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ORIGINAL PAPER

Leucoanthocyanidin dioxygenase gene (PpLDOX): a potential functional marker for cold storage browning in peach

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Abstract Enzymatic browning of the peach fruit mesocarp is a major component of the postharvest physiological disorder commonly called chilling injury or internal breakdown (IB). Previously, we detected a major quantitative trait locus (QTL; qP-Brn5.1^{m}) affecting browning in peach using two related progeny populations (Pop-DG and Pop-G). In this report, a gene encoding the leucoanthocanidin dioxygenase (PpLDOX) enzyme was identified as the gene potentially responsible for this QTL. PpLDOX has a high similarity with the LDOX gene of the anthocyanin biosynthesis pathway of *Arabidopsis thaliana*. It was co-located with qP-Brn5.1^{m} via the bin mapping technique with the *Prunus* reference T × E map. A silent SNP within the PpLDOX coding sequence was used to locate the gene more precisely on the Pop-DG map and confirm its bin assignment. These results demonstrate both the utility of comparative mapping within *Prunus* using the T × E reference map and the power of the bin mapping approach for easily mapping genes in the *Prunus* genome. An SSR polymorphism was observed in the intron of PpLDOX gene sequence. The SSR co-segregated with the SNP and was used to assess association of PpLDOX with browning in 27 peach and nectarine cultivars. Cumulative evidence obtained indicates that PpLDOX partially explains genetic variation for cold storage browning susceptibility in peach and nectarine. This functional gene has potential use in marker-assisted breeding of new cultivars with lower IB susceptibility and for genotyping current cultivars for possible differential handling during storage to reduce symptom incidence.

Keywords Leucoanthocyanidin dioxygenase · PpLDOX · qP-Brn5.1^{m}

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Introduction

Anthocyanins are phenolic compounds responsible for the main pigments in flowers and fruits, acting as insect and animal attractants (Harbone and Williams 2000). Phenolic compounds have anti-platelet, antioxidant, anti-inflammatory, antiumoral and oestrogenic activities, suggesting their potential in the prevention of coronary heart disease and cancer (Hertog et al. 1993; Jang et al. 1997; Steinberg et al. 1989; Frankel et al. 1998; Arai et al. 2000). They also play an important role in taste and aroma (flavor) as determinants of bitter, sweet, pungent or astringent taste of some products (Tomas-Barberan and Espin 2001). Additionally, they are important in plant defense against pathogens and insects. For example, phenolics have been associated with

resistance to brown rot (*Monilinia fructicola*) in peach fruit (Gradziel and Wang 1993; Lee and Bostock 2006). However, the oxidative degradation of phenolic compounds by polyphenol oxidases leads to the production of brown polymers (melanines) before or during the processing of fruit and vegetables (Kader and Chordas 1984; Chang et al. 2000; Iyidogan and Bayindirli 2003). The prevention of this enzymatic browning is a major industrial concern because it provokes a negative consumer response as a result of the alteration of the organoleptic and visual properties as well as quality loss of the products due to decreased content of phenolic compounds. It is highly desirable therefore to have fruit with high levels of phenolic compounds but low postharvest browning during and after cold storage. To achieve this goal, specific phenolic compounds that predispose fruit to browning, and the genes coding for them, need to be identified. Then, molecular markers can be designed to help manipulate such genes in breeding programs.

Enzymatic browning is a major component of the postharvest physiological disorder commonly called chilling injury or internal breakdown (IB) in peach and nectarine fruit, appearing during prolonged cold storage and/or after subsequent ripening. Brown discoloration of the flesh at harvest and those caused by bruising during postharvest handling and slicing during processing are forms of enzymatic browning that presumably share similar chemistry with cold-storage browning of IB. Other symptoms of IB are mealiness, black pit cavity, translucent flesh, red pigment accumulation (bleeding) in the flesh, and loss of flavor. During several decades of research into the problem of IB, numerous postharvest treatments of peach and nectarine fruits that produced short-term amelioration effects have been considered (Lurie and Crisosto 2005).

A genetic approach to reducing internal breakdown through breeding cultivars with reduced mealiness, browning, loss of flavor, and bleeding has been proposed based on the observation that cultivars vary in susceptibility to IB (Crisosto et al. 1999). A classical genetic analysis of resistance to IB in two segregating populations (Pop-DG and Pop-G) developed from crossing 'Dr. Davis' with 'Georgia Belle' and self-pollinating the latter showed sizeable genotypic contributions to phenotypic variability (Peace et al. 2005a, 2006). Quantitative trait loci (QTL) controlling mealiness, browning, and bleeding were localized to peach linkage groups with two QTLs (together explaining 20% of genotypic variation observed for browning) placed on linkage group G2 and a major QTL explaining ~40% of the observed genotypic variation affecting browning located on group G5 (Ogundiwin et al. 2007). This paper reports the co-location of a gene in the anthocyanin biosynthesis pathway, encoding leucoanthocyanidin dioxygenase, with the major browning QTL.

Materials and methods

Plant material

The linkage maps used for QTL analyses of peach browning were constructed from two segregating populations (Pop-DG and Pop-G) developed from crosses involving 'Dr. Davis' × 'Georgia Belle'. 'Dr. Davis' is a clingstone, non-melting, bland-flavor, non-mealy, slight-browning, yellow-flesh cultivar, whereas 'Georgia Belle' is a freestone, melting, sharp-flavor, mealiness-prone, high-browning, white-flesh cultivar. Further details of the mapping populations have been reported by Peace et al. (2005a, 2006) and Ogundiwin et al. (2007). DNA samples of the bin mapping set of the *Prunus* T × E reference map (almond parent 'Texas', peach parent 'Earlygold', F_1 and six F_2 progeny) were received from Drs. Werner Howad and Pere Arus of IRTA, Spain. Peach and nectarine cultivars used for the evaluation of the effects of PpLDOX on browning included those previously phenotyped by Crisosto et al. (1999). Others were from unpublished data. Cultivars were categorized into their freestone/clingstone and melting/non-melting flesh genotypic classes from recorded cultivar descriptions, our own observations, and confirmed with a PCR test developed by Peace et al. (2005b) for the endopolygalacturonase gene (endoPG) that controls these traits.

Browning data collection, linkage mapping and QTL analysis

Peach fruit from the progeny populations and their parents were evaluated for browning by scoring visually the extent of brownish discoloration on the mesocarp after 2 to 3 weeks storage at 5°C followed by 3days ripening at room temperature on a scale of 1 (no browning) to 6 (severe browning). For the analysis of allelic effect of PpLDOX on browning, data collected from 15 fruit for each of 27 cultivars stored at 0°C and 5°C for 3 and 4 weeks were utilized. Procedures for scoring and analyzing peach browning are described in Peace et al. (2005a) and Crisosto et al. (1999). Data were collected from 2002 through 2004. Molecular marker data collection was as described in Peace et al. (2005a, 2006), and Ogundiwin et al. (2007). Four types of markers were evaluated. These were simple sequence repeats (SSR), sequence related amplified polymorphism (SRAP), inter-microsatellite amplification (IMA) and randomly amplified DNA fingerprints (RAF). Some SSR markers were heterozygous in both parents of Pop-DG. All other markers were either present in one parent and absent in the other (1:1 segregation) or present in both parents (3:1 segregation). JoinMap® 4.0 (Van Ooijen 2006) was used to construct the linkage map. Linkage analyses

involved all markers, setting the data type as cross-pollination (CP). Kosambi mapping function was used to convert recombination fractions to map distances in centimorgans (cM) with maximum recombination frequency set at 0.4. Maximum Likelihood-based interval mapping of MapQTL® 5.0 software (Van Ooijen 2005) was used for QTL analysis, and significance LOD thresholds were obtained with 1,000 linkage group-based permutations.

