

Genetic mapping of new seed-expressed polyphenol oxidase genes in wheat (*Triticum aestivum* L.)

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Abstract Polyphenol oxidase (PPO) enzymatic activity is a major cause in time-dependent discoloration in wheat dough products. The *PPO-A1* and *PPO-D1* genes have been shown to contribute to wheat kernel PPO activity. Recently a novel PPO gene family consisting of the *PPO-A2*, *PPO-B2*, and *PPO-D2* genes has been identified and shown to be expressed in wheat kernels. In this study, the sequences of these five kernel PPO genes were determined for the spring wheat cultivars Louise and Penawawa. The two cultivars were found to be polymorphic at each of the PPO loci. Three novel alleles were isolated from Louise. The Louise X Penawawa mapping population was used to genetically map all five PPO genes. All map to the

long arm of homeologous group 2 chromosomes. *PPO-A2* was found to be located 8.9 cM proximal to *PPO-A1* on the long arm of chromosome 2A. Similarly, *PPO-D1* and *PPO-D2* were separated by 10.7 cM on the long arm of chromosome 2D. *PPO-B2* mapped to the long arm of chromosome 2B and was the site of a novel QTL for polyphenol oxidase activity. Five other PPO QTL were identified in this study. One QTL corresponds to the previously described *PPO-D1* locus, one QTL corresponds to the *PPO-D2* locus, whereas the remaining three are located on chromosome 2B.

Abbreviations

PPO	Polyphenol oxidase
QTL	Quantitative trait locus
cM	Centimorgan
T.aestivum	<i>Triticum aestivum</i> L. subsp. <i>aestivum</i>

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Introduction

Wheat products, particularly white-salted and alkaline noodles, are plagued by time-dependent discoloration of the dough during processing. This quality defect is caused in large part by the action of polyphenol oxidase (PPO) activity (Baik et al. 1994). PPO is nearly ubiquitous in plants and its activity causes browning reactions in many plant products, including those derived from cereal grains (Whitaker and Lee 1995). In addition to PPO, plant products contain phenolic compounds from cell walls such as ferulic and caffeic acid. These serve as enzymatic substrates for PPO. PPO causes the hydroxylation and dehydrogenation of these phenolic compounds to form reactive ortho-quinone products (EC 1.14.18.1, EC

1.10.3.10). These then either self-polymerize or react with accessible nucleophilic moieties to form large light-absorbing melanins which cause darkening and discoloration (Mayer and Harel 1979). Food products determined by the consumer to be discolored are considered less desirable and are therefore less valuable in the marketplace. As a result, wheat improvement efforts focused on quality and marketability have striven to reduce the levels of kernel PPO activity. These efforts have been greatly hampered by a substantial redundancy of PPO genetic loci. Wheat is an allohexaploid species, consisting of three homeologous genomes, providing on average, triple redundancy for any gene. In addition to this, it has been found that wheat also contains many paralogous PPO genes that have arisen by gene duplication and subsequent mutation (Fuerst et al. 2008; Jukanti et al. 2004; Massa et al. 2007). Until recently, only two genes belonging to one paralogous gene family were known to be expressed in developing kernels. These genes, *PPO-A1* and *PPO-D1*, have been shown to affect kernel PPO activity and noodle dough color stability (Chang et al. 2007; He et al. 2007; Sun et al. 2005). *PPO-A1* and *PPO-D1* are located on the long arm of the homeologous chromosomes 2A and 2D, respectively (Anderson et al. 2006; Chang et al. 2007; Demeke et al. 2001; He et al. 2007; Jimenez and Dubcovsky 1999; Mares and Campbell 2001; Raman et al. 2005; Simeone et al. 2002; Sun et al. 2005; Watanabe et al. 2004; Zhang et al. 2005). Recently, another paralogous PPO gene family has been identified and consists of the *PPO-A2*, *PPO-B2*, and *PPO-D2* genes (Beecher and Skinner 2011). *PPO-A2* and *PPO-D2* were found to be expressed to high levels in developing wheat kernels of the cultivar ‘Alpowa’. In fact, when all five genes (*PPO-A1*, *PPO-D1*, *PPO-A2*, *PPO-D2*, and *PPO-B2*) were measured, *PPO-A2* and *PPO-D2* together accounted for over 72% of PPO transcripts in developing wheat kernels. These new *PPO-2* genes are therefore likely to account for a substantial portion of the total PPO activity present in wheat kernels. Beecher and Skinner (2011) localized the new *PPO-2* gene family members to group 2 homeologous chromosomes, but did not genetically map their locations. Thus, details important to wheat improvement efforts, such as what if any genetic linkage exists between the *PPO-1* and *PPO-2* gene sets, were unknown. The goals of this study were fourfold: First was to clone and sequence the allele variants of the *PPO-A1*, *PPO-D1*, *PPO-A2*, *PPO-B2*, and *PPO-D2* genes present in the wheat cultivars Louise and Penawawa. Second was to develop co-dominant allele-specific markers for each of the 10 PPO sequences. Third was to determine the genetic map positions of these five genes using the 188 member Louise X Penawawa mapping population. Last was to identify genetic regions affecting PPO activity in this population.

Materials and methods

Plant materials

An $F_{5:6}$ derived RIL population of 188 individuals from a cross between Louise and Penawawa was selected for phenotypic analysis. The entire RIL population has been previously used and described by Carter et al. (2009). In short, Louise, a soft white spring wheat released in 2005, has moderate grain volume weight, low grain protein concentration, and excellent end-use quality characteristics for soft wheat products such as cookies and cakes (Kidwell et al. 2006). Penawawa, a soft white spring wheat released in 1985, has moderate grain volume weight, moderate grain protein concentration, and average end-use quality characteristics. Both Louise and Penawawa consistently show considerable kernel PPO activity as measured by in vitro L-Dopa activity assays, with Penawawa tending toward higher activity levels in a given environment (data not shown).

