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ORIGINAL PAPER

Genetic characterization and expression analysis of wheat (*Triticum aestivum*) line 07OR1074 exhibiting very low polyphenol oxidase (PPO) activity

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Abstract

Key message **Characterized novel mutations present at *Ppo* loci account for the substantial reduction of the total kernel PPO activity present in a putative null *Ppo-A1* genetic background.**

Abstract Wheat (*Triticum aestivum*) polyphenol oxidase (PPO) contributes to the time-dependent discoloration of Asian noodles. Wheat contains multiple paralogous and orthologous *Ppo* genes, *Ppo-A1*, *Ppo-D1*, *Ppo-A2*, *Ppo-D2*, and *Ppo-B2*, expressed in wheat kernels. To date, wheat noodle color improvement efforts have focused on breeding cultivars containing *Ppo-D1* and *Ppo-A1* alleles conferring reduced PPO activity. A major impediment to wheat quality improvement is a lack of additional *Ppo* alleles conferring reduced kernel PPO. In this study, a previously reported very low PPO line, 07OR1074, was found to contain a novel allele at *Ppo-A2* and null alleles at the *Ppo-A1* and *Ppo-D1* loci. To examine the impact of each mutation upon kernel PPO, populations were generated from crosses between 07OR1074 and the hard white spring wheat cultivars Choteau and Vida. Expression analysis using RNA-seq demonstrated no detectable *Ppo-A1* transcripts in 07OR1074 while *Ppo-D1* transcripts were present at less than 10 % of that seen in Choteau and Vida. Novel markers specific for the *Ppo-D1* and *Ppo-A2* mutations discovered in 07OR1074, along with the *Ppo-A1* STS marker, were used to screen segregating populations. Evaluation of lines indicated a substantial genotypic effect on PPO with *Ppo-A1* and *Ppo-D1* alleles contributing significantly to total PPO in both populations. These results show that the novel mutations in *Ppo-A1* and *Ppo-D1* present in 07OR1074 are both important to lowering overall wheat seed PPO activity and may be useful to produce more desirable and marketable wheat-based products.

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Introduction

Found extensively throughout land plants (Flurkey 1986; Tran et al. 2012) polyphenol oxidase (EC.1.14.18.1) enzymes (PPO) catalyze the formation of o-quinones through the hydroxylation of monophenolic compounds using molecular oxygen (Mayer and Harel 1979). Consequentially, o-quinones can react non-enzymatically with cellular thiol and amine groups or polymerize to form dark melanin pigments (Matheis and Whitaker 1984; Whitaker and Lee 1995) resulting in darkened and discolored food products that are perceived as spoiled and less desirable. Numerous studies have examined the contribution of PPO to the time-dependent discoloration in food products derived from common wheat (*Triticum aestivum*) (McCallum and Walker 1990; Baik et al. 1995; Morris et al. 2000; Martin et al. 2011). Both the genotype and the environment contribute to variation in kernel PPO activity in bread wheat (Park et al. 1997). Through the efforts of plant breeding, novel PPO alleles that contain mutations that effectively reduce plant PPO levels are currently being incorporated into a variety of crops to prevent enzymatic oxidation of food products with the hopes of extending shelf life and decreasing food waste (Onto 2011; Holderbaum et al. 2010; Kahn 1975).

Wheat end use quality improvement efforts to reduce kernel PPO levels have been hampered by bread wheat's hexaploid genome and lack of known low PPO activity alleles. Bread wheat possesses three unique homeologous genomes resulting in, on average, triplicate sets of any given gene. In addition to this state of genetic redundancy, bread wheat contains multiple paralogous *Ppo* genes arising from gene duplication events (Jukanti et al. 2004; Fuerst et al. 2008). Paralogous gene family *Ppo-1* members consisting of *Ppo-A1* and *Ppo-D1* are localized to the long arm of homeologous chromosomes 2A and 2D, respectively (Sun et al. 2011; He et al. 2007). *Ppo-A1* and *Ppo-D1* allelic variation is associated with kernel PPO activity variation (Sun et al. 2011; He et al. 2007; Wang et al. 2009; Martin et al. 2011; Nilthong et al. 2012). The sequence-tagged site (STS) marker PPO18, amplifies a 685 bp (*Ppo-A1a*) and 876 bp (*Ppo-A1b*) fragment corresponding to genotypes associated with high and low PPO activity, respectively (Sun et al. 2011). Complementary STS markers PPO16 and PPO29 amplify a 490 and 713 bp fragment that effectively discriminates two alleles associated with high (*Ppo-D1b*) and low (*Ppo-D1a*) PPO activity, respectively (He et al. 2007). Additional *Ppo-A1* and *Ppo-D1* alleles have been reported from common wheat and diploid wheat relative species (*Ppo-A1c–Ppo-A1 h*) and *Ppo-D1c–Ppo-D1e* (He et al. 2007; Beecher et al. 2012), none of which are identical to the *Ppo-A1i* and *Ppo-D1f* alleles we report here. Paralogous gene family *Ppo-2* consisting of the *Ppo-A2*, *Ppo-D2*, and *Ppo-B2* genes was identified and also maps to the long arm of homeologous group 2 chromosomes with *Ppo-A2* and *Ppo-D2* localized within 10 cM of their *Ppo-1* paralogous counterparts (Beecher and Skinner 2011; Beecher et al. 2012). This is in agreement with prior genetic studies demonstrating that seed-expressed *Ppo* genes are localized to the homeologous group 2 chromosomes (Jimenez and Dubcovsky 1999; Chang et al. 2007; Sun et al. 2011; He et al. 2007; Demeke et al. 2001; Zhang et al. 2005). *Ppo-A2* and *Ppo-D2* of paralogous gene family *Ppo-2* were shown to account for over 72 % of *Ppo* gene family transcripts in developing kernels in the cultivar 'Alpowa', while transcripts of *Ppo-B2* were not detected (Beecher and Skinner 2011). These findings suggest the *Ppo-1* and *Ppo-2* orthologous A genome loci contribute more transcripts and, therefore, likely more functional PPO enzyme than *Ppo* transcripts from either the B and D genome combined. This is in accordance with prior work indicating that *Ppo-A1* allelic variation has the greatest effect on kernel PPO activity (Raman et al. 2005; Sun et al. 2011; Nilthong et al. 2012) and upon Chinese raw noodle color (Martin et al. 2011) compared to *Ppo-D1* allelic variation. However, since the *Ppo-1* and *Ppo-2* orthologous genes are separated by just 10 cM (Beecher et al. 2012), genetic linkage may confound previously reported results examining the relative importance of *Ppo-A1* and *Ppo-D1* allelic variation upon kernel PPO activity. However, to date there are no characterized *Ppo-A2* and *Ppo-D2* alleles conferring reduced PPO activity.