Nomination of PpLDOX

The *Arabidopsis thaliana* leucoanthocyanidin dioxygenase (LDOX) TDS4 gene (Accession number NM_118417) was selected from the anthocyanin biosynthesis pathway on the AraCyc database (http://www.arabidopsis.org) as one of the candidate genes for browning in peach. The *A. thaliana* LDOX gene sequence was used to search for peach homologous sequences using BLASTn of the National Center for Biotechnology Information (NCBI). An NCBI peach homolog (Accession number AB097216) was used to search the Genomics Database for Rosaceae (GDR, http://www.bioinfo.wsu.edu/gdr/) via BLAST. The matching ESTs were in turn used to search the Prunus Assembly v3 of the GDR for matching contigs. ESTs with significant matches were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) and the consensus sequence was used for designing primers for further analyses.

Mapping PpLDOX to qP-Brn5.1^{m} region

Amplified fragments of the 3′-end of PpLDOX from parents and six progeny were sequenced (see below for details on sequencing). Primers Ldox1f and Ldox1r, designed for the sequence (Table 1), were used to screen for polymorphism between ‘Dr. Davis’ and ‘Georgia Belle’ and among the T × E bin-set. Observations were made for presence or absence of polymorphism for the main PCR product as well as associated single-stranded conformational polymorphisms (SSCP). The bin location of PpLDOX on the *Prunus* genome was identified by the method of Howad et al. (2005), comparing the observed segregation pattern in the T × E bin-set to the expected genotypic scores from the bin. PpLDOX was mapped to Pop-DG with JoinMap® 4 (Van Ooijen 2006) using the Kosambi mapping function.

Temperature gradient capillary electrophoresis

The Ldox1f and Ldox1r primers were used to amplify a section of the PpLDOX sequence from ‘Dr. Davis’, ‘Georgia Belle’, three F_1 high browning progeny, and three F_1 low browning progeny of Pop-DG, four high browning cultivars (‘August Red’, ‘Kaweah’, ‘O’Henry’, and ‘September Red’), and four low browning cultivars (‘Honey Kist’, ‘May Glo’, ‘Saturn’, and ‘Summer Bright’). To identify potential SNPs, heteroduplexes were formed between different pairwise combinations of the amplified sections and resolved by temperature gradient capillary electrophoresis (TGCE) (Murphy and Berg 2003) on a Spectrumedix instrument (SpectruMedix Inc., State College, PA, USA) using variations of the protocol recommended by the manufacturer. Briefly, PCR products were heated at 95°C for 5min and cooled slowly to 50°C in 1°C/min intervals, then held at 50°C for 20min, and finally cooled to 25°C at the rate of 2.5°C/min. The amplicons were diluted tenfold in 1X TM buffer (10mM Tris pH8.0, 1mM $MgCl_2$) and resolved on the 96-capillary Spectrumedix SCE 9610. Sample injection was carried out at 6kV for 50s. Empirical observations indicated that the best resolution of heteroduplexes for these samples occurred when the run temperature was set at 30°C. Analysis of data was conducted using the Revelation 2.4 image analysis software (SpectruMedix). Sample peaks were compared to the haploid ‘Lovell’ control and scored in reference to peaks of ‘Dr. Davis’ and ‘Georgia Belle’.

Primer construction, DNA amplification and polyacrylamide gel electrophoresis

All primers were designed using Primer3 software (Rozen and Skaletsky 2000; Table 1). For the initial mapping efforts, forward and reverse primers (Ldox1f and Ldox1r) were designed to produce a 289bp amplicon at the 3′-end of PpLDOX EST sequence. Subsequent primers were designed to give full-length coverage of the gene (Fig. 1). Following the TGCE and sequence analyses of the 3′-end 289bp fragment, a set of two SNP forward primer pairs (Ldox2fa–Ldox2fb and Ldox3fa–Ldox3fb) were designed to target the A/G mutation in ‘Dr. Davis’ and were used in combination with Ldox1r for mapping the PpLDOXsnp marker onto the Pop-DG linkage map.

SNP primers were designed as described by Chagne et al. (2007), Jeong and Saghai Maroof (2004), and Hansson and Kawabe (2005) with some modifications. For the first primer pair (Ldox2fa and Ldox2fb), primer Ldox2fa had its last base at the 3′-end matching the wild type nucleotide on which the putative SNP was located and a base mismatch at the fourth base from the 3′-end, and Ldox2fb had two mismatches—one mismatch at the fourth base position from the 3′-end and the other mismatch at the location of the putative SNP. The mismatch at the fourth base position was to reduce PCR amplification 100 times in the 3′-end mismatch primer (Kwok et al. 1990). Each of the primers in the second pair (Ldox3fa and Ldox3fb) matched one of the SNP variants at the 3′-end (A or G), with Ldox3fb having two 5′ bases longer than Ldox3fa. PCR conditions and polyacrylamide gel electrophoresis were as reported in

Table 1 Features of primers used in mapping and sequencing of PpLDOX

Combination	Sequence (5′–3′)[a]	Ann temp (^{0}C)[b]	Primer position[f]	Amplicon size (bp)		Purpose
				Expected [c]	Observed	
Ldox1f	CAGTTTTCTGTGAGCCACCA		1,233–1,252			
Ldox1r	TCAACAAAGCAGGTAGACAGTAGC	60	1,508–1,521	289	289[d]	Map to TxE, sequencing
Ldox2fa	GCTGAGCACATTCAGCA*G*AAG		1,331–1,351			
Ldox2fb	GCTGAGCACATTCAGCA*G*AA*T*		1,331–1,351			
Ldox1r	as above	64		191	191	Map to Pop-DG
Ldox3fa	TGAGCACATTCAGCACAAA		1,331–1,351			
Ldox3fb	GCTGAGCACATTCAGCACAAG		1,329–1,351			
Ldox1r	as above	64		189, 191	189, 191	Map to Pop-DG
Ldox4f	TCCACCATAAGAAAAGAGTATAGGC		1–24			
Ldox1r	as above	60		1,306	~1,520	Whole gene sequence
Ldox5f	TGAGCAAAAGGAGAAGTATGC		437–457			
Ldox5r	CTTGCCTTCATAGAAAAGCTG	60	1,076–1,096	448	666[e]	Intron sequence target
$LDOX_{SSR}$-f	CTGCTGATTACATGTAAGTACTCAAGG		598–623			
$LDOX_{SSR}$-r	GTAGCCTCACTGCAAAGGTAT	57	814–834	238, 240	238, 240	Association mapping

[a] Underlined nucleotides were mismatches and those in bold were added for length polymorphism in the SNP analysis (see text)
[b] Annealing temperature used in the PCR profile
[c] Expected amplification product based on EST sequence
[d] Additional fainter bands were observed
[e] This included an intron of ~218 bp
[f] Primer nucleotide position based on the sequence of the GB-allele of PpLDOX in Fig. 1.

Peace et al. (2005b), and annealing temperatures used for each primer combination are listed in Table 1. For the SNP PCR amplifications, three primers were used (two forward and one reverse). For a positive control, 0.5× forward (5′- ACACCTACCAAAGAGACTTGCTC -3′) and reverse (5′- TATCGTACCCATCACCGAAC-3′) primers of a candidate gene encoding mannan endo-1,4-beta-mannosidase (Man1) were included in each SNP PCR reaction.

