	1: 15973147 - 15976749
1997	GO:0009607, GO:0006952	*Prunus persica* hypothetical domain/motif containing AT1G24020.1 Bet v I allergen family protein, similar to major pollen allergen Bet v 1 GB:CAA96544 GI:1321726 from (*Betula pendula*); contains InterproScan IPR000916: Bet v I allergen	ppa011018m	1: 9548896 - 9551174
2260	GO:0008219	*Prunus persica* weakly similar to AtMlo-h1 [*Arabidopsis thaliana*] contains IPR004326: Mlo-related protein	ppa004508m	8: 21170244 - 21172100
2307	GO:0009607, GO:0006952	*Prunus persica* AT1G24020.1 Bet v I allergen family protein, similar to major pollen allergen Bet v 1 GB:CAA96544 GI:1321726 from (*Betula pendula*); weakly similar to contains InterproScan IPR000916: Bet v I allergen	ppa011018m	1: 9548896 - 9551174
2464	GO:0009415, GO:0006950	*Prunus persica* similar to abscicic acid response protein [*Prunus dulcis*]; contains IPR000167: Dehydrin, complete CDS 19:17:07)	ppa011637m	7: 17143214 - 17144411

Stress Response	2473	GO:0009607, GO:0006952	AT1G24020.1 Bet v I allergen family protein, major pollen allergen Bet v 1 GB:CAA96544 GI:1321726 from (*Betula pendula*);contains InterproScan IPR000916: Bet v I allergen	ppa008222m	8: 2567207 - 2570516
	2528	GO:0050832, GO:0042742, GO:0016998, GO:0006032	Identical to class 4 pathogenesis-related protein mRNA contains InterProScan IPR001153: Barwin IPR009009: Barwin-related endoglucanase	ppa012991m	6: 11409983 - 11410896
	2718	GO:0006979	*Prunus persica* AT4G11600.1 glutathione peroxidase, putative weakly similar to contains InterproScan IPR000889 : Glutathione peroxidase IPR001452: Src homology-3 IPR012336: Thioredoxin-like fold	ppa010771m	5: 10690895 - 10693157
	2785	GO:0007568	hypothetical protein |NP_188894,2| senescence-associated protein-related	ppa 009460m	1: 1141432 - 1144128
	2802	GO:0007568	hypothetical protein |NP_565167,1| senescence-associated protein-related	ppa012051m	6: 16859874 - 16861218
	2919	GO:0007568	Hypothetical domain/motif containing AT5G20700.1 senescence-associated protein-related, [*Arabidopsis thaliana*] contains InterProScan IPR007650: Protein of unknown function DUF581	ppa010802m	1: 5274461 - 5278182
	339	GO:0006979	Probable phospholipid hydroperoxide glutathione peroxidase (EC 1.11.1.12) (PHGPx) (6P229) contains InterProScan IPR000297: PpiC-type peptidyl-prolyl cis-trans isomerase IPR000889: Glutathione peroxidase IPR0014	ppa012416m	5: 10697597 - 10699370
	2131	GO:0016998, GO:0006032	AT3G12500.1 basic endochitinase, identical to basic endochitinase precursor SP:P19171 from (*Arabidopsis thaliana*) contains InterproScan IPR000726: Glycoside hydrolase, family 19 IPR001002: Chitin-binding, type 1	ppa008859m	7: 17991605 - 17993043
	1027	GO:0015979, GO:0010270	Non-cell-autonomous heat shock cognate protein 70 [*Cucurbita maxima*] contains InterProScan IPR001023: Heat shock protein Hsp70 IPR001093: IMP dehydrogenase/GMP reductase IPR013126: Heat shock protein 70	ppa002641m	2: 22075055 - 22077889