Wheat lines exhibiting very low kernel PPO was discovered through a screen of the USDA-ARS National Small Grains Collection germplasm by Onto (2011). Nilthong et al. (2012) described these wheat lines as containing a putative null genotype at the *Ppo-A1* locus but did not characterize any mutations in other *Ppo* genes. The goals of the present study were: (1) characterize mutations present at additional *Ppo* loci that explain the substantial reduction in kernel PPO activity present in a putative null *Ppo-A1* genetic background; (2) create novel functional markers encompassing mutations found within newly characterized alleles; (3) assess the allelic impact of the newly characterized alleles on kernel PPO activity; and (4) characterize the expression profile of kernel-expressed *Ppo* genes in a putative null *Ppo-A1* genetic background.

Materials and methods

Plant materials

Hard white spring line 07OR1074 derived from mating Australian white-seeded spring wheat genotypes PI 117635 and 'Seaspray'(PI 134049) (Onto 2011) was crossed with hard white spring wheat parents 'White Choteau' ('Choteau' PI 633974/5* 'Clear White' PI 365044), and 'White Vida' ('Vida' PI 642366/5* 'Clear White' PI 365044) (Talbert et al. 2013) to develop populations to assess the allelic impact of the newly characterized alleles on kernel PPO. Initial crosses were grown in the greenhouse in 2011. F_1 plants were advanced to produce segregating F_2 populations. The F_2 plants were grown in the field in 3 m rows with plants spaced 30 cm apart at the Montana State University Arthur H. Post Field Research Center near Bozeman MT in 2012 under irrigated conditions. F_3 plants were grown in the greenhouse in 2012 at Montana State University and genotyped for low-*Ppo* alleles at the *Ppo-D1*, *Ppo-A1*, and *Ppo-A2* loci. To reduce the population size *Ppo-A2* heterozygotes were discarded. One hundred twenty-one 07OR1074/White Choteau F_3:F_4 and 126 07OR1074/White Vida lines along with the parental genotypes were grown in replicated trials in 2 years. The 2013 trial was planted in single 3 m row plots spaced 30 cm apart in a randomized block design with two replications under irrigated conditions. In 2014, the same entries were grown in randomized block with two replications in separate, adjacent experiments with and without irrigation. Irrigated conditions consisted of 2–4 cm of added water 1 week before and after flowering. Trials were grown at the Arthur H. Post Field Research Center in near Bozeman MT in both years. Grain from each plot was

Table 1 *Ppo* alleles characterized in hard white spring wheat parents by gene cloning and subsequent sequencing

Ppo genes	White Choteau	White Vida	07OR1074
Ppo-A1	*A1b*[a]	*A1a*[b]	*A1i*[c]
Ppo-A2	*A2a*	*A2b*	*A2d*
Ppo-D1	*D1b*[d]	*D1b*[d]	*D1f*[e]
Ppo-D2	*D2b*	*D2b*	*D2b*
Ppo-B2	*B2c*	*B2c*	*B2c*

[a] Low-*Ppo* allele as designated by STS marker PPO18 amplifying a 876 bp fragment

[b] High-*Ppo* allele as designated by STS marker PPO18 amplifying a 685 bp fragment

[c] Null-*Ppo* allele as designated by STS marker PPO18 producing no fragment

[d] Null-*Ppo* allele as designated by CAPS marker PPOD1CAP producing a 931 bp fragment

[e] Null-*Ppo* allele as designated by CAPS marker PPOD1CAP producing 535 and 396 bp fragments

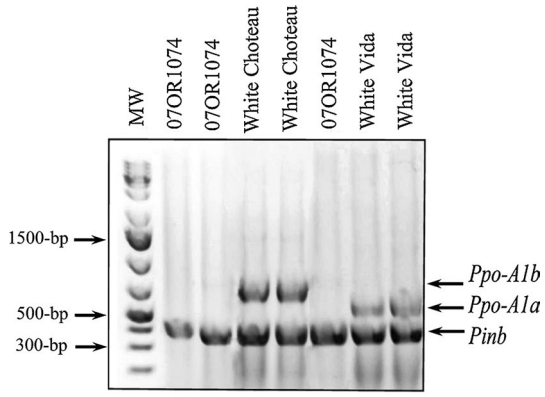

Fig. 1 STS marker analysis of lines segregating at the *Ppo-A1* locus. Electrophoresis of PCR fragments multiplexed with puroindoline-b primer combo PB5 and CAT3.4 and STS marker PPO18 producing an 469 bp product serving as a positive control and 876 or 685 bp fragments corresponding to *Ppo-A1b* and *Ppo-A1a* alleles, respectively

threshed at maturity and used for PPO activity and kernel trait measurements.

PPO gene cloning and sequencing

Genomic DNA was isolated from young leaf tissue (Riede and Anderson 1996). Gene-specific PCR was conducted for *Ppo-A1* and *Ppo-D1* using the primers presented in supplementary Table 1 of Beecher and Skinner (2011). PCRs were performed in a total volume of 25 µL containing approximately 40 ng of genomic DNA, 1× Green GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each oligonucleotide primer and 0.65 unit of GoTaq®Flexi DNA Polymerase (Promega, Madison, WI, USA). The nonspecific primers POUT5S4 and POUT53A1 (Beecher and Skinner 2011) were used to amplify a composite of the paralogous group *Ppo-2* genes using the proofreading Phusion® polymerase (New England Biolabs, Ipswich, MA, USA). Reactions were performed in a total volume of 30 µL consisting of approximately 40 ng of genomic DNA, 1x Phusion GC PCR buffer, 0.2 mM of each dNTP, 0.4 µM of each oligonucleotide primer, 3 % v/v DMSO, and 0.65 unit of Phusion Polymerase. The temperature regime for the gene-specific primers consisted of a 5 min initial denaturation step at 98 °C, followed by forty cycles of 98 °C for 50, 30 s at the annealing temperature (Beecher et al. 2012), and a 60 s extension step at 72 °C. The thermocycling program for the nonspecific primers amplifying the paralogous group *Ppo-2* genes consisted of a 60 s initial denaturation step at 98 °C, followed by forty cycles of 98 °C for 50, 30 s at 50 °C, and a 2 min extension step at 72 °C. PCR products were purified using EconoSpin Silica Membrane DNA extraction kit columns (Epoch Life Sciences,