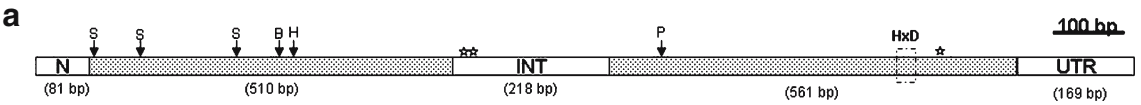
Cloning and sequence analysis of PpLDOX

For the 3′-end 289bp fragment of PpLDOX used in SNP detection, PCR products from genomic DNA templates were separated on 4% polyacrylamide gel with 7M urea. Silver stained gels were allowed to dry and a 2μl drop of sterile water was applied to each band and allowed to sit for 5min before cutting out the bands with a clean scalpel. Each excised band was placed in a 1.5ml microcentrifuge tube with 5μl of sterile water. The tube was incubated at 4°C for 2h to elute the DNA. Eluted DNA was used for a second round of PCR amplification as described above. Amplified products were separated on 1% agarose gels. Single bands were excised from the gels and DNA extracted with Montage DNA Gel Extraction Kit (Millipore Corporation, Bedford, MA). Extracted DNA was used as the template for a third and final round of PCR amplification in a 25μl reaction volume. PCR products were cleaned with MinElute PCR purification Kit (QIAGEN, Valencia, CA, USA). Samples were forward and reverse sequenced at the Division of Biological Science, UC Davis DNA Sequencing Facility. To obtain whole gene sequence from genomic DNA, two primer pairs (Ldox4f and Ldox1r for the whole gene, and Ldox5f and Ldox5r for the internal section containing the intron; Table 1) were used and amplification products separated on 1% agarose gels. Bands were excised as above, cloned into pCR2.1 vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), and sequenced. Sequence analysis was conducted using BioEdit v7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results

Characterization of PpLDOX

A *Prunus persica* mRNA for anthocyanidin synthase (pPpANS, GenBank accession number AB097216, Tsuda et al. 2004) had 74% nucleotide identity with an *A. thaliana* LDOX/TDS4 gene (NM_118417) with an *E* value of 1^{e-128}. A BLASTn search for pPpANS in the GDR database produced several matching *P. persica* ESTs, the top ten are presented (Table 2). All matching ESTs have an *E* value of zero and almost perfect (~100%) nucleotide identity with pPpANS. Nucleotide identity of these ESTs with LDOX TDS4 ranged from 73 to 74% with *E* values ranging between of 8^{e-91} and 2^{e-118}. These ESTs belong to Contig731 of the *Prunus* Assembly v3 of the GDR. However, a closer look at this contig indicated a mixture



b

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DD-allele-1      ----CCACCATAAGAAAAGAGTATAGGCCTGCACTTTTAGTGTAAAAAAGCTATCAAAGAAAGCTAGCCAAGAGTTCAGAGCTTACGAAG 86
DD-allele-2      ----CCACCATAAGAAAAGAGTATAGGCCTGCACTTTTAGTGTAAAAAAGCTATCAAAGAAAGCTAGCCAAGAGTTCAGAGCTTACGAAG 86
GB-allele        ----CCACCATAAGAAAAGAGTATAGGCCTGCACTTTTAGTGTAAAAAAGCTATCAAAGAAAGCTAGCCAAGAGTTCAGAGCTTACGAAG 86
PpLDOX-EST       CAATCCACCATAAGAAAAGAGTATAGGCCTGCACTTTTAGTGTAAAAAAGCTATCAAAGAAAGCTAGCCAAGAGTTCAGAGCTTACGAAG 90
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DD-allele-1      AGCCGGCTGCCAAAATGGTGAGCTCTGATTCAGTGAACTCAAGAGTTGAGACCTTGTCCAGCAGTGGAATTGCAACAATCCCAAAGGAGT 176
DD-allele-2      AGCCGGCTGCCAAAATGGTGAGCTCTGATTCAGTGAACTCAAGAGTTGAGACCTTGTCCAGCAGTGGAATTGCAACAATCCCAAAGGAGT 176
GB-allele        AGCCGGCTGCCAAAATGGTGAGCTCTGATTCAGTGAACTCAAGAGTTGAGACCTTGTCCAGCAGTGGAATTGCAACAATCCCAAAGGAGT 176
PpLDOX-EST       AGCCGGCTGCCAAAATGGTGAGCTCTGATTCAGTGAACTCAAGAGTTGAGACCTTGTCCAGCAGTGGAATTGCAACAATCCCAAAGGAGT 180
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DD-allele-1      ACATCAGACCCAAGGAAGAGCTCATAAACATCAGTGACATCTTCGAACAGGAGAAGAGCACTGATGGGCCTCAAGTTCCCACCATTGATT 266
DD-allele-2      ACATCAGACCCAAGGAAGAGCTCATAAACATCAGTGACATCTTCGAACAGGAGAAGAGCACTGATGGGCCTCAAGTTCCCACCATTGATT 266
GB-allele        ACATCAGACCCAAGGAAGAGCTCATAAACATCAGTGACATCTTCGAACAGGAGAAGAGCACTGATGGGCCTCAAGTTCCCACCATTGATT 266
PpLDOX-EST       ACATCAGACCCAAGGAAGAGCTCATAAACATCAGTGACATCTTCGAACAGGAGAAGAGCACTGATGGGCCTCAAGTTCCCACCATTGATT 270
                 ********************************************************************************************

DD-allele-1      TGAAGGAGATAGACTCTGAGAACGAAAATGTGAGAGAGAGATGCAGGGAGGAGTTGAAGAAGGCAGCAGTGGACTGGGGTGTCATGCACC 356
DD-allele-2      TGAAGGAGATAGACTCTGAGAACGAAAATGTGAGAGAGAGATGCAGGGAGGAGTTGAAGAAGGCAGCAGTGGACTGGGGTGTCATGCACC 356
GB-allele        TGAAGGAGATAGACTCTGAGAACGAAAATGTGAGAGAGAGATGCAGGGAGGAGTTGAAGAAGGCAGCAGTGGACTGGGGTGTCATGCACC 356
PpLDOX-EST       TGAAGGAGATAGACTCTGAGAACGAAAATGTGAGAGAGAGATGCAGGGAGGAGTTGAAGAAGGCAGCAGTGGACTGGGGTGTCATGCACC 360
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DD-allele-1      TTGTGAACCATGGCATCTCCGATGAGCTCATGGACCGTGTCAGGAAGGCCGGGAAGGCCTTTTTCGATCTTCCCATTGAGCAAAAGGAGA 446
DD-allele-2      TTGTGAACCATGGCATCTCCGATGAGCTCATGGACCGTGTCAGGAAGGCCGGGAAGGCCTTTTTCGATCTTCCCATTGAGCAAAAGGAGA 446
GB-allele        TTGTGAACCATGGCATCTCCGATGAGCTCATGGACCGTGTCAGGAAGGCCGGGAAGGCCTTTTTCGATCTTCCCATTGAGCAAAAGGAGA 446
PpLDOX-EST       TTGTGAACCATGGCATCTCCGATGAGCTCATGGACCGTGTCAGGAAGGCCGGGAAGGCCTTTTTCGATCTTCCCATTGAGCAAAAGGAGA 450
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DD-allele-1      AGTATGCCAATGACCAGGCCTCTGGCAAAATTCAAGGCTATGGAAGCAAGCTTGCAAACAATGCTTCTGGGCAGCTTGAGTGGGAGGACT 536
DD-allele-2      AGTATGCCAATGACCAGGCCTCTGGCAAAATTCAAGGCTATGGAAGCAAGCTTGCAAACAATGCTTCTGGGCAGCTTGAGTGGGAGGACT 536
GB-allele        AGTATGCCAATGACCAGGCCTCTGGCAAAATTCAAGGCTATGGAAGCAAGCTTGCAAACAATGCTTCTGGGCAGCTTGAGTGGGAGGACT 536
PpLDOX-EST       AGTATGCCAATGACCAGGCCTCTGGCAAAATTCAAGGCTATGGAAGCAAGCTTGCAAACAATGCTTCTGGGCAGCTTGAGTGGGAGGACT 540
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DD-allele-1      ACTTCTTCCACCTTGTATACCCTGAGGACAAGCGTGACTTGTCCATTTGGCCTCAAACACCTGCTGATTACATGTAAGTACTCAAGGAAA 626
DD-allele-2      ACTTCTTCCACCTTGTATACCCTGAGGACAAGCGTGACTTGTCCATTTGGCCTCAAACACCTGCTGATTACATGTAAGTACTCAAGGAAA 626
GB-allele        ACTTCTTCCACCTTGTATACCCTGAGGACAAGCGTGACTTGTCCATTTGGCCTCAAACACCTGCTGATTACATGTAAGTACTCAAGGAAA 626
PpLDOX-EST       ACTTCTTCCACCTTGTATACCCTGAGGACAAGCGTGACTTGTCCATTTGGCCTCAAACACCTGCTGATTACAT---------------- 613
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DD-allele-1      AAAAAAAAAAAAA--TCTTATCATGCTTAATTGAATGAAAATTTTGAACATATCCTCGAAAATAACACAACATTCTTTAACGAATTATCA 714
DD-allele-2      AAAAAAAAAAAAAAATCTTATCATGCTTAATTGAATGAAAATTTTGAACATATCCTCGAAAATAACACAACATTCTTTAACGAATTATCA 716
GB-allele        AAAAAAAAAAAAA--TCTTATCATGCTTAATTGAATGAAAATTTTGAACATATCCTCGAAAATAACACAACATTCTTTAACGAATTATCA 714
PpLDOX-EST       ---------------------------------------------------------------------------------------- 