Field experiments

Field trials were conducted in Pullman, WA (latitude 46°41'N, longitude 117°08'W, elevation 776.3 m) and Genesee, ID (latitude 46°33'N, longitude 116°55'W, elevation 807.7 m) in 2007, and in Moscow, ID (latitude 46°43'N, longitude 116°57'W, elevation 796.1 m), and again in Pullman, WA, in 2008. Plots were planted using randomized complete block design with three replicates per location. Before planting, the field was cultivated and fertilized with nitrogen (formulated as urea) at a rate of 101 kg ha⁻¹. Due to differences in resistance to the foliar fungal disease stripe rust (caused by *Puccinia striiformis* f. sp. *tritici*) to which Louise is resistant and Penawawa is susceptible, plots were sprayed with Tilt (propiconazole; Syngenta, Basel, Switzerland) at the rate of 0.3 l per hectare at stem extension (Feekes 1941) to prevent confounding end-use quality tests. Plots were harvested using a Wintersteiger NurseryMaster combine (Wintersteiger Co., Salt Lake City, UT, USA) and grain was collected individually for each plot.

Kernel PPO analysis

Kernel PPO activity was determined according to Approved Method 22-85 of the American Association of Cereal Chemists (AACC 2000; Bettge 2004). Briefly, whole kernels were incubated at room temperature with agitation in a buffered solution containing 10 mM L-DOPA and a surfactant (Tween-20). Colored reaction products were measured by absorbance at 475 nm using a Shimadzu BioSpec-1601 Spectrophotometer. Each replicate consisted

of five kernels, and two replications were performed per grain sample.

PPO gene cloning and sequencing

The primers described in supplementary Table 1 were used to amplify products from approximately 50 ng of a genomic DNA preparation using the proofreading Phusion[®] polymerase (Espoo, Finland). Reaction conditions consisted of 1× Phusion GC PCR buffer, 0.2 mM of each dNTP, 0.2 pmol/μl of each primer, 3% (v/v) DMSO, and 0.01 unit/μl Phusion polymerase in a 25 μl reaction volume. Thermal cycling was carried out using a MJ Research PTC-200 Thermo Cycler and consisted of a 30 s initial denaturation step at 98°C, followed by forty cycles of 98°C for 10 s, annealing temperature (see Supplementary Table 1) for 30 s, with a 2-min extension step at 72°C. The proofreading PCR products were purified using AxyPrep gel extraction kit columns (Axygen Scientific, Union City, CA, USA). Single adenosine extensions were then added to the fragments by incubation for 10 min at 72°C in 1× PCR buffer (New England Biolabs) supplemented with 0.2 mM of each dNTP and 0.02 unit per microliter *Taq* DNA polymerase (Promega, Madison, WI, USA). The products were then subcloned into pGEMT-Easy (Promega, Madison, WI, USA). Three independent clones for each gene were fully sequenced using the BigDye[®] Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequencing reactions were read using an Applied Biosystems 3130 Genetic Analyzer. Note that the primers used for the *PPO-A2*, *PPO-B2* and *PPO-D2* genes are not sequence specific, requiring multiple clones to be analyzed in order to identify representatives of each homeologous gene (supplementary Table 1).

Phylogenetic analysis

All sequences were aligned with Clustal X, version 2.0.12 (Larkin et al. 2007). Gene trees were also constructed using the Clustal X program. The entire coding region, starting with the ATG, and ending with the stop codon was used in the sequence comparisons. Introns were included in the analysis. All gaps were reset before alignments were performed. For the tree construction, the default parameters were used with the following two exceptions: the ‘exclude positions with gaps’ and ‘correct for multiple substitutions’ options were employed. Trees were generated by maximum likelihood and neighbor-joining algorithms. Bootstrap was performed using 1,000 replicates. Phylogenetic trees were displayed using Tree-View (Page 1996). Bootstrap values of greater than 70% were reported.

PPO SNP markers and analysis

SNP assays for the PPO alleles were based on allele-specific extension and fluorescent labeling of amplified products. Each PCR reaction contained five oligonucleotide primers: Two oligonucleotides containing either a 5′ FAM or HEX label (0.16 pmol/μl each), two forward allele-specific tailed primers with sequence in common with the labeled primers (0.04 pmol/μl each), and one reverse primer common to both alleles (0.2 pmol/μl). Successful amplification yields an allele-specific product labeled with either FAM or HEX. Primer sets, reaction products, and general strategy are described in supplementary Figure 2. A complete list of primers and annealing temperatures used is provided in supplementary Table 2. Markers are co-dominant, allowing for the determination of either homozygous or heterozygous condition. Reaction mixture consisted of 1× PCR buffer (New England Biolabs) supplemented with an additional 1 mM magnesium chloride, 1 M betaine, 0.2 mM of each dNTP, and 0.5 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA) in a 25 μl reaction volume. Thermocycling was conducted using a PTC-200 Thermo Cycler (MJ Research) and consisted of a temperature regimen of an initial 3 min denaturation step at 95°C, followed by forty-five cycles of 95°C for 50 s, 30 s at the annealing temperature (see Supplementary Table 1), and a 30 s extension step at 72°C. Reaction products were detected using an Applied Biosystems 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) and alleles were determined using the Gene Marker 1.90 program (Soft Genetics, State College, PA, USA).

Genetic linkage map construction

The genetic linkage map was constructed using an established map consisting of 320 SSR markers (Carter et al. 2009), with an additional 1,434 SNP markers added to the map using a subset of 94 individuals from the RIL population. SNP markers were analyzed using an Illumina GoldenGate Assay (Illumina, Inc, San Diego, CA 92121, USA). Additionally, the markers *PPO-A1*, *PPO-A2*, *PPO-B2*, *PPO-D1*, and *PPO-D2* were assayed on the population. Joinmap v4.0 (Van Ooijen 2006) was used to establish and order linkage groups. Linkage groups were first identified with the ‘groupings tree’ using the independence LOD score starting at 2.0 and ending at 10.0. Maximum likelihood mapping was used to order markers within each linkage group using the default parameters within the program. There were 29 linkage groups identified and assigned to the 21 wheat chromosomes based on previously identified linkage groups (Carter et al. 2009). Analysis of variance was performed with location and entry as main

effects using Proc GLM in the statistical package SAS v9.2 (SAS Institute, Raleigh, NC, USA). Broad-sense heritability estimates and standard errors were calculated on a progeny mean basis using the formula: $h^2 = \text{Var}(G)/\text{Var}(P)$ [in which $\text{Var}(G)$ is the variance of the genotypic effect and $\text{Var}(P)$ is the variance of the phenotypic effect] using SAS code provided by Holland et al. (2003).