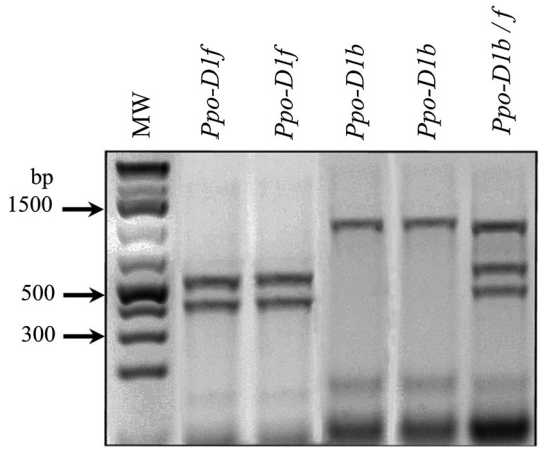

Fig. 2 Electrophoresis of PCR fragments amplified with CAPS marker PPO-D1CAP and digested with restriction endonuclease FspBI. The *Ppo-D1f* allele when cut with FspBI yields 535 and 396 bp products. An uncut 931 bp fragment with a 535 and 396 bp product denotes a *Ppo-D1b/c* heterozygote. A single uncut 931 bp fragment corresponds to the *Ppo-D1b* allele

Missouri City, TX, USA) and subcloned into pGEM®T-Easy (Promega, Madison, WI, USA). Twenty clones from each cultivar were screened by sequencing and verified by re-sequencing from an independent PCR clone using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were read using an Applied Biosystems PRISM 3730xl Genetic Analyzer.

PPO STS markers and analysis

The PCR-based makers PPO18, PPOD1CAP, and PPOA2d1074 (Table 1) were used to genotype lines in both

segregating populations at the *Ppo-A1*, *Ppo-D1*, and *Ppo-A2* loci, respectively, to identify alleles conditioning high or low PPO activity. All PCRs were performed in a total volume of 25 µL as described above. PPO18 was multiplexed with the primer combination PB5 and CAT 3.4 (Swan et al. 2006) to distinguish between the putative null allele (Nilthong et al. 2012) and alleles conditioning high and low activity at the *Ppo-A1* locus (Sun et al. 2011). The primer combination PB5 and CAT 3.4 amplifies a portion of the *Pinb-D1* gene which was used as an internal control for genomic DNA quality (Fig. 1). PPO18 and the *Pinb-D1* multiplex thermocycling program was 98 °C for 4 min, followed by 40 cycles of touchdown PCR at 98 °C for 50 s, 58 °C for 1 min decreasing 0.2 °C every cycle, 72 °C for 1 min, and a final extension of 72 °C for 7 min. The CAPS marker PPOD1CAP (Fig. 2), produces a 931 bp product that is cut once or not at all with restriction endonuclease FspBI (ThermoScientific, Pittsburgh PA, USA) yielding an uncut 931 bp product (*Ppo-D1b*), an uncut 931 bp fragment with a 535 and 396 bp product (*PPO-D1b/f* heterozygote), and a 535 and 396 bp product (*Ppo-D1f*). The thermocycling regime for PPOD1CAP consisted of a 5 min initial denaturation step at 98 °C, followed by forty cycles of 98 °C for 50, 45 s at 62 °C and 60 s at 72 °C, with a final extension at 72 °C for 7 min. Each restriction digest reaction was performed in a total volume of 25 µL containing approximately 10 µL of PCR product, 0.2 µL of FspBI enzyme, and 2.5 µL of 10× buffer. Restriction digests were incubated at 37 °C overnight. STS marker PPOA2d1074 was designed to amplify an 878 bp fragment encompassing a single nucleotide polymorphism (SNP) identified from the null-PPO parent line 07OR1074 in the *Ppo-A2* gene. Amplified products were purified using EconoSpin Silica Membrane DNA extraction kit columns (Epoch Life Sciences, Missouri City, TX, USA.) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Grand Island, NY, USA). Sequencing reactions were read using an Applied Biosystems PRISM 3730xl Genetic Analyzer. All PCR conditions were performed in a total volume of 25 µL containing approximately 40 ng of genomic DNA, 1X Green GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each oligonucleotide primer and 0.65 unit of GoTaq®Flexi DNA Polymerase (Promega, Madison, WI, USA).

Measurement of PPO activity and kernel characteristics

Polyphenol oxidase activity for all parents and $F_{3:4}$ (2013) or $F_{3:5}$ (2014) progeny lines was measured in 96-well 2 mL plates (USA Scientific, Ocala, FL, USA) with four replicates of three seeds for each genotype from each field replication following the methods described by Anderson and Morris (2001). Controls were a blank well filled only with [L-3-(dihydroxyphenyl) alanine] (L-DOPA) solution, the hard red spring 'Glenn' (Mergoum et al. 2006) as a moderate-PPO control, and the durum wheat 'Mountrail' (Elias and Miller 1998) as a low-PPO control. Each well received 1 mL of a solution that was 50 mM 3-(*N*-morpholino) propanesulfonic acid and 10 mM L-DOPA, pH 6.5. Plates were sealed with TempPlate XP inset cut sealing film (USA Scientific) and mixed on a Vari-Mix platform rocker (ThermoFisher Scientific, Waltham, MA, USA) at speed 20 and a rocking angle of 48° for 2 h at room temperature. Sample (200 µL) from each well was transferred to a flat-bottom, 96-well clear microtiter plate (ThermoFisher), and absorbance (A_{475}) was measured on a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The absorbance value of the blank control was subtracted from the absorbance value of each individual sample. Grain protein concentration was determined by near-infrared spectroscopy for whole grain using an Infratec 1241 Grain Analyzer (Foss North America, Eden Praire, MN, USA). Kernel weight and diameter were determined from 100 kernels using the Single Kernel Characterization system (SKCS) (Perten Instruments North America Inc., Springfield, IL, USA) from each field-grown replication.