Design of intron flanking EST-PCR Markers

To design molecular markers representative of each candidate gene, we used the intron-flanking PCR molecular marker approach described by Wei et al (2005). This approach consists in a local BLAST-N alignment of the candidate genes against genomic sequences that includes introns and untranslated regions (UTRs) of *Arabidopsis thaliana* (genome version TAIR10 plus introns and UTRs downloaded from www.arabidopsis.org). Taking in consideration that predictions of putative splicing sites through a comparative analysis have been demonstrate to be highly accurate between *A. thaliana* and non model species (Wei et al. 2005; De Keyser et al. 2009; Sargent et al. 2009; Tamura et al. 2009) we decide to take this approach for our analysis. In order to design intron flanking PCR molecular markers which will be representative of each candidate gene, the consensus sequence of the assembled contigs obtained from our local database were aligned using BLAST-N against the tenth version of the annotated *A. thaliana* genome which includes introns and UTRs (http://www.arabidopsis.org/wublast/index2.jsp). This sequence alignment allows putative splicing sites to be predicted (exon-exon joint). Based on the assumption that simple sequence polymorphisms are more abundant in non coding regions, we designed PCR primers that flank exon-exon junction (Figure 1). The primermaster 2.0 program was used for primer design (Proutski and Holmes, 1996). The following criteria were considered: length of primers between 16-24 mer, size of amplicon in cDNA sequence no shorter than 200 bp and no longer than 300 bp, and melting temperature limits between 55ºC - 65ºC. Designed primers and expected size of introns can be observed in Table 2. The contigs included in the molecular marker design were also physically mapped to the Peach 1.0 scaffolds available in the Genome Database for Rosaceae (Jung et al. 2004; Arús et al. 2012) and Phytozome v8.0 (Goodstein et al. 2012). The mapping was done by BLASTX alignment against the *Prunus* proteome available within these databases (Table 1).

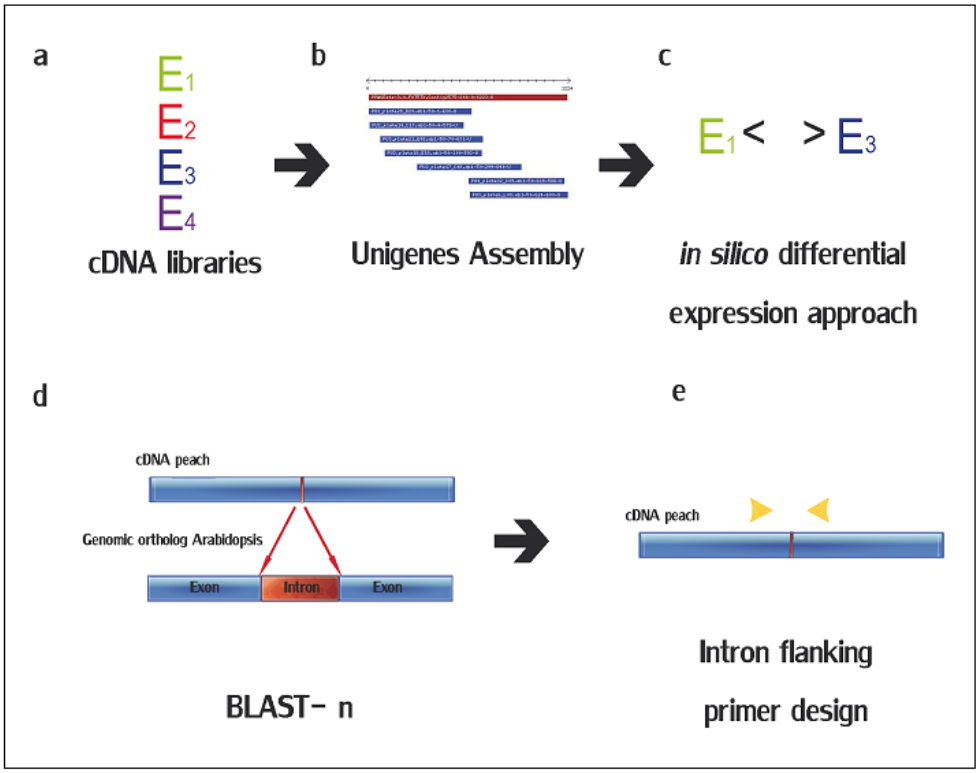

Fig. 1 Strategy for the design of intron flanking PCR markers. (a) Four cDNA libraries that represents the different post harvest conditions: packing (E1), ripe fruit (E2), cold treatment at 4ºC for 21 days (E3), and ripe fruit after cold treatment (E4) were assembled (b) and used for the *in silico* analysis in Vizoso et al. 2009; (c) those unigenes differentially expressed between stages E1 and E3 were selected considering those that were not previously described in any public database, and that also belongs to the categories associated to cell wall integrity and stress response; (d) a local BLAST-N with default parameters was performed to search for putative splicing site (exon-exon joint) as described in Wei et al. (2005); (e) primers that flank the predicted site were design using the primer master 2.0 program.

Table 2. Intron flanking EST-PCR primers. The table shows the selected unigene identity, the code number for their orthologs in the *Arabidopsis* genome. The predicted sizes of introns according to the observed in Arabidopsis are shown.