QTL analysis

Composite interval mapping (Zeng 1993, 1994) was used to identify markers with significant effects on PPO values using the software WinQTLCart V2.5.009 (Basten et al. 1997). QTL were identified using a window size of 10 cM, probability in and out of 0.1, a walking speed of 2 cM, five control markers, and the forward and backward regression method. LOD value was set for significant QTL based on a permutation test using 1,000 permutations and a significance value of 0.05. QTL were established using the map position of the peak LOD score in the interval between flanking markers. A one-LOD fall-off (from the QTL peak) was used to estimate the confidence interval of the left and right flanking markers (Chaky 2003).

Results

Identification of PPO alleles present in Louise and Penawawa

A total of ten PPO alleles (*PPO-A1*, *PPO-D1*, *PPO-A2*, *PPO-B2*, and *PPO-D2* genes) were cloned and sequenced in their entirety from the cultivars Louise and Penawawa. The cultivars were found to be polymorphic at each of these genes, which facilitated the genetic mapping of each gene in this mapping population. The hypothesized *PPO-B1* gene was not isolated despite repeated efforts to do so using degenerate primer sets (data not shown). Note that *PPO-B1* has yet to be formally described in the literature, but a few accessions are annotated as such in GenBank (GQ303713.1 and AB254804.1). Of the ten PPO sequences that were characterized, seven have been previously described whereas three alleles, all isolated from the cultivar Louise, were found to be novel. Penawawa was also found to contain the previously described *PPO-A1f*, *PPO-D1b*, *PPO-A2b*, *PPO-B2a*, and *PPO-D2b* alleles (GenBank accessions EU371654, EF070150, HQ228149, HQ228150, and HQ228153, respectively). Interestingly, the *PPO-A1* sequence isolated from Penawawa is 100% identical to that of *PPO-A1f* (GenBank accession EU371654) previously isolated from *T. dicoccoides* (He et al. 2009). Louise was found to contain the previously described *PPO-D1a* and

PPO-D2a (GenBank accessions F070149 and HQ228152, respectively) as well as three novel alleles for the *PPO-A1*, *PPO-A2* and *PPO-B2* genes. The first of these is a 1,969 bp *PPO-A1* sequence. This sequence is similar in structure to known *PPO-A1* alleles. Exons I, II, and III contain 596, 292, and 876 bp, respectively, whereas introns I and II are 102 and 125 bp in length. It encodes a 577 amino acid residue polypeptide. We propose naming this allele *PPO-A1h*. The second novel sequence identified is a 1,934 bp allele of *PPO-A2*. Like the two previously characterized *PPO-A2* alleles, it contains two exons of 557 and 1,117 bp, and a single intron of 222 bp. The exons together encode a 557 residue polypeptide. We propose naming this novel allele *PPO-A2c*. The third novel allele identified from Louise is a 2,031 bp allele of *PPO-B2*. Like *PPO-B2a* and *PPO-B2b*, this sequence has two predicted introns and three exons and encodes a 562 amino acid polypeptide. Introns I and II are predicted to be 208 and 98 bp in length, whereas exons I, II and II are 572, 262, and 855 bp, respectively. We propose naming this novel allele *PPO-B2c*. The complete sequences for *PPO-A1h*, *PPO-A2c*, and *PPO-B2c* have been deposited in GenBank under the accession numbers JN632506, JN632507, and JN632508, respectively.

Phylogenetic analysis and gene allele comparisons

Phylogenetic analysis was conducted on a dataset consisting of ten PPO sequences that included the full complement of kernel expressed PPO genes contained by both Louise and Penawawa (Fig. 1). The analysis was conducted including introns and excluding gaps. The ten sequences are well separated into the expected *PPO-1* and *PPO-2* paralogous groups. Within these, the alleles at *PPO-A1* and *PPO-A2* are much more tightly grouped than the remaining homeologous gene sets (Fig. 1). Within *PPO-A1*, the sequences *PPO-A1f* and *PPO-A1h* are over 99% identical and differ by only four base pair. The similarity is even more substantial at the amino acid level. Three of the four mutations are silent, resulting in strikingly similar 577 residue polypeptides that only differ in a relatively conservative alanine to valine substitution at position 122 of *PPO-A1f*, a mutation it shares with the *PPO-A1a* allele (see position 140, supplementary Figure 1). The alleles at *PPO-A2* are even more similar. The nucleic acid sequences of *PPO-A2b* and *PPO-A2c* differ at only two positions, one of which is silent. The encoded proteins have a single conservative valine to isoleucine substitution at position 548 of *PPO-A2b* (see position 606, supplementary Figure 1). Neither position 122 nor position 548 is particularly well conserved among PPO polypeptides (supplementary Figure 1). The situation

