THE GENETIC CHARACTERIZATION OF A NIL POLYPHENOL OXIDASE (PPO) TRAIT FOR THE IMPROVEMENT OF END PRODUCT QUALITY IN WHEAT (*Triticum aestivum*)

by

Steven Michael Hystad

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Plant Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2014

©COPYRIGHT

by

Steven Michael Hystad

2014

All Rights Reserved

ACKNOWLEDGEMENTS

I would like to acknowledge research associates Andy Hogg and Alanna Schlosser for generously providing instruction and guidance throughout this project. I also thank Petrea Hofer for all her hard work ensuring the viability and success of the crossing required for the success of this project. Members of the cereal quality lab Harvey Tesla, Jackie Kennedy, and Deanna Nash for their guidance and hard work ensuring the success in evaluating end product quality. I would like thank my committee for their guidance and my advisors Dr. John Martin and Dr. Mike Giroux for their extreme patience, support, and insight in ensuring the success in not only the research itself but ultimately the success of myself in future endeavors. Finally, I would like to thank my wife Shawn for her endearing support and patience throughout graduate school.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF TABLES - CONTINUED

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

Wheat (*Triticum aestivum*) polyphenol oxidase (PPO) contributes to the time dependent discoloration of Asian noodles. Wheat contains multiple paralogous and orthologous PPO genes expressed in wheat kernels, *Ppo-A1*, *Ppo-D1*, *Ppo-A2*, *Ppo-D2*, and *Ppo-B2*. To date, wheat 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. Thus, the discovery of novel low or null PPO alleles is critical to further reduce PPO and improve noodle color stability. The objective of this study was to 1) Characterize mutations present in a low PPO line 07OR1074 and create molecular markers associated with newly characterized alleles; 2) assess the allelic impact of the newly characterized alleles on kernel PPO activity and upon Chinese white salted noodle quality. To characterize additional mutations present and develop molecular markers, genomic clones were created for each known PPO gene and sequenced. Previously reported very low PPO line, 07OR1074, was found to contain a novel allele at Ppo-A2 and null alleles at Ppo-A1 and Ppo-D1 loci and molecular markers were created that encompassed each mutation. To determine the allelic impact of the new alleles on kernel PPO activity, $F_{3:4}$ lines were genotyped with the novel molecular markers, grown in the field in Bozeman, Montana, and assayed for kernel PPO activity. Evaluation of lines indicated a substantial genotypic effect on PPO with Ppo-A1 and Ppo-D1 loci contributing significantly ($P < .001$) to total PPO. To determine the effect of the null PPO alleles upon noodle quality Chinese white salted noodles were produced from both refined white flour and whole wheat flour. The results clearly demonstrate that the null PPO alleles at Ppo-A1 and Ppo-D1 loci produce noodles that are brighter (greater L*), more red (greater a*), and more yellow (greater b*) at 24 and 48 hours. These results show that mutations in *Ppo-A1* and *Ppo-D1* genes are important to lowering overall wheat kernel PPO activity producing a more desirable and marketable product.

CHAPTER 1

GENETIC CHARACTERIZATION AND EXPRESSION ANALYSIS OF WHEAT (*Triticum aestivum*) LINE 07OR1074 EXHIBITING VERY LOW POLYPHENOL OXIDASE (PPO) ACTIVITY

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 effort 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 aimed at reducing kernel PPO levels have been hampered by

bread wheat's hexaploid genome and lack of known low PPO 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 consisting of *Ppo-A1* and *Ppo-D1* are localized to the long arm of homeologous chromosomes 2A and 2D respectively (Sun et al. 2005; He et al. 2007;). Allelic variation at both Ppo-A1 and Ppo-D1 loci is associated with variation in kernel PPO activity (Sun et al. 2005; 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* allele) and 876-bp (*Ppo-A1b* allele) fragment corresponding to genotypes associated with high and low PPO activity, respectively (Sun et al. 2005). Complementary STS markers PPO16 and PPO29 amplify a 490-bp 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). Paralogous gene family PPO-2 consisting of *Ppo-A2*, *Ppo-D2*, and *Ppo-B2* genes was identified and mapped to the long arm of homeologous group 2 chromosomes with *Ppo-A2* and *Ppo-D2* localized to within 10cM of their PPO-1 paralogous counterparts (Beecher and Skinner 2011; Beecher et al. 2012). This is in agreement with prior genetic studies demonstrating the locations of major PPO genes are localized to the homeologous group 2 chromosomes (Jimenez and Dubcovsky 1999; Chang et al. 2007; Sun et al. 2005; 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 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 contribute more transcripts and therefore likely more functional PPO protein than PPO transcripts from either the B and D genome combined. This is in accordance with prior works indicating that *Ppo-A1* allelic variation has the greatest effect on kernel PPO activity (Nilthong et al. 2012; Raman et al. 2005; Sun et al. 2005) and upon Chinese raw noodle color (Martin et al. 2011) compared to *Ppo-D1* allelic variation. It should be noted that PPO-1 and PPO-2 orthologous genes are separated by just 10cM and therefore 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 were discovered through a screen of 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 this study were (1) characterize additional mutations present at other PPO loci that account for the substantial reduction of the total PPO activity present in a putative null *Ppo-A1* genetic background; (2) create novel functional markers encompassing mutations found in 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/ 'Seaspray'(PI134049) (Onto 2011), was crossed with hard white spring wheat parents 'White Choteau' ('Choteau' PI 633974/5* 'Clear White' PI365044), and 'White Vida' ('Vida' PI 642366/5* 'Clear White' PI365044) (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. Plants from the resulting F_1 generation were advanced to produce segregating F_2 populations. The F_2 plants were grown in the field in 3m 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 prior to planting in 2013. One hundred forty-four 07OR1074 / White Choteau F_3:F_4 and one hundred forty-three 07OR1074 / White Vida F_3:F_4 lines were planted in single 3m rows at a seeding rate of twenty seeds per row in a randomized block design with two replications at the Arthur H. Post Field Research Center in near Bozeman MT in 2013 under irrigated conditions. Each row was bundled and the grain threshed at maturity. Irrigated conditions consisted of 2-4 cm of water, applied once one week before flowering and one week after flowering.