DD-allele-1      ATTGTTTACATAACTTTCTTTTCATAGATATCAATATTCTGTCTTATCAACAATTTTGTTTTTTCAGACCCTCAATATTTCTGTTAACAT 804
DD-allele-2      ATTGTTTACATAACTTTCTTTTCATAGATATCAATATTCTGTCTTATCAACAATTTTGTTTTTTCAGACCCTCAATATTTCTGTTAACAT 806
GB-allele        ATTGTTTACATAACTTTCTTTTCATAAATATCAATATTCTGTCTTATCAACAATTTTGTTTTTTCAGACCCTCAATATTTCTGTTAACAT 804
PpLDOX-EST       ---------------------------------------------------------------------------------------- 

DD-allele-1      AGATAGTTAATACCTTTGCAGTGAGGCTACCGCCGAGTATGCTAAGGAACTGAGGGCGCTAGCAACCAAGGTACTGCGCGTGCTGTCACT 894
DD-allele-2      AGATAGTTAATACCTTTGCAGTGAGGCTACCGCCGAGTATGCTAAGGAACTGAGGGCGCTAGCAACCAAGGTACTGCGCGTGCTGTCACT 896
GB-allele        AGATAGTTAATACCTTTGCAGTGAGGCTACCGCCGAGTATGCTAAGGAACTGAGGGCGCTAGCAACCAAGGTACTGCGCGTGCTGTCACT 894
PpLDOX-EST       ----------------------TGAGGCTACCGCCGAGTATGCTAAGGAACTGAGGGCGCTAGCAACCAAGGTACTGCGCGTGCTGTCACT 682
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DD-allele-1      TGGGTTGGGATTGGAAGAAGGGAGGCTGGAGAAGGAAGTTGGGGGGCTTGAGGAGCTTCTCTTGCAAATGAAAATCAACTACTACCCACT 984
DD-allele-2      TGGGTTGGGATTGGAAGAAGGGAGGCTGGAGAAGGAAGTTGGGGGGCTTGAGGAGCTTCTCTTGCAAATGAAAATCAACTACTACCCACT 986
GB-allele        TGGGTTGGGATTGGAAGAAGGGAGGCTGGAGAAGGAAGTTGGGGGGCTTGAGGAGCTTCTCTTGCAAATGAAAATCAACTACTACCCACT 984
PpLDOX-EST       TGGGTTGGGATTGGAAGAAGGGAGGCTGGAGAAGGAAGTTGGGGGGCTTGAGGAGCTTCTCTTGCAAATGAAAATCAACTACTACCCACT 772
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DD-allele-1      TTGCCCTCAGCCAGAGCTTGCACTTGGTGTCGAAGCTCACACTGATGTCAGTGCACTCACCTTCATACTCCACAACATGGTTCCTGGCCT 1074
DD-allele-2      TTGCCCTCAGCCAGAGCTTGCACTTGGTGTCGAAGCTCACACTGATGTCAGTGCACTCACCTTCATACTCCACAACATGGTTCCTGGCCT 1076
GB-allele        TTGCCCTCAGCCAGAGCTTGCACTTGGTGTCGAAGCTCACACTGATGTCAGTGCACTCACCTTCATACTCCACAACATGGTTCCTGGCCT 1074
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Fig. 1 PpLDOX gene annotation. **a** PpLDOX gene sequence annotation. *Unshaded regions* are N-terminal sequence (*N*), Intron (*INT*), and the 3′ untranslated region (*UTR*). *Arrows* indicate restriction enzymes sites for *Sac*I (*S*), *Bst*XI (*B*), *Hind*III (*H*), *Eco*RV (*E*), and *Pst*I (*P*). The *boxed section* with *dotted lines* indicates the location of the critical residue (HxD) associated with leucoanthocyanidin dioxygenase function. Asterisk shows the position of the A/G SNP (*asterisk*) and SSR (*double asterisk*) used in mapping PpLDOX to qP-Brn5.1^{m}. **b** Sequence alignment of PpLDOX alleles from 'Dr. Davis' (*DD*) and 'Georgia Belle' (*GB*) with the PpLDOX EST consensus sequence. The microsatellite and SNP regions are in bold letters. **c** Amino acid sequence similarity between predicted protein of PpLDOX and Arabidopsis LDOX (Acc no. NM_118417). The active site motive HxD is *underlined*

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PpLDOX-EST      TTGCCCTCAGCCAGAGCTTGCACTTGGTGTCGAAGCTCACACTGATGTCAGTGCACTCACCTTCATACTCCACAACATGGTTCCTGGCCT 862
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DD-allele-1     GCAGCTTTTCTATGAAGGCAAGTGGGTCACTGCCAAGTGTGTTCCAAATTCCATTATCATGCATATTGGGGACACCATTGAGATTTTGAG 1164
DD-allele-2     GCAGCTTTTCTATGAAGGCAAGTGGGTCACTGCCAAGTGTGTTCCAAATTCCATTATCATGCATATTGGGGACACCATTGAGATTTTGAG 1166
GB-allele       GCAGCTTTTCTATGAAGGCAAGTGGGTCACTGCCAAGTGTGTTCCAAATTCCATTATCATGCATATTGGGGACACCATTGAGATTTTGAG 1164
PpLDOX-EST      GCAGCTTTTCTATGAAGGCAAGTGGGTCACTGCCAAGTGTGTTCCAAATTCCATTATCATGCATATTGGGGACACCATTGAGATTTTGAG 952
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DD-allele-1     CAATGGTAAGTACAAAAGCATTCTTCACAGAGGAATGGTGAACAAGGAGAAGGTGAGGATTTCATGGGCAGTTTTCTGTGAGCCACCAAA 1254
DD-allele-2     CAATGGTAAGTACAAAAGCATTCTTCACAGAGGAATGGTGAACAAGGAGAAGGTGAGGATTTCATGGGCAGTTTTCTGTGAGCCACCAAA 1256
GB-allele       CAATGGTAAGTACAAAAGCATTCTTCACAGAGGAATGGTGAACAAGGAGAAGGTGAGGATTTCATGGGCAGTTTTCTGTGAGCCACCAAA 1254
PpLDOX-EST      CAATGGTAAGTACAAAAGCATTCTTCACAGAGGAATGGTGAACAAGGAGAAGGTGAGGATTTCATGGGCAGTTTTCTGTGAGCCACCAAA 1042
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DD-allele-1     GGAGAAGATCATCCTTAAGCCGCTCCCGGAAACTGTGTCGGAGACTGAGCCGCCAATCTTCCCACCAAGAACTTTTGCTGAGCACATTCA 1344
DD-allele-2     GGAGAAGATCATCCTTAAGCCGCTCCCGGAAACTGTGTCGGAGACTGAGCCGCCAATCTTCCCACCAAGAACTTTTGCTGAGCACATTCA 1346
GB-allele       GGAGAAGATCATCCTTAAGCCGCTCCCGGAAACTGTGTCGGAGACTGAGCCGCCAATCTTCCCACCAAGAACTTTTGCTGAGCACATTCA 1344
PpLDOX-EST      GGAGAAGATCATCCTTAAGCCGCTCCCGGAAACTGTGTCGGAGACTGAGCCGCCAATCTTCCCACCAAGAACTTTTGCTGAGCACATTCA 1132
                **********************************************************************************