is somewhat different at the other PPO loci. Alleles at both *PPO-D1* and *PPO-D2* are particularly divergent and do not cluster, whereas *PPO-B2a* and *PPO-B2c* comprise their own cluster with a bootstrap value of 96% (Fig. 1). *PPO-B2a* and *PPO-B2c* have 97% identity at the nucleic acid level. This results in seventeen amino acid differences among the two alleles. The majority of these are conserved and/or occur at relatively non-conserved regions of the proteins (Supplementary Figure 1). However, the alanine to glycine missense mutation at position 211 *PPO-B2c* is notable (position 238 in Supplementary Figure 1). This residue is in close proximity to one of the catalytically required copper-binding sites of the protein, and is very highly conserved among not only wheat PPOs but also other PPOs from multiple plant genera (Fig. 2). *PPO-D2a* and *PPO-D2b* alleles at *PPO-D2* are less similar and have 94% and 96% identity at the nucleic acid and amino acid level, respectively. Most of the 27 amino acid differences between the encoded proteins occur at non-conserved regions. The exception is a proline to alanine substitution at position 112 of *PPO-D2a* (see position 144 in Supplementary Figure 1). Database searches reveal that with the exception of *PPO-D2a*, proline is conserved among wheat PPO sequences, and is well conserved among PPO proteins from other species such as rice and pineapple. However, it is not completely conserved among PPO sequences isolated from dicotyledonous species (data not shown, supplemental Figure 1). The *PPO-D1* sequences from both cultivars have previously been described. Penawawa was found to possess *PPO-D1b* and Louise *PPO-D1a* (GenBank accessions EF070150 and EF070149, respectively). Like the *PPO-A1* genes described above, they encode 577 residue polypeptides. However, these polypeptides are much more distinct from one another than any of the other allelic pairs in the Louise and Penawawa populations. *PPO-D1a* and *PPO-D1b* are only 95% identical at the nucleic acid level. The encoded proteins contain 38 amino acid substitutions relative to one another and are only 93% identical (Supplemental Figure 1). The *PPO-D1a* allele contains two substitutions which are noteworthy since they lie within very highly conserved catalytic regions of the encoded enzymes. The first mutation is a tyrosine to phenylalanine substitution at position 176 of *PPO-D1a*. This tyrosine is very highly conserved among wheat PPO sequences and is well conserved among sequences from divergent plant genera (Fig. 2). The second mutation in *PPO-D1a* is a leucine to valine substitution at position 202 of the *PPO-D1a* polypeptide product. Like the previously mentioned tyrosine 176, this leucine is likewise extremely well conserved among known plant PPOs. In addition it lies within a catalytic copper-binding site of the enzyme (Fig. 2).

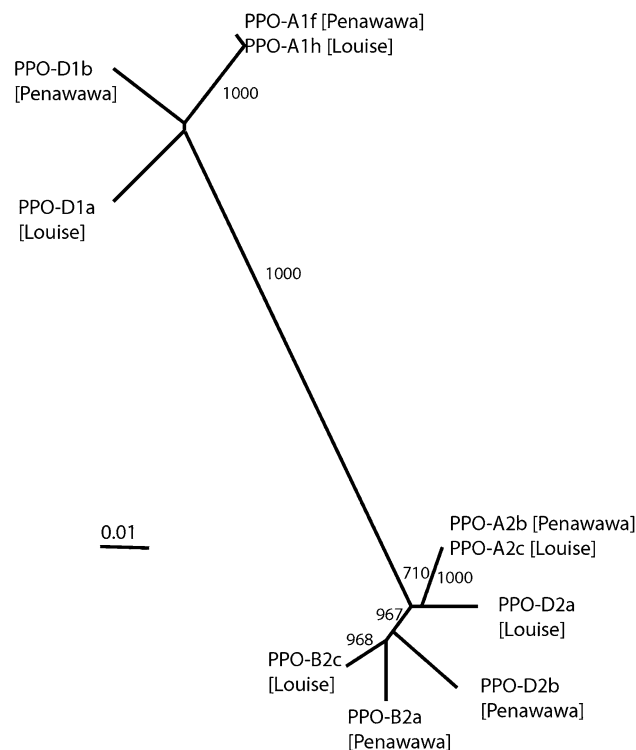


Fig. 1 Phylogenetic analysis of the Kernel-type PPO sequences of *T. aestivum* cultivars ‘Louise’ and ‘Penawawa’. The tree was derived by neighbor-joining distance analysis using the novel *PPO-A1h*, *PPO-A2c*, and *PPO-B2c* DNA sequences here isolated from Louise as well as the previously isolated *PPO-A1f*, *PPO-D1a*, *PPO-D1b*, *PPO-A2b*, *PPO-B2a*, *PPO-D2a*, and *PPO-D2b* sequences (Genbank accession numbers JN632506, JN632507, JN632508, EU371654, F070149, HQ228152, HQ228149, HQ228150, HQ228152, HQ228153, respectively). Sequences are labeled by gene name and cultivar of origin (in brackets). Scale bar indicate the number of nucleotide substitutions per site

Map location of the five PPO genes

The allelic genotypes at the *PPO-A1*, *PPO-A2*, *PPO-B2*, *PPO-D1* and *PPO-D2* loci were determined for the 188 member Louise X Penawawa recombinant inbred population used in this study. A differentially labeled PCR extension assay that exploits single nucleotide polymorphisms between alleles was used. The oligonucleotide primers, products and strategy used can be found in supplementary Table 2 and supplementary Figure 2. This population was previously described in Carter et al. (2009) with the use of 320 SSR markers to establish linkage groups. The 29 currently identified linkage groups were assigned to 21 wheat chromosomes, which covered 3,907 cM, and consist of an additional 1,434 SNP markers. Chromosome 1A was comprised of 2 linkage groups and chromosomes 2D, 5B, and 5D by four, three, and three linkage groups, respectively. The shortest chromosome was 43 cM (4D) and the longest was 235 cM (5A). The