PPO Gene Cloning and Sequencing

Genomic DNA was isolated from young leaf tissue (Riede and Anderson 1996). Allele specific PCR was conducted for *Ppo-A1* and *Ppo-D1* using the primers presented in Beecher and Skinner (2011). PCR reactions were performed in a total volume of 25µl containing approximately 40 ng of genomic DNA, 1X Green GoTaq® Flexi Buffer, 2mM $MgCl_2$, 0.2mM of each dNTP, 0.4µM of each oligonucleotide primer and 0.03 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, Espoo, Finland). Reactions were performed in a total volume of 30µl consisting of approximately 40 ng of genomic DNA, 1x Phusion GC PCR buffer, 0.2mM of each dNTP, 0.4uM of each oligonucleotide primer, 3% v/v DMSO, and 0.03 unit of Phusion Polymerase. The temperature regime for the allele specific primers consisted of a 5 min initial denaturation step at 98 ^{0}C, followed by forty cycles of 98 ^{0}C for 50 s, 30s at the annealing temperature (Beecher et al. 2012), and a 60s extension step at 72 ^{0}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 ^{0}C, followed by forty cycles of 98 ^{0}C for 50 s, 30s at 50 ^{0}C, and a 2 min extension step at 72 ^{0}C. PCR products were purified using EconoSpin Silica Membrane DNA extraction kit columns (Epoch Life Sciences, 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 Ppo-A1, Ppo-D1, and Ppo-A2 loci respectively to identify alleles conditioning high or low PPO activity. All PCR reactions were performed in a total volume of 25µl 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. 2005). The primer combination PB5 and CAT 3.4 amplifies a 476-bp portion of the *Pinb-D1* gene which was used as an internal control for genomic DNA quality. PPO18, and the *Pinb-D1* multiplex thermocycling program was 98 ^{0}C for 4 min, followed by 40 cycles of touchdown PCR at 98 ^{0}C for 50 s, 58 ^{0}C for 1 min decreasing 0.2^0 C every cycle, 72 ^{0}C for 1 min, and a final extension of 72^0 C for 7 min. The multiplex PPO18/PB5/CAT3.4 combo produces an 876-bp and 476-bp product or 685-bp and 476-bp product corresponding to Ppo-A1b and Ppo-A1a alleles respectively (Figure 1). The presence of a single 476-bp fragment corresponding to the *Pinb-D1* gene denotes the presence of a null Ppo-A1 allele (Figure 1). The CAPS marker PPOD1CAP (Figure 1), produces a 931-bp product that is cut with restriction endonuclease FspBI (ThermoScientiic, Pittsburgh PA, U.S.A.) yielding an uncut 931-bp product (*Ppo-D1b*), an uncut 931-bp fragment with a 535-bp and 396-bp

product (*PPO-D1b/c* heterozygote), and a 535-bp and 396-bp product (*Ppo-D1c).* Thermocycling regime for PPOD1CAP consisted of a 5 min initial denaturation step at 98^0 C, followed by forty cycles of 98^0 C for 50 s, 45s at 62^0 C and 60s 72^0 C, with a final extension at 72^0 C for 7 min. Each restriction digest reaction was performed in a total volume of 25µl containing approximately 10µl of PCR product, 0.2µl of FspBI enzyme, and 2.5µl of 10x Tango buffer. Restriction digests were incubated at 37 ^{0}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 directly sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were read using an Applied Biosystems PRISM 3730xl Genetic Analyzer. All PCR reaction conditions were performed in a total volume of 25µl containing approximately 40 ng of genomic DNA, 1X Green GoTaq® Flexi Buffer, 2mM $MgCl_2$, 0.2mM of each dNTP, 0.4µM of each oligonucleotide primer and 0.03 unit of GoTaq®Flexi DNA Polymerase (Promega, Madison, WI, USA).

Measurement of PPO Activity and Seed Characteristics

Polyphenol oxidase activity for all parents and $F_{3:4}$ progeny lines was measured in 96-well 2mL plates (USA Scientific, Ocala, FL.) 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.

Table 1. 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.