ContigID	BesthitinArabidopsis	PredictedIntronsize(bp)inArabidopsis	PrimerID	Sequence5'-3'	cDNAamplificationfragment(bp)
1471	AT1G13700.1	82	1471intflnkfw1 1471intflnkrv1	tttgggctgacgagcg gtgccgagtggttagg	296
1879	AT5G64860.1	153	1879intflnkfw1 1879intflnkrv1	ttcctgacccctgttt gaagctcttcctttgt	378
2768	AT1G43670.1	169	2768intflnkfw1 2768intflnkrv1	atggttgcagcaggct ccgttcctgccagtga	427
2825	AT2G01850.1	197	2825intflnkfw1 2825intflnkrv1	cactggacgaacggac gggaaagtcaccaccc	395
2828	AT1G32170.1	408	2828intflnkfw1 2828intflnkrv1	gggaagagcgttaccg cggctgggatttgctg	311
2957	AT1G02640.1	94	2957intflnkfw1 2957intflnkrv1	caagggcacaatccgt ccttgcctagcagctt	365
457	AT4G03210.1	220	457intflnkfw1 457intflnkrv1	ccaacctgggctttcg cccatagcttggtcct	448
745	AT5G18670.1	155	745intflnkfw1 745intflnkrv1	gctgcgcactactttg aaccactccccaccag	707
803	AT5G13870.1	84	803intflnkfw1 803intflnkrv1	ttcgacccaaccgcag aatttggcctccaccg	283
910	AT5G24090.1	168	910intflnkfw1 910intflnkrv1	tggtgggattgccatc tgtggagctgcagcta	472
1590	AT3G43190.1	173	1590intflnkfw1 1590intflnkrv1	cgtgccttgacccgtg gcagtgggtgcatgct	539
474	AT5G65690.1	88	474intflnkfw1 474intflnkrv1	gcctaccccatcgagt tgaagaggccagccag	558
1322	AT5G54130.2	169	1322intflnkfw1 1322intflnkrv1	cgcgaacgaacaaccg gcttttcggagctggc	573
1686	AT4G24620.1	209	1686intflnkfw1 1686intflnkrv1	tccgcatgcgctgtgg ggccagcctcacggaa	598
1737	AT1G47840.1	182	1737intflnkfw1 1737intflnkrv1	cgatctgctgccgttt ccagcccagaagcaat	363
2575	AT1G79530.1	437	2575intflnkfw1 2575intflnkrv1	atcgaagcctcgcgtg ccccaagggatctccg	490
3328	AT1G13440.1	289	3328intflnkfw1 3328intflnkrv1	ggcgactacatggttt cttttgggtggcagta	447
3749	AT4G26390.1	630	3749intflnkfw1 3749intflnkrv1	tcggtgcccatggtcg agtgccatcagcgcag	342
1190	AT3G18165.1	432	1190intflnkfw1 1190intflnkrv1	ccacttcccaagccca aggcgtcaggaccata	243
1263	AT2G31570.1	249	1263intflnkfw1 1263intflnkrv1	cccaaattatggctga cattgtttcctggctc	251

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1997	AT1G63100.1	0	1997intflnkfw1 1997intflnkrv1	cagacgagtccacctc gaccagtggccttctc	411
2260	AT4G02600.1	94	2260intflnkfw1 2260intflnkrv1	gcaaaacccacttccc gcgatcgaatcctccc	235
2307	AT1G63100.1	0	2307intflnkfw1 2307intflnkrv1	cagacgagtccacctc tctgggttggccacaa	450
2464	AT5G66400.1	81	2464intflnkfw1 2464intflnkrv1	ccaggagaaggggacg cgccattgcgttttga	317
2473	AT1G63100.1	0	2473intflnkfw1 2473intflnkrv1	ccccaagattgctccc caagccaaagcagctc	495
2528	AT3G04720.1	201	2528intflnkfw1 2528intflnkrv1	cgcaacatgggatgct ttgggcattgccttgt	237
2718	AT4G11600.1	89	2718intflnkfw1 2718intflnkrv1	caatggctagccagtc gctccaaactgattgc	234
2785	AT3G63210.1	95	2785intflnkfw1 2785intflnkrv1	ttcggctcgggttttc cacggctgcagaatgc	286
2802	AT4G39795.1	323	2802intflnkfw1 2802intflnkrv1	ctcggctgctcatgac gcggtcgtccttcttt	201
2919	AT5G20700.1	78	2919intflnkfw1 2919intflnkrv1	aggtcaagcccaatcc gccaagaagaccagcg	485
339	AT1G63460.1	159	339intflnkfw1 339intflnkrv1	tgcctccaaatgtggc tgcctgccttcaagaa	247
2131	AT3G12500.1	475	2131intflnkfw1 2131intflnkrv1	tatcgaaatgacgcga tgcgtgagttggatgg	312
1027	AT5G02500.1	351	1027intflnkfw1 1027intflnkrv1	ccgtttaggattggct ggctcatggtgcctgg	306