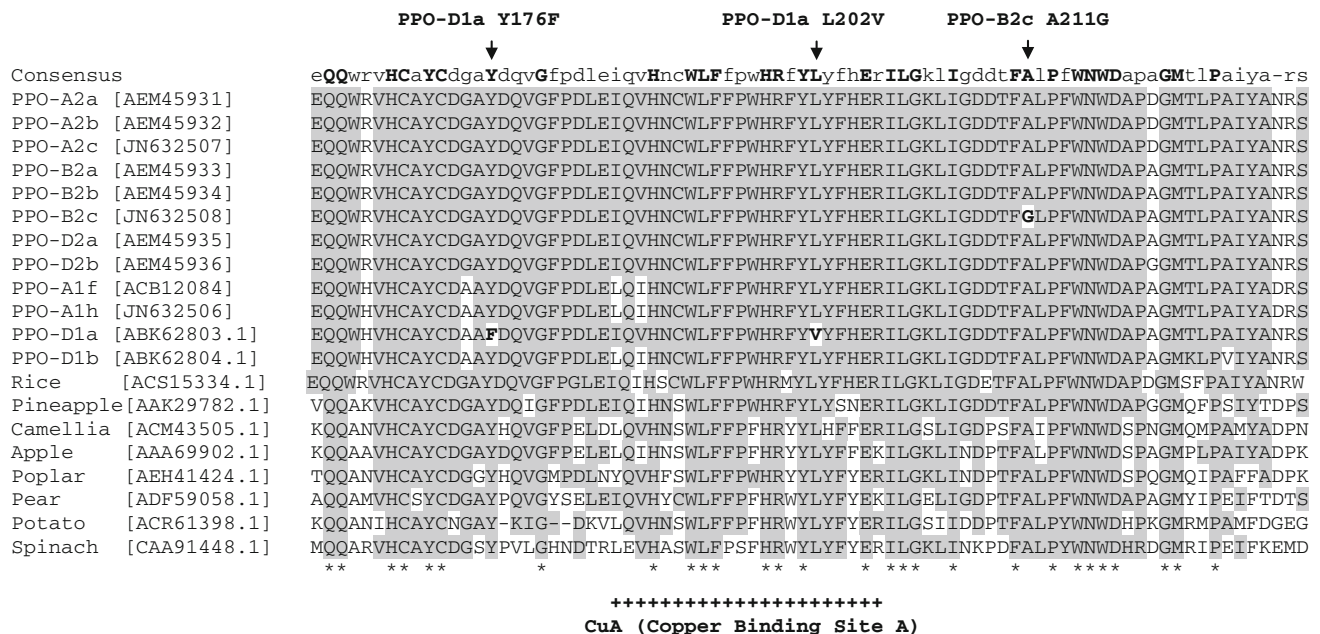


Fig. 2 Alignment of twenty PPO polypeptide sequences at the first catalytic site. Twelve sequences from wheat and eight from various unrelated genera are included. Wheat sequences are given by gene designation, non-wheat PPOs are listed by common name, all followed by Genbank accessions in brackets. Consensus line is at

top. Copper-binding site necessary for catalysis is indicated by “+” symbols (*bottom*). Conserved residues are shaded, completely conserved residues are indicated by “*” at *bottom*. Arrows are used to indicate non-conserved missense mutations found in the polypeptides encoded by the *PPO-D1a* and *PPO-B2c* alleles

A-genome chromosomes totaled 1,403 cM, the B-genome chromosomes totaled 1,394 cM, and the D-genome chromosomes totaled 1,110 cM. The averaged distance between markers on the A-, B-, and D-genome were 2.0, 1.8, and 4.9 cM, respectively. The genome-wide average distance between markers was 2.2 cM. The complete linkage map for chromosomes 2A, 2B, and 2D are shown in supplemental Figures 3, 4, and 5, respectively. All five PPO genes map to homeologous group 2 chromosomes. *PPO-A1* and *PPO-A2* both reside on the long arm of chromosome 2A (Fig. 3a). *PPO-A2* maps to position 137.4 cM and is flanked by markers *Xcfa2058* and *Xiwa174* at positions 137.0 and 137.8, respectively. *PPO-A1* maps to 146.3 cM, a position 8.9 cM distal to *PPO-A2*. *PPO-A1* is flanked by markers *Xiwa7593* and *Xwmc181* at positions 145.7 and 157.7 cM, respectively (Fig. 3a). *PPO-B2* maps to 173.5 cM on chromosome 2B (Fig. 3b). It is flanked by the proximal markers *Xiwa175* and *Xiwa4866*, both at 172.8 cM, and the distal marker *Xiwa7955* at position 175.8. Note that *PPO-B1* was not included in this study. The *PPO-D1* and *PPO-D2* genes both map to the long arm of chromosome 2D (Fig. 3c). *PPO-D2* is located at 173.0 cM on the map. It is flanked rather tightly by marker *Xcfd62* at position 172.8 and by the marker *Xcfd168* at 173.4 cM. *PPO-D1* is located 10.7 cM distal to *PPO-D2*, at position 183.7. It is flanked by markers *Xgwm608* and *Xbarc349* at positions 181.1 and 184.6 cM, respectively.

Kernel PPO activity

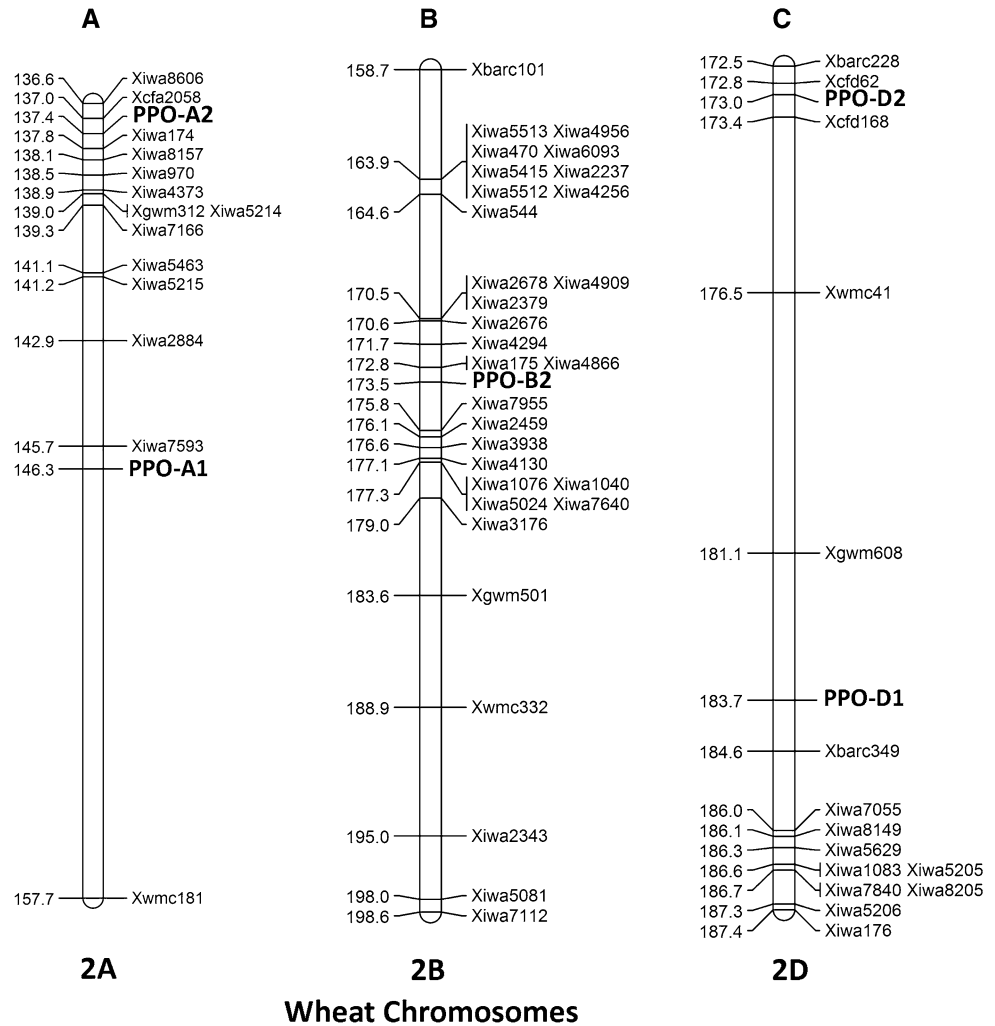
Significant differences were detected between parental lines ($P < 0.001$) and RIL ($P < 0.0001$) for PPO activity. Bloc and Bloc*Entry were not significant, although Location was significant ($P < 0.0001$). All locations demonstrated a normal distribution for PPO activity. Transgressive segregation was detected at all locations (Table 1). On average, Louise had a lower PPO value than Penawawa by 0.18 points. The ranges in RIL values were 0.4504–1.2090. The broad-sense heritability estimate for PPO activity was moderate, ranging from 0.32 to 0.43 (Table 1).