		Primer Sequence		
Marker	Gene	Forward (5'-3')	Reverse (5'-3')	Product-bp
PPO18	*Ppo-A1a* *Ppo-A1b*	AACTGCTGGCTCTTCTTCCCA	AAGAAGTTGCCCATGTCCGC	876
PB5/CAT3.4	*Pinb-D1*	ATGAAGACCTTATTCCTCCTA	GGCACGAATAGAGGCTATATCA	469
PPOD1CAP	*Ppo-D1c*	CCAGAAAAGCAAACACCGGCA	TGTTGGCGTCGGTCCCAC	935
PPOA2d1074	*Ppo-A2d*	CATCATGTACCGCCAGATGG	GCGGTGCTTCACTGGT	935

Each well received 1mL of a solution that was 50mM 3-(N-morpholino) propanesulfonic acid and 10mM 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, U.S.A.) at speed 20 and a rocking angle of 48^0 for 2hr at room temperature. Sample (200µl) 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, U.S.A.). The absorbance value of the blank control was subtracted from the absorbance value of each individual sample. Grain protein concentration was determined by near-infared spectroscopy for whole grain using the infratec 1241 Grain Analyzer (Foss North America, Eden Praire, MN). Kernel

weight and diameter were determined from 100 kernels using the Single Kernel Characterization system (SKCS) (Perten Instruments North America Inc., Springfield, IL) from each field grown replication.

PPO Gene Expression Analysis via RNA-Seq

To analyze expression levels of PPO genes, developing seeds were collected from 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 ^{0}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 2.0mL tube and 0.5mL of RNA extraction buffer (100mM Tris pH 8.0, 150mM LiCl, 50mM EDTA, 1.5% (w/v) SDS, 0.15% (v/v) BME) was added and samples were vortexed until homogenous. Next, 0.25mL 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 a RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.) Total RNA was quantified and its quality assessed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA). 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). Amplicons from cDNA libraries were sequenced as single 100bp reads using an Illumina HiSeq 2000. RNA-Seq data was analyzed using Q-seq in

ArrayStar v5.0 (DNASTAR, Madison, WI.) Genes of interest were elected from the NCBI database for analysis with the match settings in QSeq 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) 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 house keeping 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, data were analyzed via analysis of variance using R (R Core Development Team, 2013). A mixed effects model was used where genotype classes were fixed and replications and entries within genotype class were random effects using the nlme (Pinhero et al. 2013) in R. 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 average of replications using the lm package in R. Segregation ratios were tested using Chi-square tests.

Results

Characterization of Novel Alleles

Due to high sequence similarity among the PPO-2 paralogous gene family, the nonspecific primers POUT5S4 and POUT53A1 (Beecher and Skinner 2011) were used to amplify and clone a composite of the paralogous group PPO-2 genes from each cultivar. The resulting PCR products were examined by sequencing of individual clones. A total of nine different PPO alleles were cloned and sequenced from the cultivars White Choteau, White Vida, and line 07OR1074 (Table 2). Of the nine PPO alleles identified, two were novel, and seven were previously described. The two novel alleles were isolated from the low PPO line 07OR1074. The cultivar White Choteau contained the *Ppo-A1b, Ppo-D1b, Ppo-A2a, Ppo-B2C,* and *Ppo-D2b* alleles (GenBank accessions EF070148, EF070150, HQ228148, JN632508, HQ228153). White Vida contained the *Ppo-A1a, Ppo-D1b, Ppo-A2b, Ppo-B2c,* and *Ppo-D2b* alleles (GenBank accessions EF070147, EF070150, HQ228149, JN632508, HQ228153). The very low PPO line 07OR1074 contained *Ppo-B2c* and *Ppo-D2b* and a putative null allele at the Ppo-A1 loucs (no PCR fragments for PPO18), as well as new alleles for the *Ppo-D1* and *Ppo-A2* genes. The 2,067-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-D1c* (Genbank accession KJ567059). The nucleic acid sequences of *Ppo-D1b* and *Ppo-D1c* differ by a SNP resulting in a nonsense mutation at position 171 of *Ppo-D1c*. Position 171 resides in a highly conserved peptide

region near the first catalytic site. The second novel allele identified is a 1,934-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 1,117-bp respectively and a single intron I containing 222-bp. We propose naming this novel allele *Ppo-A2d* (Genbank accession KJ567060).

Table 2. 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* [b]	*D1b* [b]	*D1c* [d]
Ppo-D2	*D2b*	*D2b*	*D2b*
Ppo-B2	*B2c*	*B2c*	*B2c*

[a] low PPO allele as designated by STS marker PPO18 amplifying an 876-bp fragment.
[b] high PPO allele as designated by STS marker PPO18 amplifying an 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 PPOD1CAPS producing a 535-bp and 396-bp fragments.

Creation and Inheritance of Functional DNA Markers

The PPO18 STS marker fails to amplify the 685-bp product associated with *Ppo-A1a* or the 876-bp product associated with *Ppo-A1b* therefore, the primers PB5 and CAT 3.4 were multiplexed with the PPO18 marker to ascertain a failed PCR reaction from a deletion associated with null *Ppo-A1* allele. The results (Figure 1) are interpreted as follows: single 469-bp product

(homozygous null *Ppo-A1*), 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).