PPO gene expression analysis via RNA-seq

To analyze expression levels of *Ppo* genes, developing seeds were collected from greenhouse-grown 07OR1074, White Choteau, and White Vida at 16 days post-anthesis (dpa), the time at which kernel *Ppo* expression levels peak (Beecher and Skinner 2011). Collected seeds were immediately frozen in liquid N_2, and stored at −80 °C. For each genotype, developing seeds were collected from three separate plants, with each plant sample composed of three seeds from the middle of three different developing heads (9 seeds total). One hundred mg of seed powder, ground in liquid N_2, was transferred to a pre-chilled 2-mL tube and 0.5 mL of RNA extraction buffer [100 mM Tris pH 8.0, 150 mM LiCl, 50 mM EDTA, 1.5 % (w/v) SDS, 0.15 % (v/v) BME] was added and samples were vortexed until homogenous. Next, 0.25 mL of 1:1 (v/v) phenol–chloroform (pH 4.7) was added and samples were mixed by inversion followed by centrifugation at 13,000*g* for 15 min at room temperature. The supernatant was transferred to a QIAshredder spin column and total RNA was extracted using an RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was quantified and its quality assessed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For RNA-seq analysis, one µg of total RNA was used for the creation of cDNA libraries using TruSeq RNA-seq library kits (Illumina, San Diego, CA, USA). Amplicons from cDNA libraries were sequenced as single 100 bp reads using an Illumina HiSeq 2000. RNA-seq data were analyzed using Q-seq in ArrayStar v5.0 (DNASTAR, Madison, WI, USA) Genes

of interest were selected from the NCBI database for analysis with the match settings in Q-Seq set to 100 % for at least 90 bp with mer minimization turned off. All other settings were left at default and sequences were normalized using the reads per kilobase of exon model per million mapped reads (RPKM) method. Non-detectable transcripts (nd) designations were given to samples that did not contain transcripts aligning to gene-coding sequences under RPKM normalization. Resultant linear counts were then further normalized to the expression levels of the housekeeping gene Cyclophilin A (Cyp18-2) (Nicot et al. 2005). Student's *t* tests were used to compare expression levels between 07OR1074, White Choteau, and White Vida.

Statistical analysis

To evaluate the allelic impact of *Ppo-A1*, *Ppo-D1*, and *PpoA2* on kernel PPO activity and other kernel traits data were analyzed via analysis of variance using PROC MIXED procedure with the SAS/STAT software version 9.3 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Data from each cross-population and year were analyzed separately. In 2013, a mixed effects model was used where genotype classes were fixed and replications and entries within genotype class were random effects. The analysis was the same for 2014 except that it was combined over the rain-fed and irrigated environments. The proportion of phenotypic variation explained by *Ppo-A1*, *Ppo-D1, Ppo-A2* and genotypes was determined as the sum of squares explained by the model divided by the total sum of squares (r^2) using the entry means. Segregation ratios were tested using Chi-square tests.

Results

Characterization of novel alleles

Previous work by Nilthong et al. (2012) suggested the possibility of unique mutations present in major genes controlling kernel seed-expressed PPO activity. Due to high sequence similarity among the *Ppo-2* paralogous gene family, the non-specific primers POUT5S4 and POUT53A1 (Beecher and Skinner 2011) were used to amplify and clone a composite of the paralogous group *Ppo-2* genes from breeding line 07OR1074 and the two cultivars. The resulting PCR products were examined by sequencing of individual clones. Nine different *Ppo* alleles were cloned and sequenced from the cultivars White Choteau, White Vida, and the breeding line 07OR1074 (Table 2). Of the nine *Ppo* alleles sequenced, two were novel, and seven were previously described. The cultivar White Choteau contained the *Ppo-A1b*, *Ppo-D1b*, *Ppo-A2a*, *Ppo-B2c*, and the *Ppo-D2b* alleles (GenBank accessions EF070148, EF070150, HQ228148, JN632508, HQ228153). White Vida contained *Ppo-A1a*, *Ppo-D1b*, *Ppo-A2b*, *Ppo-B2c*, and *Ppo-D2b* alleles (GenBank accessions EF070147, EF070150, HQ228149, JN632508, HQ228153). There were three novel alleles found in the low-PPO line 07OR1074. This line contained *Ppo-B2c* and *Ppo-D2b* and a putative null allele at *Ppo-A1* (no PCR fragments for PPO18), as well as new alleles for the *Ppo-A1*, *Ppo-D1* and *Ppo-A2* genes. The 2067 bp, *Ppo-D1* sequence isolated from line 07OR1074 encoding a 577 amino acid polypeptide is nearly identical in sequence and structure to the *Ppo-D1b* allele. Exons I, II, and III contain 596, 261, and 875 bp, respectively, while introns I and II contain 105 and 148 bp, respectively. We propose naming this novel allele *Ppo-D1f* (Genbank accession KJ567059). The nucleic acid sequences of *Ppo-D1b* and *Ppo-D1f* differ by an SNP resulting in a nonsense mutation at position 171 of *Ppo-D1f*. The second novel allele identified is a 1934 bp *Ppo-A2* sequence isolated from line 07OR1074 encoding a 577 amino acid polypeptide. The sequence is identical to the *Ppo-A2c* allele with the exception of an aspartate to glycine substitution at position 400 of the 577 residue polypeptide. Exons I and II contain 557 and 1117 bp, respectively, and a single intron I containing 222 bp. We propose naming this novel allele *Ppo-A2d* (Genbank accession KJ567060).

Creation and inheritance of functional DNA markers

In parental genotype 07OR1074, the PPO18 STS marker fails to amplify the 685 bp product associated with

Table 2 PCR markers for Wheat *Ppo* genes used to identify *Ppo* alleles in hard white spring wheat recombinant inbred populations White Choteau/07OR1074 and White Vida/07OR1074

Marker	Gene	Database accession	Primer sequence		
			Forward (5′–3′)	Reverse (5′–3′)	Product bp
PPO18	*Ppo-A1a*	EF070147	AACTGCTGGCTCTTCTTCCCA	AAGAAGTTGCCCATGTCCGC	685
	Ppo-A1b	EF070148			876
PB5/CAT3.4	*Pinb-D1*	DQ363913	ATGAAGACCTTATTCCTCCTA	GGCACGAATAGAGGCTATATCA	469
PPOD1CAP	*Ppo-D1f*	KJ567059	CCAGAAAAGCAAACACCGGCA	TGTTGGCGTCGGTCCCAC	935
PPOA2d1074	*Ppo-A2d*	KJ567060	CATCATGTACCGCCAGATGG	GCGGTGCTTCACTGGT	878