QTL analysis

QTL analysis identified six loci for PPO activity (Table 2). In all cases, the Penawawa allele contributed to higher PPO activity values. Four QTL were located on chromosome 2B and two on chromosome 2D. A QTL was identified from the Genesee, ID location in 2007 with a LOD score of 2.44 found in a 2.0 cM region flanked by SSR markers *Xgwm410* and *Xgwm148*. This QTL explained 10% of the phenotypic variation. Another QTL was identified from the Moscow, ID location in 2008. This QTL had a LOD score of 2.41, accounted for 9% of the phenotypic variation, and was in a 2.5 cM region flanked by SNP markers *Xiwa905* and *Xiwa3908*. The third QTL identified was in a 5.9 cM region flanked by SNP markers *Xiwa554* and *Xiwa2678*.

Fig. 3 Chromosomal location of PPO sequences known to be expressed in wheat kernels.

a Partial linkage map showing the distal end of the long arm of chromosome 2A. *PPO-A2* is located at 137.4 cM, *PPO-A1* is located 8.9 cM distal to that at 146.3 cM. **b** Partial linkage map showing the distal end of the long arm of chromosome 2B. *PPO-B2* is located at 173.5 cM. **c** Partial linkage map showing the distal end of the long arm of chromosome 2D. *PPO-D2* is located at 173.0 cM and *PPO-D1* is located 10.7 cM distal to that at 183.7 cM

**Table 1** Range and heritability of polyphenol oxidase (PPO) activity in a Louise X Penawawa recombinant inbred line (RIL) population over 2 years and three locations in the Pacific Northwest region of the United States

Location	Year	Parents				RIL				Heritability	
		Louise	Penawawa	Mean	SE	Minimum	Maximum	Mean	SE	h^2	SE
Genesee, ID	2007	0.7365	0.9321	0.8343	0.14	0.4501	1.0871	0.8512	0.08	0.41	0.05
Moscow, ID	2008	0.6798	0.8146	0.7422	0.14	0.6147	1.0724	0.8070	0.08	0.35	0.05
Pullman, WA	2007	0.8044	0.9871	0.8913	0.12	0.6904	1.2090	0.9124	0.08	0.32	0.05
	2008	0.6927	0.9177	0.8052	0.17	0.5342	1.1492	0.8170	0.08	0.43	0.04

The fourth QTL identified was in a 0.7 cM region flanked by SNP marker *Xiwa4866* and *PPO-B2*. Since this locus is coincidental with the marker *PPO-B2* (Fig. 4a), it is suspected that this gene is conferring the associated phenotype. Both the third and fourth QTL on chromosome 2B were identified from the Genesee, ID location and the combined location values and accounted for 12–13% of the variation.

The two loci identified on chromosome 2D corresponded to the *PPO-D1* and *PPO-D2* markers. The first

locus, identified in Genesee, ID in 2007 as well as the combined average from all locations, spanned a 0.2 and 0.4 cM region, respectively, with the peak LOD value near the *PPO-D2* locus. The percentage variation explained by this QTL was 11 and 7%, respectively. The second locus spanned 2.7 cM between marker *Xgwm608* and *PPO-D1* (Fig. 4b) and had a higher LOD score. This QTL also was identified in Genesee, ID in 2007 and using the combined averages from all locations. The percentage variation explained by this QTL was 11 and 7%, respectively. Both

Table 2 Significant quantitative trait loci (QTL) identified through composite interval mapping in a Louise X Penawawa recombinant inbred line (RIL) population for polyphenol oxidase (PPO) activity

Chromosome	Location	LOD cutoff ^a	LOD score	Peak position	Flanking markers	R ²	Additive effect ^b
2B	Kam2007	2.2	2.44	92.3	<i>Xgwm410-Xgwm148</i>	0.10	−0.07
	Mos2008	2.2	2.41	112.3	<i>Xiwa905-Xiwa3908</i>	0.09	−0.05
	Kam2007	2.2	2.51	165.8	<i>Xiwa554-Xiwa2678</i>	0.12	−0.04
	Combined	2.0	2.54	165.8	<i>Xiwa554-Xiwa2678</i>	0.13	−0.03
	Kam2007	2.2	2.34	173.0	<i>Xiwa4866-PPO-B2</i>	0.12	−0.04
	Combined	2.0	2.50	173.0	<i>Xiwa4866-PPO-B2</i>	0.13	−0.03
2D	Kam2007	2.4	2.60	172.9	<i>Xcfd62-PPO-D2</i>	0.11	−0.04
	Combined	2.5	2.90	173.3	<i>PPO-D2-Xcfd168</i>	0.07	−0.02
	Kam2007	2.4	3.31	181.9	<i>Xgwm608-PPO-D1</i>	0.11	−0.04
	Combined	2.5	3.37	182.9	<i>Xgwm608-PPO-D1</i>	0.07	−0.02