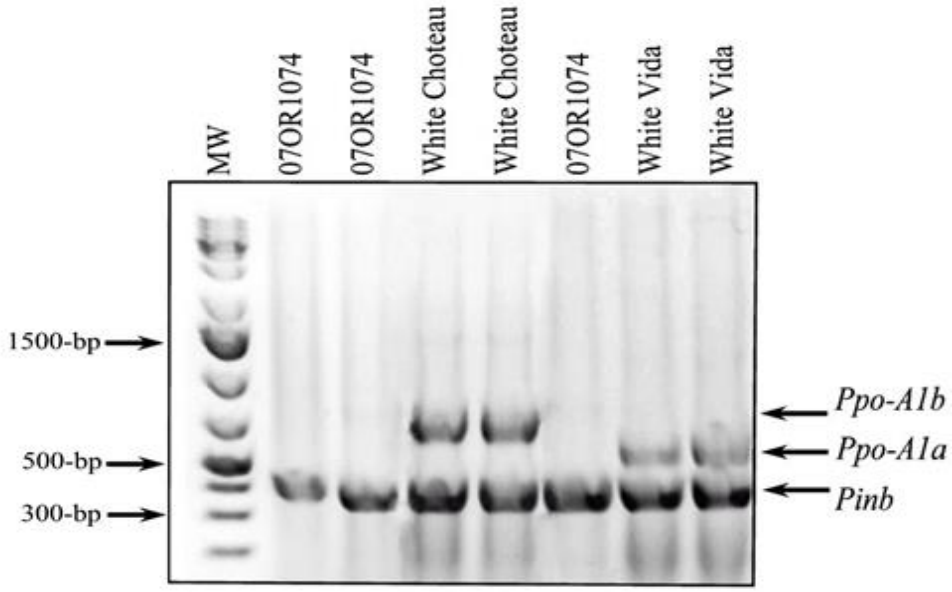

Figure 1. STS marker analysis of lines segregating at the Ppo-A1 locus. Electrophoresis of PCR fragments multiplexed with primer combo PB5 and CAT3.4 and STS marker PPO18 producing an 469-bp product serving as a positive control and 876-bp or 685-bp fragement corresponding to *Ppo-A1b* and *Ppo-A1a*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

Using the STS marker PPO29, the cultivars White Choteau, White Vida, and line 07OR1074 were found to be nonpolymorphic at the *Ppo-D1* locus. A new functional CAPS marker PPOD1CAP, based on parental *Ppo-D1* sequence dissimilarities was designed to distinguish between the *Ppo-D1c* null allele and the *Ppo-D1b* allele. The CAPS marker PPOD1CAP, produces a 931-bp product that is cut with restriction endonuclease FspBI (ThermoScientiic, Pittsburgh PA, U.S.A.) yielding an uncut 931-bp product (*Ppo-D1b*), an uncut 931-bp fragment with a 535-bp and 396-bp product (*PPO-D1b/c* heterozygote), and a 535-bp and 396-bp product (*Ppo-D1c)* (Figure 1**)**. Expected and observed segregation ratios are presented for each F_4 population in Table 3.

Dominant marker PPO18 prevented recognition of heterozygote genotypes at the *Ppo-A1* locus in each population. With a few exceptions, the observed F_3 genotypic ratios did not deviate from expected ratios based on $\chi 2$ 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 the *Ppo-A2* locus in both F_3 populations. This is likely due to heterozygotes observed at the *Ppo-A2* locus being discarded prior to planting in the field in 2013 as described in the materials and methods.

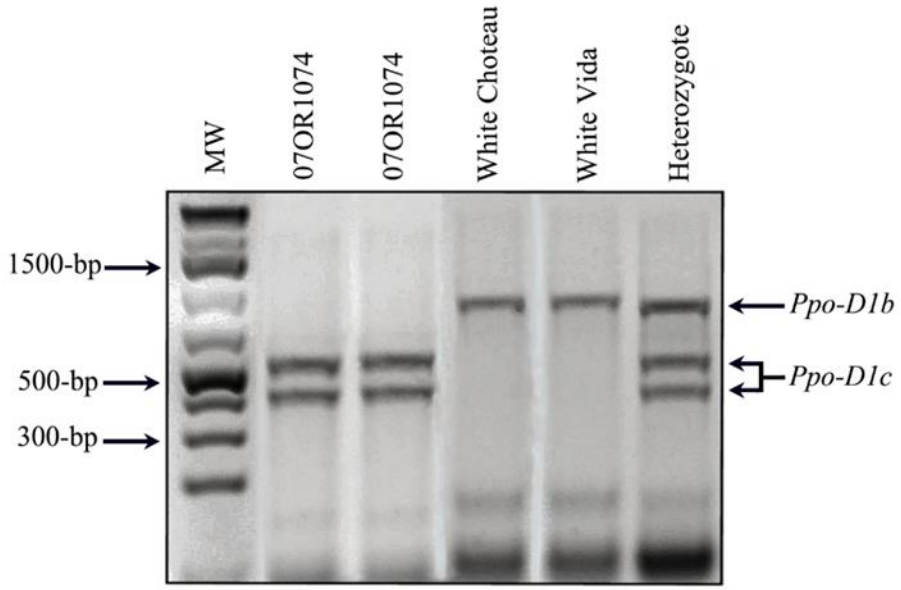

Figure 2. Electrophoresis of PCR fragments amplified with CAPS marker PPO-D1CAP and digested with restriction endonuclease FspBI. The Ppo-D1c allele when cut with FspBI yields a 535-bp and 396-bp product (lanes 01-03 and 08). An uncut 931-bp fragment with a 535-bp and 396-bp product Ppo-D1b/c heterozygote (lane 05). A single uncut 931-bp fragment corresponds to PPO-D1b allele.