Ppo-A1a or the 876 bp product associated with *Ppo-A1b*, therefore, the puroindoline-b gene primers PB5 and CAT 3.4 were multiplexed with the PPO18 marker to discriminate a failed PCR from the partial or complete *Ppo* gene deletion associated with the null-*Ppo-A1* allele. The results (Fig. 1) are interpreted as follows: single 469 bp product (homozygous null-*Ppo-A1* allele termed *Ppo-A1i*), a 469 bp product and 685 bp product (*Ppo-A1a*), a 469 bp product and 876 bp product (*Ppo-A1b*), no PCR bands (PCR failed). Using the STS marker PPO29, no *Ppo-D1* polymorphisms were detected between the cultivars White Choteau, White Vida. Therefore, a new functional CAPS marker PPOD1CAP, based on parental *Ppo-D1* sequence dissimilarities was designed to distinguish between the *Ppo-D1f* null allele found in 07OR1074 and the *Ppo-D1b* allele found in White Choteau and White Vida. The CAPS marker PPOD1CAP, produced a 931 bp product that was cut with restriction endonuclease FspBI (Thermo Scientific, Pittsburgh PA, USA) yielding an uncut 931 bp product (*Ppo-D1b*), an uncut 931 bp fragment with a 535 bp and 396 bp product (*PPO-D1b/f* heterozygote), and a 535 bp and 396 bp product (*Ppo-D1f)* (Fig. 2). Expected and observed segregation ratios are presented for each F_4 population in Table 3. Dominant marker PPO18 prevented recognition of *Ppo-A1* heterozygote genotypes in each population. With a few exceptions, the observed F_3 genotypic ratios did not deviate from expected ratios based on χ^2 analysis. The major exception was in finding a reduced number of genotypes homozygous wild-type at the *Ppo-A1* locus in the White Chouteau/07OR1074 population and at the *Ppo-A2* locus in both F_3 populations. This is likely due to selections made at the *Ppo-A2* locus prior to planting in the field in 2013 as described in the materials and methods.

Table 3 Chi-square analysis of observed F_3 segregation ratios of alleles at *Ppo-A1*, *Ppo-D1*, *Ppo-A2*

Population		*N*	Observed ratio	χ^2 test	Pr > F
#07OR1074/White Choteau		127			
Ppo-A1	5:3[a]		68:59	4.35	0.037*
Ppo-A2	5:3		65:62	6.94	0.008*
Ppo-D1	3:2:3[b]		55:21:51	5.02	0.081
#07OR1074/White Vida		129			
Ppo-A1	5:3		73:56	1.92	1.93
Ppo-A2	5:3		63:66	10.27	0.001*
Ppo-D1	3:2:3		44:21:57	5.86	0.053

* Significantly different at $P = 0.05$

[a] 5:3 Genotypic ratio based on a dominant marker giving a 3:1 ratio in the F_2

[b] 3:2:3 Genotypic ratio based on co-dominant marker giving a 1:2:1 ratio in the F_2

Parental line PPO activity and seed traits

Parental line 07OR1074 had substantially lower PPO activity than White Choteau and White Vida in both years (Table 4). White Choteau was higher in grain protein in both years than either White Vida or 07OR1074 and was substantially smaller in kernel weight in 2014.

PPO gene expression analysis

Expression levels for *Ppo* genes were compared for three parents using whole transcriptome sequencing (RNA-seq). No measureable *Ppo-A1* transcripts were detected in line 07OR1074 possessing the null-*Ppo-A1* allele confirming previous reports of a putative null-*Ppo-A1* allele in this genetic background (Table 5). We propose naming this null allele *Ppo-A1i*. Alignments of transcripts to multiple alleles of the same gene within a genetic background are likely due to similar sequences aligning to highly homologous regions within the *Ppo* genes. A nonsense mutation in the *Ppo-D1f* allele was associated with considerable reduction in the number of *Ppo-D1* transcripts in 07OR1074 compared to White Vida and White Choteau. *Ppo-D2b* was expressed in levels comparable to *Ppo-A1* and *Ppo-D1* genes in the cultivar White Vida but was noticeably lower in the cultivar White Choteau and line 07OR1074. Transcripts were detected for *Ppo-A2a* in White Choteau and for *Ppo-A2b* for White Vida, but levels were much lower than for the *Ppo-A1* alleles from these parent lines. *Ppo-B2c* was expressed in all cultivars although the levels detected were lower than the *Ppo-A1* and *Ppo-D1* loci which together have been shown to account for most of the variation in kernel PPO activity.

Impact of novel PPO-A1, PPO-D1 and PPO-A2 alleles on kernel PPO activity

The *Ppo-D1* allelic difference impacted kernel PPO activity for both years ($P < 0.01$) for the White Choteau/OR1074 population (Table 6). The *Ppo-A1* allelic difference reached statistical significance ($P < 0.05$), however, only for 2013, while the *Ppo-A2* locus had no effect on kernel PPO activity in either year. *Ppo-A1*, *Ppo-A2* and *Ppo-D1* allelic status significantly impacted kernel PPO activity ($P < 0.05$) in the White Vida/OR1074 population in both years (Table 7). The allelic difference was greatest for *Ppo-A1*. The *Ppo-A1* and *Ppo-A2* effects are not independent since these loci are closely linked. To better determine the independent contribution of *Ppo-A1* and *Ppo-A2* we examined the effect of substituting the White Choteau or White Vida allele for the OR1074 allele at the *Ppo-A1* or *Ppo-A2* loci while the remaining two *Ppo* loci had the OR1074 allele.

Table 4 Mean kernel PPO activity and kernel traits of parents

Parent	Mean kernel PPO activity, Δ475		Kernel weight, Mg[a]		Kernel diameter, Mm[a]		Grain protein, %[b]	
	2013	2014	2013	2014	2013	2014	2013	2014
White Choteau	0.281	0.207	38.1	32.5	3.07	2.870	16.1	14.6
White Vida	0.351	0.304	36.8	34.0	3.15	2.834	15.7	13.9
07OR1074	0.043	0.011	37.2	41.7	3.21	3.246	15.4	13.3
LSD (0.05)	0.097	0.045	3.8	1.6	0.14	0.062	0.4	0.3

[a] Kernel weight and diameter was determined using the single kernel characterization system (SKCS) on three reps of 200 seeds each

[b] Kernel protein concentration was determined by near-infrared spectroscopy

Table 5 RNA-seq expression analysis of *Ppo* genes in developing kernels 16 dpa from 07OR1074, White Choteau, and White Vida parents