Genotype screening, genetic anchored map position assignment and PCR amplification

To position these candidate genes within the TxE *Prunus* reference map, we used the Bin mapping approach, described by Howad et al. (2005). The approach consists in a PCR profiling on the 8 most informative plants of the reference map population (2 parental plants and 6 F_2 population plants). The different genetic profile of this small set of plants describes 67 spaces across the 8 linkage groups of the *Prunus* genetically anchored map (Joobeur et al. 1998), which are determined by the presence of female, male or heterozygous alleles; allele A represents the female parental; B represents the male parental (Earlygold line) and H is the heterozygote (TxE line). PCR amplification of genomic DNA was performed as follow: 25 µL of PCR mixture consisting of 10 ng of genomic DNA; 6.3 µL of nuclease free H_2O; 2.5 µL of 10X RBC® reaction buffer (with 15 mM Mg^{2+}); 1 µL of RBC ® 50 mM $MgCl_2$ (to reach a 3.5 mM final Mg^{2+} concentration); 1 mM of Invitrogen® dNTPs mixture; 0.5 mM of forward and reverse primers and 1 U of RBC ® Taq DNA Polymerase. The mixtures were incubated for 5 min at 94ºC, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing for 1 min at selected temperatures for each set of primers, and elongation at 72ºC for 1 min, followed by a final extension of 10 min at 72ºC. The PCR amplification products were analyzed by electrophoresis in a 7% acrylamide gel, at 120 V for 2 hrs, and revealed by ethidium bromide staining.

Identification of orthologs in the *Arabidopsis* genome

The closest orthologs mapped into the genetically anchored TxE Bin mapping genes were located within the *Arabidopsis* genome through the chromosome map tool on the TAIR website (http://www.arabidopsis.org). The orthologs names were used as input into the chromosome map and their positions were displayed using the whole genome view.

RESULTS

Alignment gaps were localized and compared to the intron position in *Arabidopsis's* sequences for prediction of exon-exon joint putative position within the peach sequences (Figure 1). Primers that flank one or maximum two consecutive introns were designed (Table 2). Additionally, primers that amplify a large fragment within sequences available were designed in those unigenes that present lack of introns in their respective *Arabidopsis* orthologs (Table 2).

Amplification from genomic DNA was performed on the Binset samples (Figure 2). 72% of the Unigenes analyzed showed evidence of size polymorphism on the Binset TxE population, from which 34% were successfully mapped to a Bin position (Table 3 and Figure 3) and 38% of them showed a polymorphic profile not described in any Bin. The unigene 803 similar to a xyloglucan endotransglycosilase of *A. thaliana*, and unigene 1686 which is similar to a putative glucose-6-phosphate isomerase from *A. thaliana* were mapped to the Bin 1:78, which is delimited by RFLP markers BF08A and TubA3. Both unigenes were selected from groups associated with cell wall integrity and carbohydrate metabolism. A group of two candidate genes related with cell wall integrity and carbohydrate metabolism, unigene 2575, similar to a glyceraldehyde-3-phosphate dehydrogenase from *Capsicum annuum* and unigene 474 that is a *Prunus persica* phosphoenolpyruvate carboxykinase homolog to one from *Solanum lycopersicum*, were placed to the Bin 4:18 which is delimited by the RFLP marker BG05A and the SSR marker BPPCT040. The unigene 2131 (from the stress response related group) similar to an endochitinase precursor from *A. thaliana* was positioned within the Bin 7:48, which is delimited by RFLP marker AG60A and PMS2 SSR marker. In the Bin 7:56 (contiguous to Bin 7:48) was positioned the unigene 1590 (cell wall and carbohydrate metabolism group) which has a domain of a sucrose-UDP glucosyltransferase. The unigene 2785, a hypothetical protein associated to senescence was positioned to the Bin 1:14 delimited by the EST-SSR EPPCU0027 and the SSR UDAp-471. The unigene 1027, similar to the heat shock protein 70 was mapped to the Bin 2:50, delimited by two SSR markers, which are UDA-023 and UDAp-462, both from *Prunus amygdalus* (Struss et al. 2003). Within the linkage group number 3, just one of the selected candidate genes was mapped to the Bin 3:12, unigene 2957, similar to a protein from the glycosyl hydrolase family 3. The marker designed from the unigene 1879 which showed a weak similitude to the precursor for 4-alpha-glucanotransferase was mapped to Bin 6:39, delimited by the SSR type markers M19a and UDAp-497 from *P. persica* and *P. amygdalus* respectively. Within the linkage group 8, a new marker designed from the unigene 2473 sequence, from the cell wall integrity group of candidate genes, was mapped to the Bin 8:41, delimited by the EST-SSR EPPB4226 and the SSR marker MA013a (Yamamoto et al.