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

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

Table 4. Mean kernel PPO activity and kernel traits of parents.

Parent lines	Mean Kernel PPO activity Δ_{475}	Kernel [a] weight mg	Kernel [a] diameter mm	Kernel [b] Protein
White Choteau	0.281	38.14	3.07	16.1
White Vida	0.351	36.82	3.15	15.7
07OR1074	0.043	37.16	3.21	15.4

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

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

Impact of Novel *Ppo-A1*, *Ppo-D1* and *Ppo-A2* Alleles on Kernel PPO Activity

Parental line 07OR1074 had substantially lower mean PPO activity (0.043 Δ_{475}) compared to White Choteau (0.281 Δ_{475}) and White Vida (0.351 Δ_{475}) (Table 4). In each population both Ppo-A1 and Ppo-D1 loci were shown to affect kernel PPO activity ($P < .01$) while the effect of the Ppo-A2 locus was negligible ($P > .05$) (Tables 5,6). The

estimated effect for Ppo-D1 was slightly larger than Ppo-A1 (0.072 Δ_{475} vs 0.057 Δ_{475}) in the White Chouteau/07OR1074 population. However, the effect of Ppo-A1 was larger (0.154 Δ_{475} vs 0.117 Δ_{475}) than that of Ppo-D1 in the White Vida/07OR1074 population (Tables 5, 6). Furthermore, the effect of Ppo-D1 was greater in the White Vida/07OR1074 population (0.117 Δ_{475}) compared to the White Choteau/07OR1074 population (0.072 Δ_{475}). This is likely due to White Vida possessing the high (*Ppo-A1a*) allele resulting in a higher total kernel PPO activity and more total variation in kernel PPO activity compared to White Chouteau which possesses the low (*Ppo-A1b*) allele associated with low PPO activity. In each population, lines possessing the putative null Ppo-A1 and Ppo-D1c alleles exhibit drastically reduced levels of kernel PPO. These findings suggest kernel PPO activity is controlled by Ppo-A1 and loci and the *Ppo-A2* locus does not have a major effect.

PPO Gene Expression Analysis

Expression levels for PPO genes were compared for the three parents using whole transcriptome sequencing (RNAseq). 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 7). 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-D1c* allele was associated with considerable reduction in the number of *Ppo-D1* transcripts in 07OR1074 compared to White Vida and White Choteau,

Table 5. Means for kernel PPO activity, kernel protein and kernel diameter 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)**		**Kernel PPO activity Δ_{475}**	**Kernel protein**	**Kernel diameter mm**	**Kernel weight mg**
Ppo-A1a		0.176	16.5	3.12	38.4
Ppo-A1i		0.119	16.1	3.13	38.1
P value [c]		0.009	0.22	0.84	0.72
Ppo-D1b		0.184	16.2	3.11	37.8
Ppo-D1c		0.111	16.4	3.14	38.7
P value [d]		<.001	0.53	0.314	0.27
Ppo-A2b		0.127	16.0	3.13	38.2
Ppo-A2d		0.168	16.5	3.12	38.4
P value [e]		0.07	0.12	0.784	0.80
PPO loci interaction					
Ppo-A1 x *Ppo-D1*	*P* value [f]	0.11	0.11	0.89	0.54
Ppo-A1 x *Ppo-A2*	*P* value [g]	0.35	0.24	0.97	0.91
Ppo-D1 x *Ppo-A2*	*P* value [h]	0.24	0.54	0.40	0.32
R^2 *Ppo-A1*		0.03	-	-	-
R^2 *Ppo-D1*		0.46	-	-	-
R^2 *Ppo-A2*		0.001	-	-	-
R^2 Genotypes		0.51	-	-	-

PPO-A1 was genotyped with the STS marker PPO18.
[a] Ppo-D1 was genotyped using the CAPS marker PPOD1CAP.
[b] Ppo-A2 was genotyped using the STS marker PPOA2d1074.
[c] *P* value for comparison of *Ppo-A1b* vs *Ppo-A1c* class means.
[d] *P* value for comparison of *Ppo-D1b* vs *Ppo-D1c* class means.
[e] *P* value for comparison of *Ppo-A2c* vs *Ppo-A2d* class means.
[f] *P* value for *Ppo-A1* x *Ppo-D1* interaction.
[g] *P* value for *Ppo-A1* x *Ppo-A2* interaction.
[h] *P* value for *Ppo-A1* x *Ppo-D1* interaction.