DD-allele-1     GCACAAATTGTTCAGGAAGAGTCAAGAAGCTCTGCTCAACAAGTGAAGCTGCTTATATTAGTTATCATATTATTGTGAAATTATCGTACT 1434
DD-allele-2     GCACAAGTTGTTCAGGAAGAGTCAAGAAGCTCTGCTCAACAAGTGAAGCTGCTTATATTAGTTATCATATTATTGTGAAATTATCGTACT 1436
GB-allele       GCACAAATTGTTCAGGAAGAGTCAAGAAGCTCTGCTCAACAAGTGAAGCTGCTTATATTAGTTATCATATTATTGTGAAATTATCGTACT 1434
PpLDOX-EST      GCACAAGTTGTTCAGGAAGAGTCAAGAAGCTCTGCTCAACAAGTGAAGCTGCTTATATTAGTTATCATATTATTGTGAAATTATCGTACT 1222
                ****** **********************************************************************************

DD-allele-1     TATGAAGCCATTTATGGCTTAGTTTGGATGATATATCTTGGTTGCCATGTATCTGCTCTTCTAGCTACTGTCTACCTGCTTTGTTG---- 1520
DD-allele-2     TATGAAGCCATTTATGGCTTAGTTTGGATGATATATCTTGGTTGCCATGTATCTGCTCTTCTAGCTACTGTCTACCTGCTTTGTTG---- 1522
GB-allele       TATGAAGCCATTTATGGCTTAGTTTGGATGATATATCTTGGTTGCCATGTATCTGCTCTTCTAGCTACTGTCTACCTGCTTTGTTGA--- 1521
PpLDOX-EST      TATGAAGCCATTTATGGCTTAGTTTGGATGATATATCTTGGTTGCCATGTATCTGCTCTTCTAGCTACTGTCTACCTGCTTTGTTGAATA 1312
                **********************************************************************************

DD-allele-1     --------------------------------
DD-allele-2     --------------------------------
GB-allele       --------------------------------
PpLDOX-EST      ATATTTATTTTATTTCCCTCGTAAAAAAAAA 1343
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C

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PpLDOX          MVSSDSVNSRVETLSSSGIATIPKEYIRPKEELINISDIFEQEKSTDGPQVPTIDLKEIDSENENVRERCREELKKAAVDWGVMHLVNHG 90
NM_118417       MVAVE----RVESLAKSGIISIPKEYIRPKEELESINDVFLEEKKEDGPQVPTIDLKNIESDDEKIRENCIEELKKASLDWGVMHLINHG 86
                **: :    ***:*:.*** :************ .*.*:* :**. ***********:*:*::*::**.* ******::*******:***

PpLDOX          ISDELMDRVRKAGKAFFDLPIEQKEKYANDQASGKIQGYGSKLANNASGQLEWEDYFFHLVYPEDKRDLSIWPQTPADYIEATAEYAKEL 180
NM_118417       IPADLMERVKKAGEEFFSLSVEEKEKYANDQATGKIQGYGSKLANNASGQLEWEDYFFHLAYPEEKRDLSIWPKTPSDYIEATSEYAKCL 176
                *. :**:**:***: **.*.:*:*********:*****************************.***:********:**:******:**** *

PpLDOX          RALATKVLRVLSLGLGLEEGRLEKEVGGLEELLLQMKINYYPLCPQPELALGVEAHTDVSALTFILHNMVPGLQLFYEGKWVTAKCVPNS 270
NM_118417       RLLATKVFKALSVGLGLEPDRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVSALTFILHNMVPGLQLFYEGKWVTAKCVPDS 266
                * *****::.**:***** .********************** ********************************************:*