2002), both from *P. persica*. Additionally, the orthologs from *A. thaliana* of the candidate genes successfully mapped in the *Prunus* reference map, through the Bin mapping approach, were identified using the TAIR chromosome map (Figure 4).

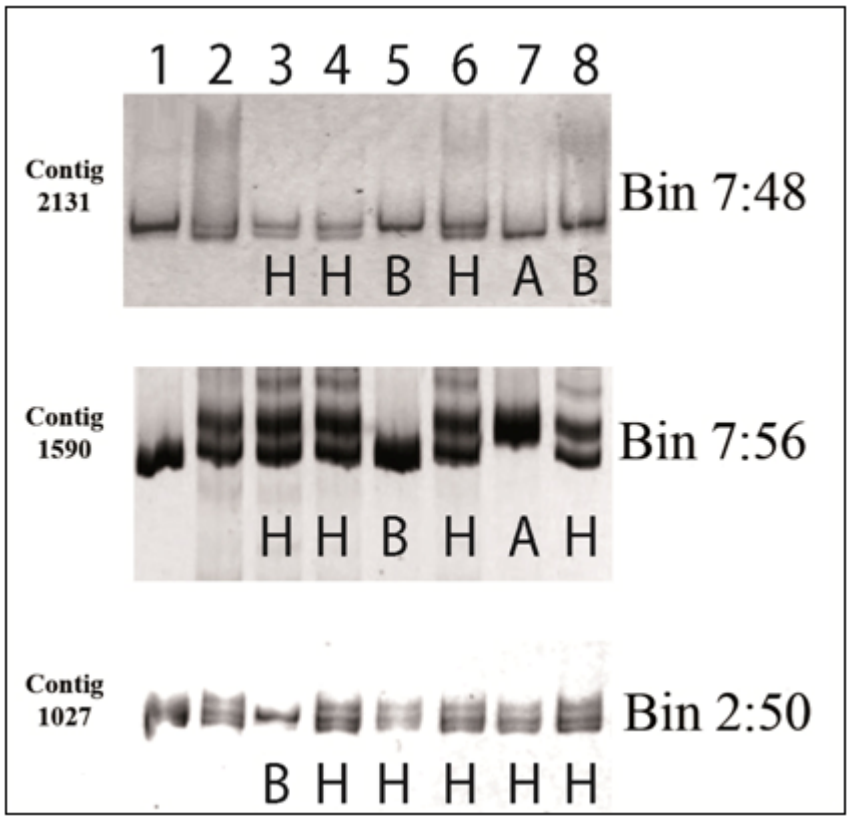

Fig. 2 Genotype profiling of three intron flanking markers on the Binset population. The figure shows PCR amplifications of markers designed from contigs 2131 (basic chitinase); 1590 (Sucrose-UDP glucosyltransferase) and 1027 (heat shock cognate protein 70) separated in a 7% Polyacrilamide gel. Lane 1 shows the genotype profile for Earlygold (peach) male parental; lane 2 the TxE heterozygous F_1 plant which determine the respective male and female alleles. The following F2 plants 5, 12, 23, 30, 34 and 83 (lanes 3 to 8 respectively) describe the genotype for unigenes. Genotyping was done as described in Howad et al. (2005). A represents the female parental, and H is the heterozygote (TxE line), B represents the male parental (Earlygold line).

DISCUSSION

We did analyze a set of genes potentially involved in abiotic stress mediated by low temperature which also could be involved in cell wall integrity. The criteria used to select genes include those unigenes that in the first place were differentially transcribed after 21 days of cold treatment as described in Vizoso et al. (2009), taking in account that the triggering of the chilling injury is considered to be irreversible after 15 days (Fernández-Trujillo and Artés, 1997). The selection of the candidate genes, based on their GO categories, considered for cell wall integrity the genes that were associated with synthesis or degradation of cell wall and additionally those unigenes with GO category included in any part of the carbohydrate metabolism, since the available pool of carbohydrates could affect the cell wall metabolism (Fischer and Bennett, 1991; Dawson et al. 1992; Trainotti et al. 2003; Brummell et al. 2004). Also those unigenes which were categorized by their GO number within pathways associated with response to abiotic stimuli were also included, taking in account the cross talk between biotic and abiotic responses (Fujita et al. 2006). Therefore an attempt to position genes functionally associated to chilling injury in the *Prunus* reference map was made.