Kam2007 Kambitsch Farm, Genesee, ID, 2007; *Mos2008* Parker Farm, Moscow, ID, 2008; *Pul2008* Spillman Farm, Pullman, WA, 2008; *Combined* data averaged over the previous four locations for each RIL and analyzed as a single value

^a As determined by a 1,000 permutation test in QTL Cartographer

^b Additive effect of the Louise allele

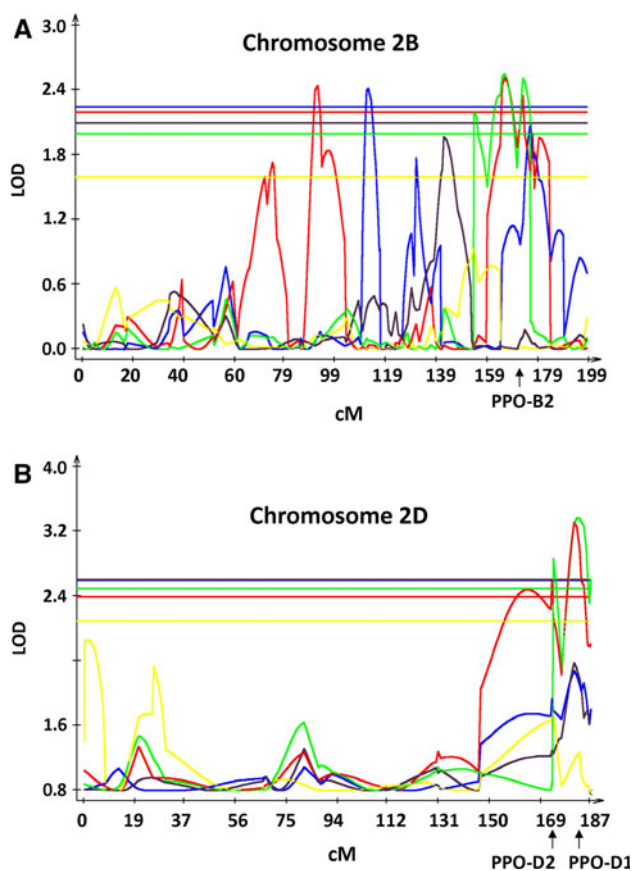


Fig. 4 QTL analysis of PPO activity across four environments. LOD plots are labeled by environment as follows: *red* (Genesee, ID, 2007), *yellow* (Pullman, WA, 2007), *blue* (Moscow, ID, 2008), *brown* (Pullman, WA, 2008), *green* (combined). LOD cutoff scores as indicated by a 1,000 permutation test are indicated in the *horizontal lines* (color figure online)

identified loci on chromosome 2D have peak positions very near the established gene locations for PPO activity. It is therefore proposed that the observed phenotype is being controlled by these genes.

Discussion

Wheat end-use quality can be severely negatively impacted by PPO activity. It is well established that PPO exists as a multigene family in wheat (Jukanti et al. 2004; Massa et al. 2007). Until recently, however, only two genes were known to be expressed in developing wheat kernels. They are *PPO-A1* identified by Sun et al. (2005) and *PPO-D1* identified by He et al. (2007). Recently three novel PPO genes were described. Two of these, *PPO-A2* and *PPO-D2*, appear to be more highly expressed in developing seeds than the previously known *PPO-A1* and *PPO-D1* (Beecher and Skinner 2011). However, their location was not determined beyond the chromosomal arm. The overall goal for this study was to determine the genetic locations and distances between these five important PPO genes.

This study characterized all the PPO genes currently known to be expressed in the kernel for the cultivars Louise and Penawawa. The sequences for *PPO-A1*, *PPO-D1*, *PPO-A2*, *PPO-B2* and *PPO-D2* were therefore cloned and sequenced from both cultivars. The cultivar Penawawa was found to contain only previously characterized alleles. Curiously, the *PPO-A1* allele carried by Penawawa was previously isolated from *T. dicoccoides*, not *T. aestivum* (He et al. 2009). However this is perhaps not so unexpected given the well known use of wide outcrosses in wheat

breeding programs for the purpose of incorporating disease and pest resistance genes. Louise was found to contain novel alleles at three loci, here named *PPO-A1h*, *PPO-A2c*, and *PPO-B2c*, as well as the previously described *PPO-D1a* and *PPO-D2a*. None of the ten sequences characterized contained any obvious function-abolishing characteristics such as splice site mutations or premature stop codons. However, it should be noted that *PPO-D1a* and to a lesser extent *PPO-B2c* contained non-conserved missense mutations either directly within or in proximity to core catalytic regions of the encoded proteins. These mutations have the potential to perturb the function or stability of the encoded enzymes, although some functionality is most likely retained. Comparisons between alleles within a locus revealed that alleles of both A-genome genes, *PPO-A1* and *PPO-A2*, showed by far the most similarity. In contrast, the greatest degree of divergence was found for the *PPO-D1a* and *PPO-D1b* alleles, followed by the a and b alleles of the *PPO-D2* locus. The *PPO-B2a* and c alleles were more similar, but not nearly identical as was the case for the f and h alleles at *PPO-A1* and the b and c alleles at *PPO-A2*.