Genbank accession	Gene	070R1074[a]	White Choteau[a]	White Vida[a]	070R1074/White Choteau	070R1074/White Vida
EF070147	*Ppo-A1a*	nd	nd	2812 ± 403	0	0*
EF070148	*Ppo-A1b*	nd	1460 ± 88	Nd	0*	0
EF070150	*Ppo-D1b*	209 ± 36[c]	2347 ± 97	2382 ± 1103	0.090**	0.087
HQ228148	*Ppo-A2a*	nd	206 ± 21	nd	0**	–
HQ228149	*Ppo-A2b*	nd	nd	385 ± 220	–	0
JN632507	*Ppo-A2c*	107 ± 1	nd	nd	–	–
JN632508	*Ppo-B2c*	613 ± 58	335 ± 2	579 ± 268	1.83*	1.06
HQ228153	*Ppo-D2b*	342 ± 138	109 ± 28	2101 ± 99	3.14	0.16
X69913.1	Puroindoline-a (*Pina*)	470,089 ± 39,797	488,032 ± 42,400	208 ± 10	0.96	2260**
AB180737	Puroindoline-b (*Pinb*)	266,432 ± 10403	260,374 ± 36412	132,199 ± 26063	1.02	2.02*
EF592180	Glyceraldehyde-3-phosphate dehydrogenase (GAPC)	12,945 ± 390	13,759 ± 442	14,342 ± 6047	0.94	0.9
AJ292521	Starch Synthase (*SsI-1*)	3208 ± 509	2616 ± 172	2356 ± 780	1.23	1.36
AJ292522	Starch Synthase (*SsI-2*)	4629 ± 375	3129 ± 3	2693 ± 659	1.48*	1.72
AY456122	Cyclophilin A (CYP18-2)	10,107	10,107	10,107	–	–

Significance was determined from a two-tailed students *t* test comparing counts from 07OR1074 and White Chouteau and White Vida *<0.05, **<0.01, ***<0.001

[a] Mean linear counts ± standard deviations from two biological replicates after normalization to Cyclophilin A

[b] Value is the ratio of 07OR1074 transcript counts to White Choteau and White Vida counts

[c] Count unable to distinguish between *Ppo-D1b*b and *Ppo-D1f*

nd Non-detectable

These comparisons show that substituting *Ppo-A2a* from White Choteau or *Ppo-A2b* from White Vida for *Ppo-A2d* had no effect on kernel PPO activity (Tables 6, 7). On the other hand, substituting *Ppo-A1a* from White Vida or *Ppo-A1b* from White Choteau for *Ppo-A1i* increased kernel PPO activity ($P < 0.01$). These comparisons indicate *Ppo-A1* had significant impact on kernel PPO activity, but the impact of *Ppo-A2* was negligible. Allelic class means were not different for the PPO loci for the kernel traits grain protein, kernel weight and kernel diameter (Tables 6 and 7). The *Ppo-A1i Ppo-A2b Ppo-D1f* class mean, however, was lower in kernel weight and diameter than the *Ppo-A1i Ppo-A2d Ppo-D1f* indicating the *Ppo-A2b* allele may be associated with smaller kernels (Table 7).

Discussion

The creation and use of functional or perfect markers in plant breeding have enabled breeding programs to select favorable alleles for a desired trait (Anderson and Lubberstedt 2003). While a functional marker is developed based upon SNPs within a gene, linked markers are subject to recombination between the marker and causative mutation in successive generations rendering them less useful. Functional markers for major *Ppo* genes *Ppo-A1* (Sun et al. 2011) and *Ppo-D1* (He et al. 2007) have enabled breeders to select favorable low-PPO alleles in early generations thus improving the efficiency of selection for traits amenable to noodle quality. However, wheat

Table 6 Means for kernel PPO activity and kernel traits for *Ppo-A1*, *Ppo-D1*, and *Ppo-A2* allelic classes in a hard spring wheat recombinant inbred population segregating for *Ppo-A1, Ppo-D1*, and *Ppo-A2*

White Choteau/07OR1074 *PPO Gene*(s)	No. lines	Kernel PPO activity, Δ475		Grain protein, %		Kernel weight, Mg		Kernel diameter, Mm	
		2013	2014	2013	2014	2013	2014	2013	2014
Ppo-A1b[a]	64	0.148	0.0811	16.5	14.4	38.4	36.7	3.12	3.052
Ppo-A1i	57	0.119	0.0697	16.1	14.3	38.1	37.1	3.13	3.064
P Value[b]		0.05	0.16	0.22	0.34	0.72	0.48	0.84	0.50
Ppo-D1b[c]	51	0.197	0.109	16.2	14.3	37.8	36.6	3.11	3.048
Ppo-D1f	51	0.075	0.042	16.4	14.6	38.7	37.1	3.14	3.107
P value[d]		<0.001	<0.001	0.53	0.42	0.27	0.92	0.314	0.93
Ppo-A2a[d]	53	0.132	0.080	16.0	14.4	38.2	36.9	3.13	3.064
Ppo-A2d	58	0.137	0.072	16.5	14.3	38.4	36.7	3.12	3.051
P value		0.71	0.36	0.12	0.22	0.80	0.58	0.784	0.46
Ppo-A1b Ppo-A2d Ppo-D1f	8	0.225	0.101	17.4	15.4	38.7	39.4	3.13	3.18
Ppo-A1i Ppo-A2d Ppo-D1f	19	0.063	0.007	15.9	14.1	38.3	36.7	3.12	3.05
P value		0.01	0.04	0.11	0.13	0.86	0.28	0.90	0.18
Ppo-A1i Ppo-A2a Ppo-D1f	1	0.072	0.011	15.9	14.4	39.5	38.3	3.17	3.13
Ppo-A1i Ppo-A2d Ppo-D1f	19	0.063	0.007	15.9	14.1	38.3	36.7	3.12	3.05
P value		0.70	0.65	0.83	0.45	0.22	0.11	0.13	0.05
R^2 *Ppo-A1*		0.03	0.02	0.00	0.01	0.00	0.00	0.00	0.00
R^2 *Ppo-D1*		0.46	0.49	0.01	0.01	0.02	0.01	0.02	0.01
R^2 *Ppo-A2*		0.001	0.01	0.00	0.01	0.01	0.00	0.01	0.01
R^2 genotypes		0.51	0.53	0.13	0.12	0.06	0.08	0.06	0.10