Table 6. Means for kernel PPO activity, kernel protein and kernel diameter 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)**		**Kernel PPO activity** Δ_{475}	**Kernel protein**	**Kernel diameter mg**	**Kernel weight mg**
Ppo-A1a		0.302	15.4	3.07	37.6
Ppo-A1i		0.148	15.0	3.04	37.0
P value [c]		<.00001	0.13	0.44	0.483
Ppo-D1b		0.283	15.2	3.06	37.4
Ppo-D1c		0.166	15.3	3.05	37.2
P value [d]		<.00001	0.87	0.60	0.85
Ppo-A2b		0.249	14.9	3.03	37.2
Ppo-A2d		0.2004	15.5	3.07	37.5
P value [e]		0.071	0.014	0.79	0.74
PPO loci interaction					
Ppo-A1 x *Ppo-D1*	*P* value [f]	0.046	0.78	0.46	0.56
Ppo-A1 x *Ppo-A2*	*P* value [g]	0.070	0.47	0.06	0.09
Ppo-D1 x *Ppo-A2*	*P* value [h]	0.611	0.31	0.80	0.61
R^2 *Ppo-A1*		0.517	-	-	-
R^2 *Ppo-D1*		0.091	-	-	-
R^2 *Ppo-A2*		0.360	-	-	-
R^2 Genotypes		0.677	-	-	-

PPO-A1 was genotyped with the STS marker PPO18.
[a] Ppo-D1 was genotyped using the CAPS marker PPOD1CAP.
[b] Ppo-A2 was genotyped using the STS marker PPOA2d1074.
[c] *P* value for comparison of *Ppo-A1b* vs *Ppo-A1c* class means.
[d] *P* value for comparison of *Ppo-D1b* vs *Ppo-D1c* class means.
[e] *P* value for comparison of *Ppo-A2c* vs *Ppo-A2d* class means.
[f] *P* value for *Ppo-A1* x *Ppo-D1* interaction.
[g] *P* value for *Ppo-A1* x *Ppo-A2* interaction.
[h] *P* value for *Ppo-A1* x *Ppo-D1* interaction.

Ppo-D2b was shown to be 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. *Ppo-B2c* was shown to be 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.

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 across varying genetic backgrounds (Anderson and Lubberstedt 2003). While a functional marker is developed based upon SNPs within a gene, linked markers are subjected to recombination between the marker and causative mutation in successive generations among breeding populations rendering them less useful. Functional markers for major PPO genes *Ppo-A1* (Sun et al. 2005) and *Ppo-D1* (He et al. 2007) have enabled breeders to select favorable low PPO alleles in early generations thus improving the efficiency at which breeders select for traits amenable to noodle quality. However wheat improvement efforts aimed at developing genotypes with minimal levels of PPO activity have been hampered by the lack of known mutant alleles conferring minimal or no kernel PPO activity. Nilthong et al. (2012) described populations derived from mating the low PPO 1930's Australian white-seeded spring wheat cultivars PI117635 (NSGC received 1936 cultivar release)/ 'Seaspray' (PI134049) as containing a putative null genotype at the Ppo-A1 locus but did not characterize other major PPO loci for potential mutations.

Table 7
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[b] Choteau**	**070R1074/White[b] Vida**
EF070147	Ppo-A1a	nd	nd	2812 ± 403	0	0*
EF070148	Ppo-A1b	nd	1460 ± 88	nd	0*	0
EF070150	Ppo-D1b	209 ± 36	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
AY456122	Cyclophilin A	10107	10107	10107		

[a] Mean linear counts ± standard deviations from two biological replicates after normalization to Cyclophilin A
[b] Value is the ratio of 07OR1074 transcript counts over White Choteau and White Vida counts *(P <0.05), **(P <0.01)
(nd) non detectable

In the present study, we have 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 *Ppo-D1c* and *Ppo-A2d,* and now confirmed null allele *Ppo-A1i*. The *Ppo-D1c* allele contains a single nonsense mutation at position 171, the site of a highly conserved peptide region near the first catalytic site. The resulting mutation at position 171 results in a stop codon which seemingly prevents the copper binding site necessary for enzyme catalysis from being translated, effectively resulting in a nonfunctional protein. In contrast to the detrimental mutation found in the *Ppo-D1c* gene, the *Ppo-A2d* gene lacks any obvious deleterious mutation. Ppo-A2d contains a single mutation in the second exon at position 400 of the 577 residue polypeptide resulting in aspartate to glycine substitution which may or may not impact protein function. Note that the use of complimentary STS markers PPO16/STS01 and PPO29 which discriminates between *Ppo-D1* alleles associated with high (*Ppo-D1b*) and low (*Ppo-D1a*) PPO activity, cannot distinguish the high PPO allele (*Ppo-D1b*) and null allele (*Ppo-D1c)* in the 07OR1074 genetic background, as the *Ppo-D1c* allele is identical in structure and nearly identical in sequence to the *Ppo-D1b* allele with the exception of a single SNP (Onto 2011; Nilthong et al. 2012; Nilthong et al. 2013). The CAPS marker, PPOD1CAP was designed to distinguish between the *Ppo-D1c* null allele and the *Ppo-D1b* allele at Ppo-D1 locus. The single C/G SNP at 538bp of the *Ppo-D1c* gene is the recognition site for restriction endonuclease FspBI yielding a 535-bp and 396-bp product (Figure 1**)**.To distinguish low and high alleles (*Ppo-A1b* and *Ppo-A1a*) from null alleles (*Ppo-A1i*) STS