PpLDOX          IIMHIGDTIEILSNGKYKSILHRGMVNKEKVRISWAVFCEPPKEKIILKPLPETVSETEPPIFPPRTFAEHIQHKLFRKSQEALLNK--- 357
NM_118417       IVMHIGDTLEILSNGKYKSILHRGLVNKEKVRISWAVFCEPPKDKIVLKPLPEMVSVESPAKFPPRTFAQHIEHKLFGKEQEELVSEKND 356
                *:******:***************:******************:**:****** **  .*. *******:**:**** *.** *:.:
```

Fig. 1 (continued)

of two different genes. Contig731 was 1,984 nucleotides long while the consensus sequence of the top ten ESTs matching LDOX was 1,343 nucleotides. Alignment of consensus sequence to Contig731 started at nucleotide 534 of Contig731. Primers designed to amplify the first 564 nucleotides of Contig731 (connecting the 533-nucleotide overhang to the consensus sequence) did not yield any PCR product. A BLASTn search of this sequence overhang

Table 2 *Prunus persica* ESTs showing high similarity with *A. thaliana* LDOX TDS gene and pPpANS

EST	pPpANS[a]			LDOX TDS4		
	Align length (bp)	PID (%)	*E* value	Align length (bp)	PID (%)	*E* value
BU039202	696	99	0	519	73	1^{e-114}
BU045579	629	100	0	453	73	3^{e-96}
BU041015	622	99	0	467	74	5^{e-112}
BU041521	622	99	0	437	74	2^{e-99}
BU047831	614	100	0	448	73	4^{e-94}
BU039495	617	99	0	523	74	2^{e-118}
AJ872805	615	99	0	473	74	1^{e-113}
BU047869	617	99	0	466	74	7^{e-111}
BU045355	608	100	0	439	72	8^{e-91}
BU048737	596	100	0	441	74	9^{e-103}

[a] pPpANS, (Acc no. AB097216, Tsuda et al. 2004) had 74% nucleotide identity with *A. thaliana* LDOX/TDS4 gene (NM_118417) with *E* value of 1^{e-128}.

showed 97% identity (*E* value = 0) with phenylalanine ammonia-lyase mRNA (PAL1) of *Prunus avium* (Acc. no. AF036948). Using another set of primers specific to the sequence overhang, we bin mapped this section of Contig731 to linkage group G6.

Therefore, the consensus sequence of the top ten ESTs matching LDOX was used in this study instead of the Contig731 sequence. The *P. persica* genomic leucoanthocyanidin dioxygenase gene, obtained via PCR using primers Ldox4f and Ldox1r designed from the consensus sequence was named PpLDOX (Fig. 1) following LDOX/TDS4 notation. The PpLDOX gene sequence was ~1,520bp in length. It had two exons and one intron (Fig. 1a and b). The intron was 216–218bp and the two exons encode a predicted protein of 357 amino acids which shared 78% identity with the *Arabidopsis* LDOX (NM_118417) gene (Fig. 1c). The active site motif HXD characteristic of *Arabidopsis* LDOX (His232 and Asp234) was located in the second exon at positions His236 and Asp238. Also located in the second exon, a single nucleotide polymorphism (SNP) was observed between the two alleles of PpLDOX in 'Dr. Davis' (Fig. 1a and b). This SNP used for initial mapping of PpLDOX (see below for details), was located on a codon of the ORF that was either 'AAA' or 'AAG' for 'Dr. Davis'. Lysine is produced from both forms; therefore, the SNP is a silent mutation. 'Georgia Belle' was homozygous for the 'AAA' allele. In addition, the intron included a microsatellite $(A)_{16–18}$ (Fig. 1a). An SSR marker developed from this sequence feature was heterozygous for 'Dr. Davis', with two alleles, $(A)_{16}$ (238bp) and $(A)_{18}$ (240bp), and homozygous in 'Georgia Belle' for the $(A)_{16}$ (238bp) allele (Fig. 1b). The 238bp SSR allele was part of the same haplotype as the 'AAA' SNP allele, while the 240bp SSR allele was associated with the 'AAG' SNP allele. In Pop-DG, '238-AAA' and '240-AAG' segregated in the expected 1:1 ratio (data not shown). The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession numbers EU292217–EU292219.

Co-location of PpLDOX and qP-Brn5.1^{m}

The products from screening 'Dr. Davis' and 'Georgia Belle' with primers Ldox1f and Ldox1r designed for the 3′-end of PpLDOX gene fragment were monomorphic between the two cultivars. When tested on the T × E bin set, the primers produced the expected 289bp main band (Fig. 2) and a few other faint bands in both parents and progeny. The main amplicon was monomorphic in the T × E bin-set but there were shadow bands around this amplicon with a distinct pattern suggesting the presence of single strand conformational polymorphism (SSCP). Based on the T × E bin set scoring scheme (Howad et al. 2005), the SSCP

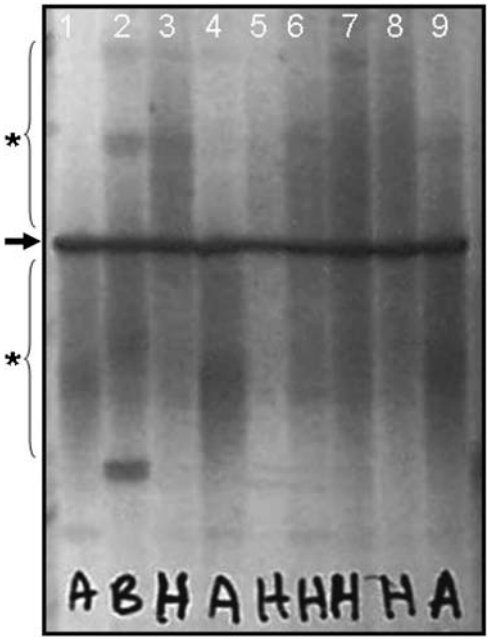

Fig. 2 Polyacrylamide gel profile of the 3′-end of PpLDOX amplified from the T × E bin-set. *Lane 1*='Texas', *lane 2*='Earlygold', *lane 3*= F_1 hybrid, and lanes 4 to 9= six F_2 progeny comprising the bin-set. Arrow indicates the expected main amplicon of PpLDOX. Brackets with asterisk indicate associated single stranded conformational polymorphism-like smears. Lanes were scored (letters at the base of lanes) according to Howad et al. (2005)

polymorphism mapped PpLDOX to bin 5:21. Two of the fainter bands (approximate sizes = 580 and 400bp) were also polymorphic as dominant markers. The 580bp band mapped to multiple possible bins (5:21, 5:41, 5:46, 6:39, 6:45, 6:49, and 8:60), while the 400bp band mapped to a single bin (4:63). The bin 5:21 on which the expected 289bp fragment mapped is 5.8cM long on the TxE map and bounded by markers AG114 and BF11D (Howad et al. 2005). Linkage group G5 of Pop-DG and Pop-G was collinear with that of the T × E map, sharing four SSR markers (Fig. 3). The region on G5 of Pop-DG and Pop-G corresponding to bin 5:21 of T × E is delimited by two SSR markers, UDP97-401 and BPPCT017, common to all maps. The qP-Brn5.1^{m} QTL was located in this window with its peak LOD closer to BPPCT017, supporting the hypothesis that PpLDOX is responsible for or at least tightly linked to this QTL.

For single nucleotide polymorphism (SNP) discovery in the PCR amplified section of PpLDOX, TGCE showed polymorphic peaks indicating presence of a SNP (Fig. 4). 'Georgia Belle' displayed a homoduplex peak, whereas 'Dr. Davis' had a split peak characteristic of a heteroduplex at the same location. Three high browning progenies of Pop-DG also had a single homoduplex peak as in 'Georgia Belle' (the high browning parent), and three low browning progenies showed heteroduplex peaks as observed for 'Dr. Davis' (the low browning parent). 'August Red', 'Kaweah',

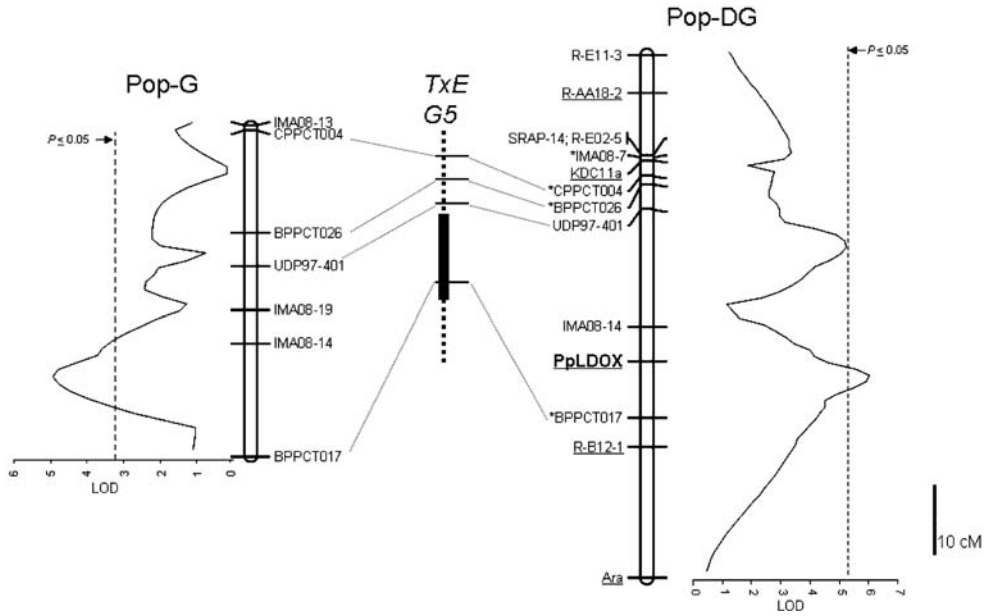

Fig. 3 Linkage group G5 of Pop-DG and Pop-G showing the LOD peaks of the peach flesh browning QTL (qP-Brn5.1^{m}) and the location of PpLDOX (*in bold*). Open vertical bars represent linkage groups. Markers are to the left while LOD chart is to the right of the linkage group in Pop-DG, and vice versa for Pop-G. For Pop-DG integrated map, underlined markers were from 'Dr. Davis', markers with asterisks were heterozygous in both parents, and all other markers were from 'Georgia Belle'. The dashed vertical lines represent LOD thresholds determined for 3 years average data ($P \leq 0.05$) calculated based on 1,000 permutations (see text). A section of G5 of T × E *Prunus* reference map (Dirlewanger et al. 2004; Howad et al. 2005) is represented between Pop-G and Pop-DG showing bin 5:21 (*solid bar*) and the positions of common SSR markers connected to other two maps by *solid lines*