QTL analysis identified six chromosomal regions affecting kernel PPO activity. No QTL were found on chromosome 2A, although several previous studies have identified this region as impacting PPO activity and/or noodle dough color stability (Anderson et al. 2006; Chang et al. 2007; Demeke et al. 2001; He et al. 2007; Jimenez and Dubcovsky 1999; Mares and Campbell 2001; Raman et al. 2005; Simeone et al. 2002; Sun et al. 2005; Watanabe et al. 2004; Zeven 1972; Zhang et al. 2005). However, the lack of QTL on 2A was not unexpected in this study, given: (1) The substantial PPO activity exhibited by the parental cultivars and members of the population, and (2) the extreme similarity for the alleles of both *PPO-A1* and *PPO-A2*. The enzymes encoded by both alleles at both loci are likely to be fully active and functionally indistinguishable from one another. Two major QTL were identified on chromosome 2D at the *PPO-D1* and *PPO-D2* loci. These were the largest QTL identified in this study, but were only identified in the Genesee, ID location and when using the combined averages over all years and locations. This replicates previous results implicating *PPO-D1a* and *PPO-D1b* as low and high activity alleles, respectively (He et al. 2007). Careful analysis of the *PPO-D1a* polypeptide here shows that it contains two non-conservative substitution mutations which could potentially explain its low-activity relative to *PPO-D1b*. Four additional QTL were found in the analysis, all on chromosome 2B. The fourth corresponds to *PPO-B2*. This is somewhat surprising given the results of a previous study by Beecher and Skinner, in which the expression of *PPO-B2* was not detected (Beecher and Skinner 2011). However, that study measured expression of yet another *PPO-B2* allele (*PPO-B2b*) in

another cultivar (Alpowa), and so perhaps those results might not transfer to the genetic materials used in the current study. Previous studies have detected PPO activity QTL on chromosome 2B (Demeke et al. 2001; Fuerst et al. 2008; Watanabe et al. 2004). The current study is only the fourth known time this has been documented, and is the first time a PPO coding sequence has been directly implicated in PPO activity on chromosome 2B. In this Louise X Penawawa population, the *PPO-B2c* allele of Louise was associated with lower measured PPO activity compared to that of the *PPO-B2a* allele donated by Penawawa. This is consistent with polypeptide analysis showing that the *PPO-B2c* allele has a non-conserved missense mutation immediately downstream of the first copper-binding region of the enzyme. This may negatively impact its catalytic activity.

The QTL identified on chromosome 2B flanked by SNP markers *Xiwa905* and *Xiwa3908* also corresponds to previously identified QTL in this population. Carter et al. (2009) identified a major QTL for stripe rust resistance at this locus. At this locus, increased stripe rust resistance is associated with decreased levels of PPO activity. Carter et al. (2011) also identified a QTL for seed kernel hardness at this locus. Smith et al. (2011) found a QTL for softness equivalent in a soft white RIL population located on chromosome 2B in a similar location as identified here. It is possible that kernel hardness or factors affecting kernel hardness may influence PPO activity. Grain hardness in wheat is associated with mutation in either of the linked *Pina-D1* or *Pinb-D1* loci (Beecher et al. 2002; Giroux and Morris 1998). Since these loci are not located on chromosome 2B, nor are these genes segregating in this population, this association with kernel hardness and PPO activity could be due to other seed components such as pentosans, polar lipids, and protein in the endosperm (Chang et al. 2006).

The third and fourth chromosomal regions identified on chromosome 2B are located 7.2 cM from each other. The second region corresponds to *PPO-B2*, whereas the first does not correspond to a PPO marker. Since *PPO-B1* is not on the linkage map, it is possible that this QTL corresponds to *PPO-B1*. The PPO markers on chromosome 2A map 9 cM from each other and the PPO markers on chromosome 2D map 10 cM from each other. In both cases, *PPO-A1* and *PPO-D1* are more distal than *PPO-A2* and *PPO-D2*. If the assumption is correct that the third QTL found on chromosome 2B is *PPO-B1*, this would be more proximal than *PPO-B2*, indicating a previous inversion event.

Five sets of allele-specific markers suitable for automated fluorescent detection were designed, validated and used to determine allelic status for members of the 188 member Louise and Penawawa mapping population. This

enabled the newly identified *PPO-A2*, *PPO-B2* and *PPO-D2* loci to be genetically mapped for the first time. The three genes map to the long arm of group 2 chromosomes. *PPO-A2* and *PPO-D2* map 8.9 and 10.7 cM proximal to their paralogous counterparts *PPO-A1* and *PPO-D1*, respectively. Group 2 chromosomes have been implicated in controlling PPO activity levels numerous times in the literature (Anderson et al. 2006; Chang et al. 2007; Demeke et al. 2001; He et al. 2007; Jimenez and Dubcovsky 1999; Mares and Campbell 2001; Raman et al. 2005; Simeone et al. 2002; Sun et al. 2005; Watanabe et al. 2004; Zhang et al. 2005). Based on these results as well as the 2011 study by Beecher and Skinner, it appears as though *PPO-B2b* and *PPO-B2c* have the potential to be low-activity allelic markers to be used in wheat improvement efforts. However, *PPO-B2c* versus *PPO-B2a* explained a minimal amount (0.05) of observed variation in PPO activities. The preponderance of the literature as well as previous expression analysis studies (Jukanti et al. 2006; Beecher and Skinner 2011) indicate that the major gene targets would be *PPO-A2*, *PPO-A1*, *PPO-D2*, and *PPO-D1*. Low-activity alleles have been described for *PPO-A1* and *PPO-D1*. A major impediment to wheat quality improvement efforts appears to be a lack of known low alleles for *PPO-A2* and *PPO-D2*. Efforts to identify useful alleles at these loci are currently underway. The results of this study indicate that the paralogous *PPO-1* and *PPO-2* loci are separated by approximately 10 cM. Therefore, once identified, low-activity alleles at all loci could easily be combined by plant breeders in wheat improvement efforts.

Conclusion

The main goal of this study was to determine the genetic location of the five genes known to be expressed in developing wheat kernels using the Louise and Penawawa mapping population. To accomplish this, the sequences at each of these loci were first determined. Three novel PPO alleles were identified during this process. Allele-specific markers were designed and used to determine allele composition of each of the 188 members of the population. The genetic location of each PPO gene was then determined. In addition a QTL analysis was performed to determine which genetic regions impact PPO activity. These results are the first determination of the chromosomal locations for the newly described, *PPO-A2*, *PPO-B2*, and *PPO-D2* genes, and reconfirmed the locations of the previously characterized *PPO-A1* and *PPO-D1* genes. The simultaneous mapping of all five loci in one population allowed the distance between the paralogous *PPO-1* and *PPO-2* gene members to be identified. In addition, QTL analysis here identified four novel QTL on chromosome 2B as well one on

chromosome 2D in addition to confirming the previously characterized *PPO-D1*.

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