[a] *Ppo-A1* was genotyped with the STS marker PPO18

[b] *P* values compare allelic class means

[c] *Ppo-D1* was genotyped using the CAPS marker PPOD1CAP

[d] *Ppo-A2* was genotyped using the STS marker PPOA2d1074

improvement efforts aimed at developing genotypes with minimal levels of PPO activity have been hampered by the lack of known alleles conferring minimal kernel PPO. Nilthong et al. (2012) described populations derived from mating the low-PPO historical Australian white-seeded spring wheat breeding line PI 117635 and the cultivar 'Seaspray' (PI 134049) as containing a putative null genotype at the *Ppo-A1* locus but did not characterize other major *Ppo* loci for potential mutations. Our objective(s) were to characterize *Ppo* mutations present at other *Ppo* loci, create functional markers that could explain the substantial reduction in kernel PPO activity, and to confirm the putative null-*Ppo-A1* locus by examining *Ppo* gene expression. In the present study, we characterized *Ppo-A1*, *Ppo-A2*, *Ppo-D1*, *Ppo-D2*, and *Ppo-B2* by cloning and subsequent sequencing. The cultivars White Choteau and White Vida contained only previously annotated alleles, while the null-PPO line O7OR1074 had novel alleles at two loci, *Ppo-D1f* and *Ppo-A2d*, and the now confirmed null allele *Ppo-A1i*. The *Ppo-D1f* allele contains a single nonsense mutation at position 171, the site of a highly conserved peptide region near the first catalytic site. In contrast to the nonsense mutation found in the *Ppo-D1f* gene, *Ppo-A2d* gene lacks any obvious deleterious mutation. *Ppo-A2d* contains a single aspartate to glycine mutation in the second exon at position 400 of the 577 residue polypeptide. Note that the use of complimentary STS markers PPO16/STS01 and PPO29 which discriminate between *Ppo-D1* alleles associated with high (*Ppo-D1b*) and low (*Ppo-D1a*) PPO activity will give conflicting results in the 07OR1074 genetic background (Onto 2011; Nilthong et al. 2012, 2013). As the *Ppo-D1f* allele is identical in structure and nearly identical in sequence to the *Ppo-D1b* allele with the exception of a single SNP, PPO29 is unable to distinguish the high-PPO allele (*Ppo-D1b*) and null allele (*Ppo-D1f*) explaining erroneous results obtained in prior reports. The CAPS marker, PPOD1CAP was designed to distinguish between the *Ppo-D1f* null allele and the *Ppo-D1b* allele at the *Ppo-D1* locus (Fig. 2). To distinguish low and high alleles (*Ppo-A1b* and *Ppo-A1a*) from null alleles (*Ppo-A1i*) STS marker PPO18 alone cannot be reliably used with populations derived from line 07OR1074 because the 685 or 876 bp fragments

Table 7 Means for kernel PPO activity and kernel traits for *Ppo-A1*, *Ppo-D1*, and *Ppo-A2* allelic classes in a hard spring wheat recombinant inbred population segregating for *Ppo-A1*, *Ppo-D1*, and *Ppo-A2*

White Vida/07OR1074 PPO gene(s)	No. lines	Kernel PPO activity, Δ475		Grain protein, %		Kernel weight, Mg		Kernel diameter, Mm	
		2013	2014	2013	2014	2013	2014	2013	2014
Ppo-A1a[a]	73	0.334	0.224	15.4	13.8	38.2	36.9	3.07	3.03
Ppo-A1i	53	0.139	0.1	15.4	13.9	37.6	36.7	3.08	3.04
P value[b]		<0.001	<0.001	0.73	0.77	0.23	0.59	0.78	0.54
Ppo-D1b[c]	44	0.289	0.197	15.4	13.8	37.4	36.1	3.06	3.01
Ppo-D1f	54	0.202	0.134	15.4	13.9	37.2	37.2	3.05	3.05
P Value		0.02	<0.001	0.75	0.89	0.85	0.83	0.6	0.52
Ppo-A2b[d]	63	0.334	0.223	15.3	13.8	38.0	36.9	3.07	3.02
Ppo-A2d	63	0.166	0.121	15.5	13.9	37.6	36.7	3.08	3.04
P value		<0.001	<0.001	0.17	0.9	0.11	0.58	0.09	0.25
Ppo-A1a Ppo-A2b Ppo-D1f	5	0.215	0.135	15.4	13.7	37.0	35.6	3.04	3.03
Ppo-A1i Ppo-A2d Ppo-D1f	21	0.082	0.050	15.5	13.9	38.2	37.6	3.09	3.08
P value		<0.01	<0.01	0.87	0.50	0.36	0.07	0.27	0.31
Ppo-A1i Ppo-A2b Ppo-D1f	3	0.043	0.017	14.7	13.6	35.1	34.9	2.95	2.92
Ppo-A1i Ppo-A2d Ppo-D1f	21	0.082	0.05	15.5	13.9	38.2	37.6	3.09	3.08
P value		0.42	0.34	0.10	0.22	0.05	0.05	0.01	0.01
R^2 *Ppo-A1*		0.52	0.45	0.00	0.00	0.01	0.00	0.00	0.00
R^2 *Ppo-D1*		0.09	0.13	0.01	0.01	0.01	0.04	0.01	0.03
R^2 *Ppo-A2*		0.39	0.32	0.02	0.02	0.02	0.00	0.00	0.01
R^2 genotypes		0.68	0.67	0.08	0.09	0.11	0.15	0.13	0.14

[a] *PPO-A1* was genotyped with the STS marker PPO18

[b] *P* values compare allelic class means

[c] *Ppo-D1* was genotyped using the CAPS marker PPOD1CAP

[d] *Ppo-A2* was genotyped using the STS marker PPOA2d1074

corresponding to the *Ppo-A1a* allele and *Ppo-A1b* alleles fail to amplify. However, here we multiplexed *Pinb*-specific primers with the PPO18 marker to function as a positive control to ensure adequate DNA quality (Fig. 1). The *Ppo-A2d* sequence isolated from line 07OR1074 is identical in structure and nearly identical in sequence with an exception of a single SNP. The primer combination PPOa21026f and PPOa21904r amplifies an 878 bp fragment encompassing an SNP in the second exon that can be identified by subsequent sequencing.

Whole transcriptome sequencing or RNA-seq was utilized to characterize the expression profile of wheat lines possessing very low (07OR1074), low (White Choteau), and moderate (White Vida) PPO levels. Results of this study confirm that the 07OR1074 genotype possesses a null allele (*Ppo-A1i*) based on no detectable transcripts. Although transcript levels are not a direct measure of the protein they encode, it has been documented that *Ppo* transcript level closely correlates with PPO protein levels (Jukanti et al. 2006). The expression of *Ppo-A1b* in White Choteau is lower than that of *Ppo-A1a* in White Vida (Table 5). The first intron of *Ppo-A1b* has a 191 bp insertion sequence relative to that of *Ppo-A1a* and as a consequence could result in alternative splicing of premature mRNA, therefore, obstructing PPO gene expression (Sun et al. 2011). Transcript levels of *Ppo-D1b* in White Choteau and White Vida were similar, while expression of *Ppo-D1f* was severely down regulated in line 07OR1074. Transcript levels of housekeeping genes (Cyp18-2, GAPDH, APD-GLC PPase ss), starch metabolism genes (SSI-1, SSI-2), and grain hardness genes (*Pinb*) were not significantly different ($P = 0.5$) between very low-PPO line 07OR1074, White Choteau, and White Vida. White Vida carries the *Pina-D1b* allele which is a deletion of the *Pina* coding sequence (Giroux and Morris 1998). With the exception of *Ppo-D2b* in White Vida, transcript levels of the *Ppo-2* genes, *Ppo-A2* and *Ppo-D2* were lower than expected in this study. Beecher and Skinner (2011) using qPCR reported that expression of *Ppo-2* genes contributed on average, 72 % of the *Ppo* gene transcripts over a 37-day after-flowering period and that the total number of *Ppo* transcripts peak at 16 dpa. However, it is important to note that RNA-seq was performed only on developing kernels at a single time point in this study at 16 dpa due to its relatively high cost. In the present study, *Ppo-D2b* expression is up-regulated in the high-PPO parent White Vida while the low-PPO parent