'O'Henry', and 'May Glo' peach and nectarine cultivars (all high browning except 'May Glo') showed homoduplex peaks while 'Honey Kist', 'Saturn', 'Summer Bright', and 'September Red' cultivars (all low browning except 'September Red') showed heteroduplex peaks. Therefore, the Pop-DG association between TGCE genotype and browning susceptibility (high browning = homoduplex, low browning = heteroduplex) was consistent for six out of eight additional cultivars.

Sequence analysis of the PpLDOX section amplified by Ldox1f and Ldox1r primers confirmed the TGCE results for 'Dr. Davis', 'Georgia Belle', three high-browning and three low-browning progenies. A SNP (A/G) was revealed at a position near the 5′-end of PpLDOX (Fig. 1b). 'Georgia Belle' and the three high-browning full-sib progenies were homozygous (A/A) while 'Dr. Davis' and the three low-browning full-sib progenies were heterozygous (A/G). The four SNP primers designed for this mutation (Ldox2fa, Ldox2fb, Ldox3fa, and Ldox3fb) used in combination with Ldox1r (Table 1) confirmed PpLDOX sequence data of 'Georgia Belle' and 'Dr. Davis' as well as the six progeny subset (Fig. 5). The same primers were used to genotype the

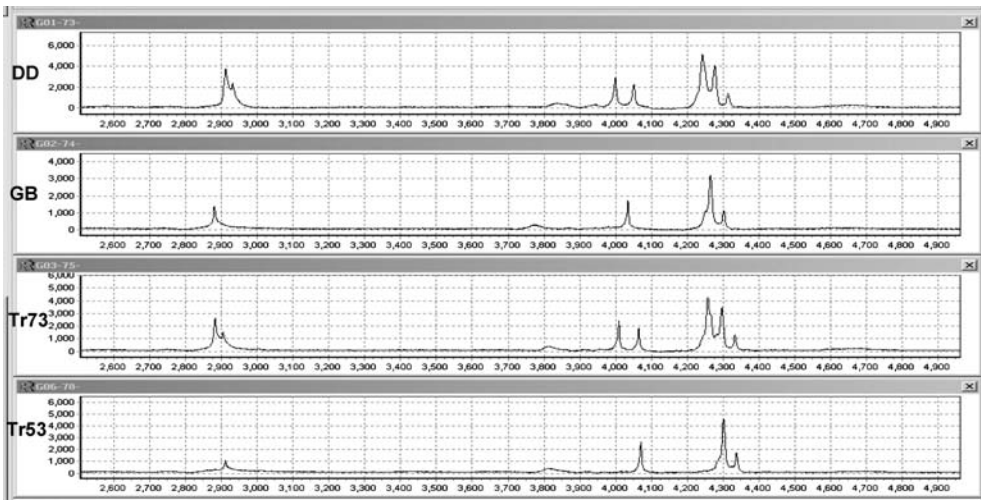

Fig. 4 Representative data captured with the use of Reveal(™) temperature gradient capillary electrophoresis of SpectruMedix instrumentation on unlabeled PCR products of PpLDOX from the parents (DD='Dr. Davis', GB='Georgia Belle') and 2 progeny (Tr53 and Tr73) of Pop-DG. DD and Tr73 are low browning while GB and Tr53 are high browning genotypes. Time=*x*-axis, fluorescence intensity=*y*-axis

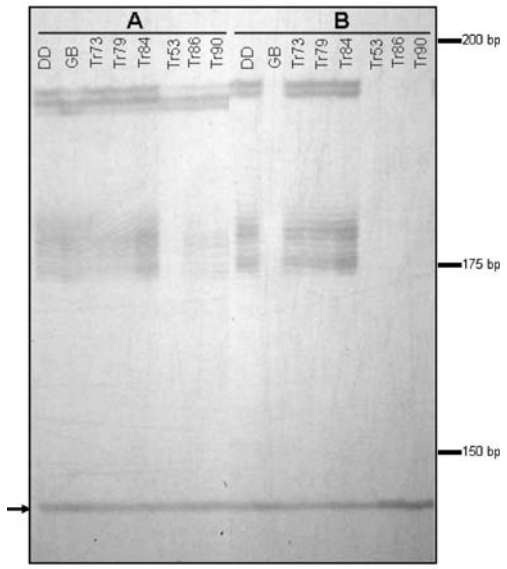

Fig. 5 Polyacrylamide gel profile of the amplicons of PpLDOXsnp primers Ldox3fa+Ldox3fb+Ldox1r (*A*), and Ldox2fa + Ldox2fb + Ldox1r (*B*) from 'Dr. Davis' (*DD*), 'Georgia Belle', low-browning progenies (Tr73, Tr79 & Tr84), and high-browning progenies (Tr53, Tr86 & Tr90). *Arrow* shows Man1 (mannan endo-1,4-beta-mannosidase) used as the positive control

entire Pop-DG population. Linkage analysis mapped PpLDOX to G5, between BPPCT017 and UDP97-401 (Fig. 3). The map location of PpLDOX on Pop-DG therefore confirmed its bin assignment on the T × E reference map.

Relationship between PpLDOX and qP-Brn5.1^{m} QTL

PpLDOX mapped ~5cM away from the SSR marker BPPCT017 on the integrated linkage group G5 of Pop-DG (Fig. 3). This integrated linkage group contained five 'Georgia Belle' markers and five 'Dr. Davis' markers. The remaining four markers were heterozygous in both parents. The peak LOD (6.01) for the qP-Brn5.1^{m} QTL was nearly midway between BPPCT017 and PpLDOX in Pop-DG, very close to PpLDOX. However, PpLDOX and the QTL peak could not be directly compared because qP-Brn5.1^{m} was heterozygous in and inherited from 'Georgia Belle' whereas PpLDOX segregated from 'Dr. Davis'. As no sequence polymorphism within the 'Georgia Belle' PpLDOX sequence could be found, PpLDOX could not be mapped to Pop-G, but the position of the qP-Brn5.1^{m} LOD peak (4.92) between markers BPPCT017 and IMA08-14 corresponded with that of Pop-DG (Fig. 3). There seemed to be another QTL from 'Georgia Belle' on LG5 of Pop-DG with peak LOD of 5.24, close to the LOD 5.3 threshold (Fig. 3). However, this putative QTL, unlike qP-Brn5.1^{m}, was not consistently significant when individual year data were considered.

PpLDOX allelic effects among peach and nectarine cultivars

Three SSR genotypes, 238:238, 238:240, and 240:240, were observed for PpLDOX among 27 peach and nectarine cultivars using the PpLDOX$_{SSR}$ primers (Table 3). The largest genotypic group was 240:240, with two-thirds of the cultivars and including both peach and nectarine. Half of this group was freestone melting flesh (FMF) and the other half clingstone melting flesh (CMF) genotypes. Genotypic group 238:238 had six cultivars (three peach and three nectarine). This group contained one clingstone non-melting flesh (CNMF), three FMF and two CMF genotypes. The smallest group was the heterozygous genotypic group 238:240, with three peach cultivars (one CNMF and two FMF). The genotypes of the mapping population parents were 238:238 for 'Georgia Belle' and 238:240 for 'Dr. Davis'. Assessment of the effects of these genotypes on browning in diverse peach and nectarine genetic backgrounds was based on data collected on the 27 cultivars in 2003 and 2004 (Table 3). The 238:238 genotype was associated with reduced browning of fruit stored for 4weeks at 5°C by 41 and 38% when compared to the 238:240 and 240:240 groups, respectively. This trend, although not statistically significant, was similar for all storage conditions (Fig. 6). The portion of browning controlled by PpLDOX/qP-Brn5.1^{m} appears to be independent of the Glabrous (*G*) locus controlling the peach/nectarine trait (located distantly on the same linkage group G5) and the Freestone–Melting flesh (F–M) locus controlling the freestone/clingstone and melting/non-melting flesh traits (located on G4), as occurrence of nectarine and peach fruit types and the three available F–M genotypic classes (FMF, CMF and CNMF) was random among PpLDOX$_{SSR}$ genotypic classes.

Discussion

A gene encoding leucoanthocyanidin dioxygenase (PpLDOX) enzyme was identified as the potential gene responsible for the qP-Brn5.1^{m}, a major QTL for browning in peach fruit. Anthocyanidin synthase is a synonym for LDOX. It is the penultimate enzyme in the anthocyanin biosynthesis pathway that converts leucocyanidin to cyanidin. Cyanidin in turn is converted to anthocyanin by UDP glucose-flavonoid 3-*O*-glucosyl transferase.

A suite of techniques was deployed in establishing the association between PpLDOX and qP-Brn5.1^{m}. These techniques include linkage, bin, comparative and association mapping, candidate gene approach, SSCP analysis, TGCE SNP detection and genotyping, and gene cloning and sequence analysis. Of particular importance were bin mapping and comparative mapping. Bin mapping with the

Table 3 Peach and nectarine cultivars with phenotypic values for cold-storage browning and genotypes of the qP-Brn5.1^{m} putative functional marker—PpLDOXssr

Cultivar	Fruit type[a]	3 weeks		4 weeks		PpLDOXssr
		0°C	5°C	0°C	5°C	
Honey Blaze	N, CMF	1.00	1.00	1.00	1.00	238:238
Honey Kist	N, FMF	1.00	1.00	1.00	1.00	238:238