Table 3. Bin position assigned to the candidate genes. The Bin position for unigenes based on the genotype exhibited on the Bin set population after intron flanking PCR profiling are shown.

Unigene ID	Annotation	Bin Assigned	Bin length (cM)
2785	hypothetical protein |NP_188894,2| senescence-associated protein-related	**1:14**	13.6
803	*Prunus persica* similar to AT5G13870.1 xyloglucan:xyloglucosyl transferase/xyloglucan endotransglycosylase/endo-xyloglucan transferase (EXGT-A4), identical to endoxyloglucan transferase EXGT-A4 GI:5533315 from (*Arabidopsis thaliana*) contains InterProScan IPR000757: Glycoside hydrolase, family 16 IPR000873: AMP-dependent synthetase and ligase IPR008263: Glycoside hydrolase, family 16, active site IPR008985: Concanavalin A-like lectin/glucanase IPR010713: Xyloglucan endo-transglycosylase, C-terminal	**1:78**	2.8
1686	*Prunus persica* identical to cDNA clone PP_YEb0033G12 and similar to putative glucose-6-phosphate isomerase [*Arabidopsis thaliana*] contains IPR001093: IMP dehydrogenase/GMP reductase IPR001672: Phosphoglucose isomerase (PGI)	**1:78**	2.8
1027	Non-cell-autonomous heat shock cognate protein 70 [*Cucurbita maxima*] contains InterProScan IPR001023: Heat shock protein Hsp70 IPR001093: IMP dehydrogenase/GMP reductase IPR013126: Heat shock protein 70	**2:50**	2.7
2957	AT5G49360.1 glycosyl hydrolase family 3 protein contains InterProScan IPR001093: IMP dehydrogenase/GMP reductase IPR001764: Glycoside hydrolase, family 3, N-terminal IPR002772: Glycoside hydrolase, family 3, C-terminal	**3:12**	4.3
2575	Glyceraldehyde-3-phosphate dehydrogenase [*Capsicum annuum*] contains InterProScan IPR000173: Glyceraldehyde 3-phosphate dehydrogenase IPR001093: IMP dehydrogenase/GMP reductase IPR011596: Glyceraldehyde 3-phosphate dehydrogenase	**4:18**	18.4
474	*Prunus persica* phosphoenolpyruvate carboxykinase homolog [*Solanum lycopersicon*] contains IPR001093: IMP dehydrogenase/GMP reductase IPR001272: Phosphoenolpyruvate carboxykinase (ATP) IPR007087: Zinc finger, C2H2-type	**4:18**	18.4
1879	*Prunus persica* weakly similar to 4-alpha-glucanotransferase precursor [*Solanum tuberosum*]	**6:39**	9.2
2131	AT3G12500.1 basic endochitinase, identical to basic endochitinase precursor SP:P19171 from (*Arabidopsis thaliana*) contains InterproScan IPR000726: Glycoside hydrolase, family 19 IPR001002: Chitin-binding, type 1	**7:48**	5.3
1590	Sucrose synthase (EC 2.4.1.13) (Sucrose-UDP glucosyltransferase) contains InterProScan IPR000368: Sucrose synthase	**7:56**	7.1
2473	AT1G24020.1 Bet v I allergen family protein, major pollen allergen Bet v 1 GB:CAA96544 GI:1321726 from (*Betula pendula*); contains InterproScan IPR000916: Bet v I allergen	**8:41**	10.8