marker PPO18 alone cannot be used with populations derived from line 07OR1074 because the 685-bp or 876-bp fragments corresponding to the *Ppo-A1a* allele and *Ppo-A1b* alleles fail to amplify. The primers PB5 and CAT 3.4 which amplify a 469-bp product corresponding to the *Pinb* gene which influences grain hardness (Swan et al. 2006). Primers PB5 and CAT 3.4 were multiplexed with the PPO18 marker to function as a positive control to ensure adequate DNA quality (Figure 2**)**. The *Ppo-A2d* sequence isolated from line 07OR1074 is identical in structure and nearly identical in sequence to previously annotated alleles Ppo-A2a, Ppo-A2b, and Ppo-A2c with an exception of a single SNP (Beecher and Skinner 2011; Beecher et al. 2012). The primer combination PPOa21026f and PPOa21904r amplifies an 878-bp fragment encompassing a SNP in the second exon that can be identified by subsequent sequencing.

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. (2005) and He et al. (2007) described QTLs on chromosome 2A and 2DL in a double haploid population derived from Zhongyou9507/CA9632 that co-segregated with PPO18 and PPO16/PPO29 explaining 28 to 43% and 9.6 to 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 6) explaining 51% 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 5) where allelic variation at *Ppo-A1* explained just 3.1% of the

phenotypic variation in kernel PPO activity. The small proportion of phenotypic variation explained by the *Ppo-A1* locus in the White Choteau/07OR1074 population suggests minimal variation exists at the *Ppo-A1* locus between a low allele (*Ppo-A1b* and null allele (*Ppo-A1i*). However, the incorporation of the null *Ppo-A1i* allele in the White Choteau/07OR1074 population has a significant effect (*P*-value =.009) on kernel PPO activity (Table 5). The allelic effect of *Ppo-D1* was greater (Table 5) in the White Choteau/07OR1074 population, explaining 46% of the phenotypic variation in kernel PPO activity compared to the White Vida/07OR1074 population (Table 6) where allelic variation at *Ppo-D1* explained simply 9% of the phenotypic variation in kernel PPO activity. Similar results were obtained in Nilthong et al. (2012) and Martin et al. (2011) demonstrating 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 just 0.1% of the phenotypic variation in kernel PPO activity compared to the White Vida/07OR1074 population (Table 6) where allelic variation at *Ppo-A2* explained 36% of the phenotypic variation in kernel PPO activity. A greater amount of kernel PPO variation exists 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 proximity between Ppo-A1 and Ppo-A2

on chromosome 2A (10cM) ensues 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. It is important to note that difference in kernel PPO levels is not significant (P=.07) when comparing genotypic classes at the *Ppo-A2* locus. Furthermore, the increase in kernel PPO activity observed when comparing *Ppo-A2* allelic class means in the White Choteau/07OR1074 population (Table 5) likely represents random sampling variation due to the small number of lines obtained that differed in alleles at Ppo-A1 and Ppo-A2 loci. Beecher et al. (2012) mapped the PPO-2 genes (*Ppo-A2* and *Ppo-D2*) within a 10cM region to the PPO-1 genes (*Ppo-A1* and *Ppo-D1*) on chromosomes 2A and 2D respectively. Due to the close proximity of *Ppo-A1* and *Ppo-A2* a small number of recombinants were obtained in which to evaluate the allelic impact of *Ppo-A2* in this study. For marker-assisted selection to be successful for traits impacting kernel PPO activity, it is important to understand if any pleiotropic effects or negative traits exist due to linkage. In the present study the Ppo-A1, Ppo-D1, and Ppo-A2 loci exhibited no significant interaction. Kernel weight, size and protein content showed no correlation with kernel PPO activity (data not shown). With the exception of kernels exhibiting higher protein content in genotypic classes containing the *Ppo-A2d* allele in the 07OR1074/White Vida population, kernel protein and diameter did not differ significantly (P = 0.05) across genotypic groups(Tables 5,6). The three parents did not differ significantly for kernel weight, protein, and diameter (Table 4).

Whole transcriptome sequencing or RNA-seq was utilized to characterize the expression profile of wheat lines possessing very low (07OR1074 $0.043\Delta_{475}$), low (White Choteau $0.281\Delta_{475}$), and moderate (White Vida $0.351\Delta_{475}$) PPO levels. Results of this study confirm that the 07OR1074 genotype possesse a null allele (*Ppo-A1i*) based on no detectable transcripts observed. It is important to note that although transcript levels are not always a direct measure of the protein they encode, it has been documented that PPO transcript levels closely correlate 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 7). 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. 2005). Transcript levels of *Ppo-D1b* in White Choteau and White Vida were similar, while expression of *Ppo-D1c* was severely down regulated in line 07OR1074. We can expect no PPO protein encoded by *Ppo-D1i* allele to be present in developing kernels due to a nonsense mutation in the first exon of the gene; however one would not expect a single mutation in the coding region to have an effect on gene expression. Additional mutations may be present in line 07OR1074 upstream of the PPO gene coding region in conserved non-coding regulatory elements and could explain the severe down regulation of *Ppo-D1c*. 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 period and that the total number of PPO transcripts peak at 16dpa.