Summer Bright	N, CMF	1.00	1.54	1.00	1.86	238:238
Super Rich	P, FMF	1.00	1.67	1.17	1.94	238:238
Crimson Lady	P, CNMF	1.00	1.07	1.02	1.37	238:238
Saturn	P, FMF	1.00	1.51	1.00	1.17	238:238
Brittney Lane	P, FMF	1.00	1.00	1.00	1.00	238:240
Summer Sweet	P, FMF	1.39	2.62	1.64	3.20	238:240
September Flame	P, CNMF	1.40	2.72	1.31	2.92	238:240
Arctic Star	N, CMF	1.00	1.00	1.00	1.00	240:240
Arctic Snow	N, FMF	1.03	3.60	1.05	4.25	240:240
August Glo	N, CMF	1.00	2.07	1.27	3.73	240:240
Autumn Flame	P, FMF	1.70	3.44	1.76	4.25	240:240
Diamond Bright	N, CMF	1.00	1.00	1.00	1.00	240:240
Diamond Ray	N, CMF	1.00	1.00	1.00	1.03	240:240
Elegant Lady	P, FMF	1.38	2.33	2.35	4.25	240:240
Fire Pearl	N, CMF	1.00	1.22	1.09	1.59	240:240
Grand Pearl	N, CMF	1.00	1.20	1.00	1.13	240:240
Ivory Princess	P, FMF	1.00	1.40	1.00	1.60	240:240
Kaweah	P, FMF	1.31	1.95	1.27	1.90	240:240
Royal Glo	N, FMF	1.00	1.00	1.00	1.00	240:240
Ruby Pearl	N, CMF	1.00	1.08	1.10	1.07	240:240
Ruby Sweet	N, FMF	1.00	1.00	1.00	1.00	240:240
September Free	N, FMF	1.00	2.33	1.18	3.73	240:240
Spring Bright	N, CMF	1.00	1.00	1.00	1.00	240:240
Summer Blush	N, CMF	1.00	3.04	1.02	4.47	240:240
Sweet Dream	P, FMF	1.00	2.46	1.00	2.50	240:240

[a] *N* nectarine, *P* peach, *FMF* freestone melting flesh, *CMF* clingstone melting flesh, *CNMF* clingstone non-melting flesh.

Prunus reference T × E map, developed by Howad et al. (2005), was instrumental in locating PpLDOX to the 5:21 bin. Subsequent comparative mapping among the G5 linkage groups of the T × E, Pop-DG and Pop-G maps, made possible by common SSR markers, revealed the co-localization of PpLDOX and qP-Brn5.1^{m} to the 5:21 bin. Linkage mapping of PpLDOX in Pop-DG within the qP-Brn5.1^{m} confirmed its bin assignment on the T × E reference map. These results clearly demonstrate both the value of the TxE as the reference map for *Prunus*, and the

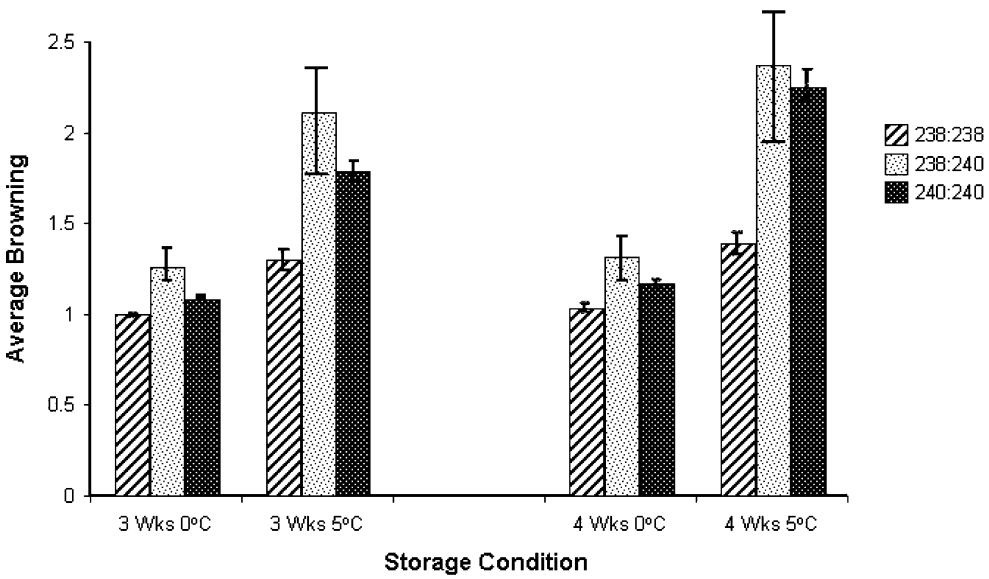

Fig. 6 Average cold storage browning associated with the three SSR allelic groups (238:238, 238:240 and 240:240) of PpLDOX observed in 27 peach and nectarine cultivars. Bar chats with associated error bars (standard error) are grouped according to the four storage conditions tested

utility of bin mapping technology, for mapping and cross-referencing with other *Prunus* maps for any gene with polymorphism between 'Texas' and 'Earlygold', the T × E population parents.

The SSCP-like polymorphism observed for PpLDOX on the polyacrylamide gel profile made its bin mapping possible. This SSCP-like polymorphism was indicative of a SNP in the gene sequence. TGCE and subsequent sequence analysis of PpLDOX supported the initial hypothesis that the PCR, polyacrylamide gel, and silver-staining protocol could detect SSCPs. TGCE analysis supported the SSCP-like polymorphism and further strengthened the likelihood that the gene sequence contained a SNP. TGCE is an established technology for SNP discovery, and its usefulness for gene-trait association discovery is demonstrated here.

Of the 16 genotypes (ten cultivars and six full-sib progenies) tested by TGCE for PpLDOX, only the TGCE-defined genotypes of two cultivars ('May Glo' and 'September Red') did not match expected browning phenotypes. Sequence analysis of PpLDOX partly explained the disparity in TGCE result for these cultivars. The SNP responsible for both the SSCP and TGCE profiles was a silent mutation. The codon at the SNP region was either 'AAA' or 'AAG' producing Lysine for both forms. Therefore, the association of PpLDOX with browning in peach may be due to variation in parts of this gene other than the detected SNP. Exploration of the upstream control region of the PpLDOX gene through a genome walk may identify the functional mutation.

The possibility that the co-localization of PpLDOX and the qP-Brn5.1^{m} browning QTL is due to linkage and not by functional identity cannot be completely discounted. However, results of preliminary association studies here further support the role of PpLDOX and warrant further studies into its role in browning resistance in peach and nectarine cultivars. When SSR variation within the intron of this gene was used to assess its effect on browning in 27 peach and nectarine cultivars, the 238:238 genotypic group was associated with low browning incidence across all storage conditions compared to their 240:240 and 238:240 counterparts.

The absence of statistical significance among the mean browning scores of the allelic groups could be attributed to two factors—the large standard deviations within the 240:240 and 238:240 groups, and the fact that PpLDOX is associated with a QTL that controls only a fraction of the browning variation. The SNP and SSR polymorphisms employed in the linkage mapping and association analysis are presumably not causal of functional differences—the SSR was in the intron and the SNP was a silent mutation, and neither was heterozygous in 'Georgia Belle' from which the QTL was inherited. Variation upstream of the gene in one of the '238-AAA' allele of 'Georgia Belle' may better explain the functional role of PpLDOX in browning.

Secondly, browning is a polygenic trait that is controlled by at least three genes and has a large environmental effect (Peace et al. 2005a, 2006; Ogundiwin et al. 2007). Its broad-sense heritability in Pop-DG and Pop-G was earlier estimated at 34 to 47% (Peace et al. 2006). The qP-Brn5.1^{m} itself to which PpLDOX is associated controls only ~40% of this genotypic variation in Pop-DG (Ogundiwin et al. 2007). Therefore, it is not unexpected that the genetic background of a cultivar will have an impact on the putative role of PpLDOX in browning control.

While the search for other functional genes controlling browning continues, the PpLDOX SNP or SSR markers reported here may be useful in marker-assisted breeding and for genotyping current peach and nectarine cultivars for possible differential handling during storage to reduce IB. The apparent independence of PpLDOX/qP-Brn5.1^{m} from the fruit types of peach/nectarine (*G* locus), freestone/clingstone, and melting/non-melting flesh (F–M locus) gives freedom to the use of the PpLDOX marker as a predictive or diagnostic tool for both fresh market and canning peach and nectarine cultivars. The F–M locus is controlled by the endopolygalacturonase gene (endoPG) (Peace et al. 2005b) and associated with a major QTL controlling mealiness and bleeding (Peace et al. 2005a, 2006; Ogundiwin et al. 2007). There exists, therefore, the opportunity to combine endoPG and PpLDOX functional markers in breeding superior cultivars with reduced incidence of mealiness, browning and bleeding—three major symptoms of IB.

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