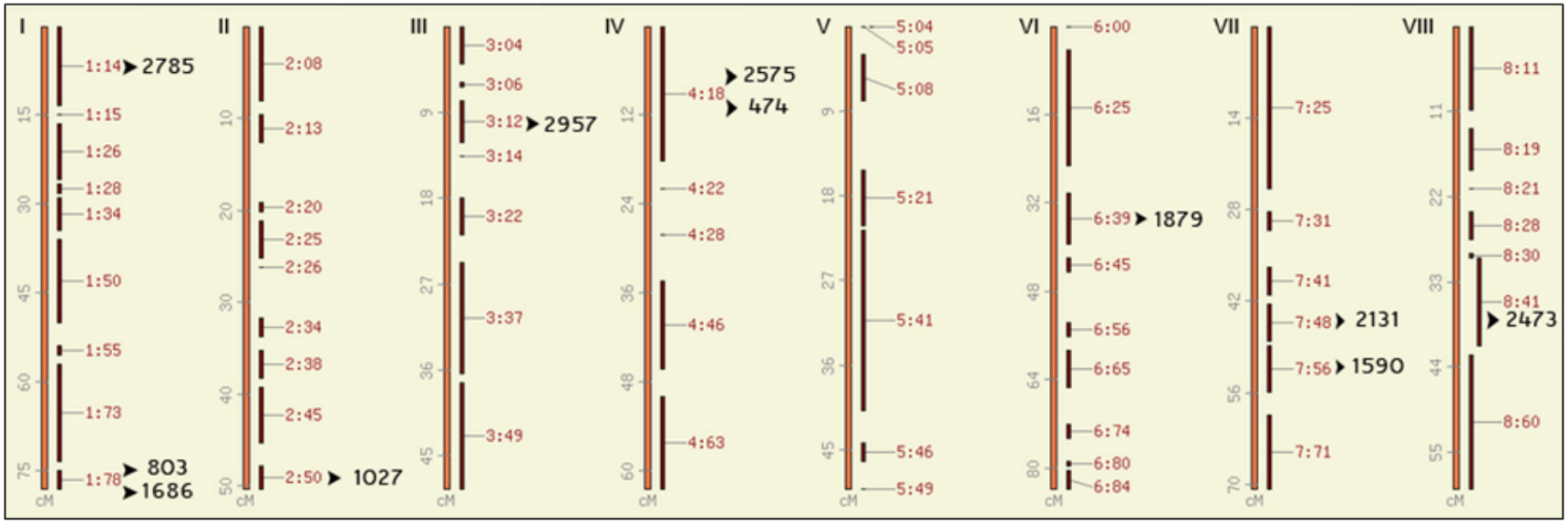

Fig. 3 Bin mapping of 11 candidate genes differentially expressed under cold treatment. Bin position is displayed. Markers representative of each unigene successfully mapped to the Binmap (Howad et al. 2005) of the *Prunus* reference map (Joobeur et al. 1998) as it is available in GDR (Jung et al. 2004). In the long bars can be observed the 8 linkage groups, in the short darker bars are showed the 67 Bins. Head black arrows shows the markers positioned and the unigene number in which they are based.

As previously has been described, the intron flanking approach showed to be a fast and efficient methodology to design polymorphic markers (Wei et al. 2005; De Keyser et al. 2009; Sargent et al. 2009; Tamura et al. 2009). This approach takes advantage of the *Arabidopsis* reference genome to predict potential polymorphic sites within candidate genes from different species. In the case of this study showed a 72% of polymorphic markers within the Binset population of the TxE *Prunus* reference map designed with this strategy. These results could be explained taking in consideration that the Bin mapping approach does not cover the whole TxE *Prunus* reference map, and the polymorphic profiles found for the non positioned markers could be associated to regions within the genetic map uncovered by the Bin mapping approach. On the other hand, from the successfully positioned genes, 2 pairs of genes were mapped to a same Bin. The first case was found in the Bin 1:78 that have a xyloglucan endotransglycosilase and a putative glucose-6-phosphate isomerase within its 2.8 cM genetic distance. In the second case, the genes glyceraldehyde-3-phosphate dehydrogenase and a unigene similar to a phosphoenolpyruvate carboxykinase were mapped together to the Bin 4:18. Additionally a similar result was found for the unigene pair composed by unigene 2131 similar to an endochitinase which was mapped to the Bin 7:48 adjacent to the Bin 7:56 where was mapped the unigene 1590 similar to a sucrose-UDP glucosyltransferase. This could indicate that these regions in the *Prunus* reference map may be potential regulations zones for genes associated with carbohydrate metabolism, cell wall integrity control and stress response. In the scaffold 1 of the *Prunus* v1.0 were positioned 12 unigenes (38%), 7 of them belonging to the cell wall and carbohydrate metabolism group and 5 of them from the stress response group of genes, suggesting that the genes involved in the regulation of these pathways can be clustered within this Chromosome. Therefore these genes could be suggested to be used as potentially markers in a molecular assisted breeding program.