(White Choteau) and very low PPO parent (07OR1074) display a near equal low number of transcripts. *Ppo-D2* was found to be non-polymorphic in our parents. Beecher and Skinner (2011) identified a QTL derived from a 'Louise' and 'Penawawa' cross on chromosome 2D corresponding to the *Ppo-D2* locus which explained 11 % of the phenotypic variation in kernel PPO activity. We hypothesize that if *Ppo-D2b* does indeed contribute to kernel PPO activity then lines 07OR0174 and White Choteau may contain mutations that reside in upstream non-coding regulatory elements. In this study, RNA-seq reveals that *Ppo-B2c* gene was expressed in all parents. These findings conflict with prior reports showing no detectable expression of the *Ppo-B2* gene (Beecher and Skinner 2011). However, the prior study measured the expression of another allele (*Ppo-B2b*) in the cultivar "Alpowa". Previous studies have shown kernel PPO activity is associated with regions on chromosome 2B (Fuerst et al. 2008; Watanabe et al. 2004; Demeke et al. 2001; Beecher et al. 2012) but the effect has been considered minor.

Previous reports have shown that *Ppo-A1* and *Ppo-D1* loci affect kernel PPO activity with *Ppo-A1* having the larger effect. (Beecher and Skinner 2011; Jukanti et al. 2006; Nilthong et al. 2012). Sun et al. (2011) and He et al. (2007) described QTLs on chromosome 2A and 2DL in a double-haploid population that co-segregated with PPO18 and PPO16/PPO29 explaining 28–43 and 9.6–24 % of variation in kernel PPO activity, respectively, across multiple environments. In the White Vida/07OR1074 population, segregating for high (*Ppo-A1a*) and null (*Ppo-A1i*) alleles, the allelic effect of *Ppo-A1* was greater (Table 7) explaining 52 and 45 % of the phenotypic variation in kernel PPO activity compared to the White Choteau/07OR1074 population segregating for the low allele (*Ppo-A1b*) and the null allele (*Ppo-A1i*) (Table 6) where allelic variation at *Ppo-A1* explained just 3 and 2 % of the phenotypic variation in kernel PPO activity in the 2 years. However, the incorporation of the null-*Ppo-A1i* allele in the White Choteau/07OR1074 population had a significant effect on kernel PPO activity in one of 2 years ($P < 0.05$) (Table 6). The allelic effect of *Ppo-D1* was greater (Table 6) in the White Choteau/07OR1074 population, explaining 46 % in 2013 and 49 % in 2014 of the phenotypic variation in kernel PPO activity compared to the White Vida/07OR1074 population (Table 7) where allelic variation at *Ppo-D1* explained just 9 % in 2013 and 13 % in 2014 of the phenotypic variation in kernel PPO activity. Similar results were obtained in Nilthong et al. (2012) and Martin et al. (2011) who demonstrated that a greater reduction in kernel PPO activity is achieved when incorporating a low-PPO allele at *Ppo-D1* locus when the population is fixed for the low-*Ppo-A1b* allele.

Our results suggest that once genotypes are fixed for a low- or null-PPO allele at *Ppo-A1*, kernel PPO activity can be further reduced by incorporating a low- or null-*Ppo-D1* allele. The allelic impact of *Ppo-A2* offers conflicting results when comparing the two populations. The allelic effect of *Ppo-A2* was trivial in the White Choteau/07OR1074 population, explaining no more than 0.1 % of the phenotypic variation in kernel PPO activity in either year compared to the White Vida/07OR1074 population (Table 7) where *Ppo-A2* allelic variation explained 39 and 32 % of kernel PPO activity variation in 2013 and 2014, respectively. A greater amount of kernel PPO variation existed in the White Vida/07OR1074 population segregating for the high (*Ppo-A1a*) and null allele (*Ppo-A1i*) compared to the White Chouteau/OR1074 population. The close linkage between *Ppo-A1* and *Ppo-A2* on chromosome 2A (10 cM) ensures that the wild-type and mutant alleles will be frequently inherited together. The majority of the phenotypic variation in kernel PPO activity explained by the *Ppo-A2* locus is likely confounded by the *Ppo-A1* locus due to linkage. As described in Table 6, substituting a *Ppo-A1a* or *Ppo-A1b* allele for the *Ppo-A1i* allele increased kernel PPO activity, whereas substituting a *Ppo-A2a* or *Ppo-A2b* allele for *Ppo-A2d* had no effect on kernel PPO activity. These results plus low expression levels of *Ppo-A2* compared to *Ppo-A1* (Table 5) indicate *Ppo-A2* contributes far less to kernel PPO activity than does *Ppo-A1*.

For marker-assisted selection to be successful for traits impacting kernel PPO activity, it is important to determine whether loci under selection affect other traits (Tables 6, 7). In the present study, the *Ppo-A1*, *Ppo-D1*, and *Ppo-A2* allelic differences did not impact grain protein, kernel weight or kernel diameter. Kernel weight, size, and protein content showed no correlation with kernel PPO activity (data not shown).

In conclusion, null-PPO alleles *Ppo-A1i* and *Ppo-D1f* characterized in hard white spring wheat line 07OR1074 can be integrated using markers PPO18, PPOD1CAP and PPOA2d1074, into wheat varieties from the hard white, hard red, and soft white spring and winter wheat market classes to allow the direct incorporation of the very low PPO trait into wheat breeding programs. Very low PPO lines were recovered from populations 07OR1074/White Choteau and 07OR1074/White Vida containing the null-*Ppo* alleles, *Ppo-A1i* and *Ppo-D1f*, indicating that selection for null alleles at these two loci is sufficient to allow the development of wheat cultivars having low PPO activity.

Author contribution statement S. M. H, J. M. M, R. A. G. and M. J. G. designed the experiments, S. M. H. carried out the PCR, sequencing, and segregation analysis. S. M. H., J. M. M., R. A. G., and M. J. G. analyzed the data, S. M. H. drafted the manuscript which was edited by all authors.

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Conflict of interest The authors declare no conflict of interest.

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