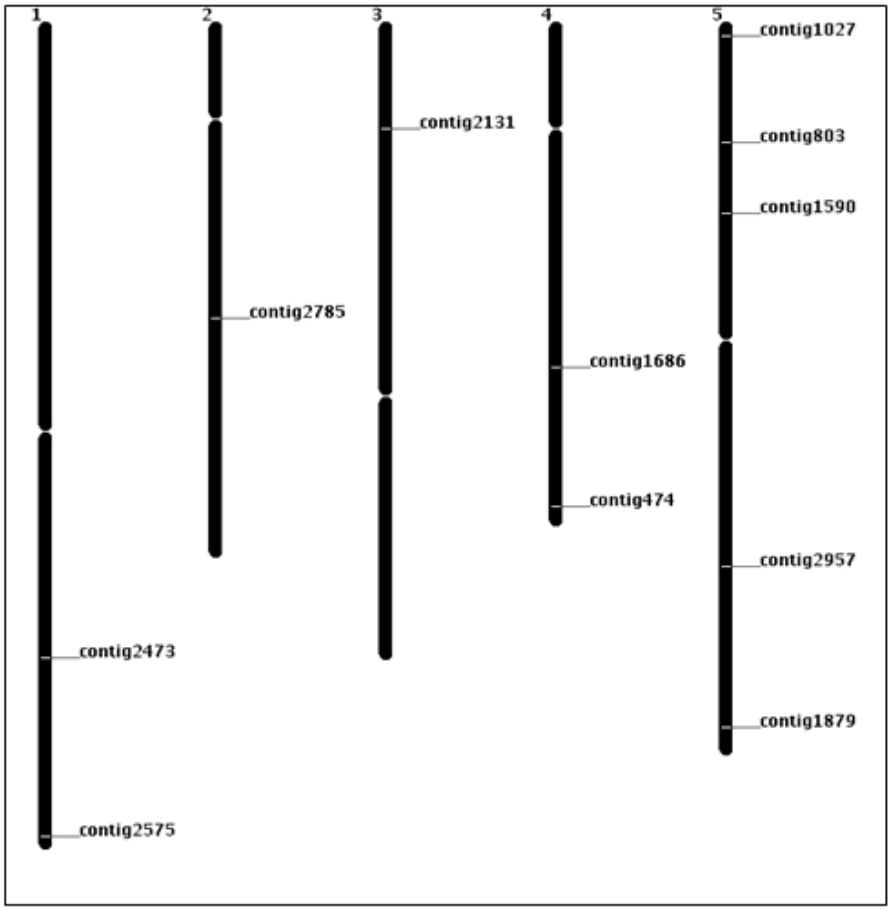

Fig. 4 Position in the *Arabidopsis* linkage groups of the orthologs mapped candidate genes from *Prunus persica*. The tags indicate the number of the unigene according to the local database and displayed in the whole genome view of SeqViewer from TAIR (http://www.arabidopsis.org).

The approach used in this work appears to be highly effective to design a simple and informative type of molecular marker. These markers can be used to address the role of selected genes against a stress condition. According to the percentage of successfully mapped genes, we suggest that the average position accuracy is directly related to the low number of candidates assigned to a genetic map position. When compared the position of the closest orthologs to the *Arabidopsis* genome, it can be observed a dispersed distribution of them on the positioning, similar to what has been reported in previous studies (Dominguez et al. 2003; Georgi et al. 2003; Jung et al. 2006; Sargent et al. 2009). However the homology at the transcript level between *P. persica* and *A. thaliana* has been shown to be informative for the prediction of putative splicing sites which can be used as intron flanking tool. This homology have been observed as well in a previous study (Tittarelli et al. 2009) to be conserved in the transcription regulation between these two species.

Recently, new molecular findings related to chilling injury traits have been described (Cantín et al. 2010; Martínez-García et al. 2012) therefore the molecular markers described in our work are a new set that can be useful for a Molecular Assisted Selection program. The intron flanking EST-PCR markers described in this work as well as new ones are projected to be used in the evaluation of different peach genotypes as well as segregating populations for traits related to chilling injury.

CONCLUDING REMARKS

The majority of the approaches that look for molecular markers associated with biologically relevant phenotypes search for a pattern that can be related to a trait segregating within a large population. The methodology used in this work melt several approaches in search for the positioning of markers designed on the base of functionally linked genes. The *in silico* profiling of candidate genes obtained from cDNA libraries of mesocarp tissue from different post harvest condition treatments allowed us to assign a position on the *Prunus* reference map to a group of new genes that also were differentially transcribed under cold treatment. The search for putative polymorphisms in non-coding regions within these genes is a very effective way to identify molecular markers representative of specific candidate genes, which could be useful for screening segregating populations. This, in addition to the Bin mapping approach makes a fast and efficient way to choose regions that could play an important role in the triggering of differential gene expression under a stress condition. Nevertheless for this last assumption an evaluation of segregant populations of *P. persica* is needed. As a final remark, it is important to mention that the results obtained in this work are intended to be used in Molecular Assisted Selections programs for the development of new cultivars with good post harvest behaviour under chilling injury conditions.

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