However, it is important to note that RNA-seq was performed only on developing kernels at a single time point in this study at 16dpa due to its relatively high cost. Beecher and Skinner (2011) showed expression of *Ppo-A1* and *Ppo-D2* to peak at 16dpa with *Ppo-A1* and *Ppo-A2* genes expressed the greatest and displayed nearly equal numbers of copies of transcripts levels. In the present study expression of the *Ppo-D2b* gene is up regulated in the high PPO parent White Vida while the low PPO parent (White Choteau) and very low PPO parent (07OR1074) display near equally low number of transcripts. Ppo-D2 was found to be non-polymorphic in our parents. Beecher and Skinner (2011) identified a major QTL derived from a 'Louise' and 'Penewawa' $F_{5:6}$ RIL population 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 copy numbers 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" and thus those prior results may not directly transfer to the genetic materials used in this study. 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 affect has been considered minor.

In conclusion, null PPO alleles *Ppo-A1i* and *Ppo-D1c* characterized in hard white spring wheat line 07OR1074 can be integrated using markers PPO18, PPOD1CAP and

PPOA2d1074, into wheat varieties to allow the direct incorporation of the very low PPO trait into wheat breeding programs. Lines recovered from population's 07OR1074/White Choteau and 07OR1074/White Vida containing the null PPO alleles *Ppo-A1i* and *Ppo-D1c* exhibited very low PPO activity suggesting selection for null alleles at these two loci is sufficient to allow the development of wheat cultivars conferring superior end use noodle quality. RNA-seq on developing kernels at 16dpa reveal transcript levels of *Ppo-A1i* and *Ppo-D1c* were severely down regulated in line 07OR1074. Low transcript levels of PPO-2 genes, *Ppo-A2* and *Ppo-D2* suggest these genes may be minor contributors to kernel PPO activity conflicting with prior results of Beecher and Skinner (2011), however the expression of *Ppo-D2* was upregulated in the high PPO parent White Vida suggesting this locus may be a large contributor to kernel PPO activity and that additional uncharacterized mutations affecting gene expression of *Ppo-D2* in line 07OR1074 and White Choteau may be present. While this is the first study to confirm expression of the PPO-B2 gene in developing kernels of bread wheat, its role in contributing to overall kernel PPO activity is not yet certain.

CHAPTER 2

THE IMPACT OF A VERY LOW POLYPHENOL OXIDASE TRAIT UPON END PRODUCT QUALITY

Introduction

Fresh noodles prepared from wheat (*Triticum aestivum*) are widely consumed throughout the world and are particularly an important food staple consumed throughout the countries of Eastern Asia. An interminable variety of noodles, produced across geographic regions and cultures may each have its own unique attributes, but it is generally accepted that noodles must possess desirable color traits such as a bright, lustrous, mottled free appearance (Hou 2010). The overall darkening of oriental noodles, particularly white-salted and yellow alkaline noodles is considered undesirable and often perceived as spoiled by consumers. Polyphenol oxidase (PPO) is largely responsible for the enzymatic discoloration and general darkening of food products. Polyphenol oxidase enzymes (PPO) oxidize phenolic compounds resulting in the formation of dark melanin pigments (Matheis and Whitaker 1984; Whitaker and Lee 1995). Noodle discoloration is especially problematic for whole grain products as PPO enzymes and their phenolic substrates have been shown to be localized to the bran (Rani et al. 2006; Okot-Kotber et al. 2001), specifically the aleurone layer of the kernel (Jerkovic et al. 2010) and therefore flour PPO activity and noodle discoloration intensifies with increasing flour extraction rate and amount of fiber fortification (Baik et al. 1994; Hatcher and Kruger 1993). The renewed interest by consumers in the potential health benefits of increasing their intake

of whole-grain foods relative to refined-grained products has prompted producers to explore the possibilities of increasing dietary fiber content in various forms of foods made from cereal grains. An increased dietary intake of whole-grain foods is associated with a reduced risk of coronary heart disease (Jacobs et al. 1998; Jensen et al. 2004), type II diabetes (Meyer et al. 2000; Salmeron et al. 1997; Ventura et al. 2009), some colorectal cancers (Slavin et al. 2000) and lower rates of obesity (Mckeown et al. 2010; Saltzmen et al. 2001). However, taste and textural properties of whole grain noodles are not as favored and their color profile is often perceived as less attractive compared to their refined white flour counterparts. Improvements in textural, taste, and color properties is necessary to better consumer acceptability of whole-grain noodles.

Plant breeding efforts have focused on incorporating novel PPO alleles that contain mutations that effectively reduce plant PPO levels 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). Martin et al. (2011) showed that low PPO alleles at PPO loci, Ppo-A1 and Ppo-D1 produced brighter (greater L*), more yellow (greater b^*), and more red (greater a^*) after a period of 24 hours. Hence, minimizing kernel PPO levels can lead to an improved and more consumer accepted color profile. PPO content of wheat kernels has been shown to be influenced by genotype and the environment (Park et al. 1997). 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 consisting of *Ppo-A1* and *Ppo-D1* are localized to the long arm of homeologous chromosomes 2A and 2D respectively (Sun et

al. 2005; He et al. 2007;). Paralogous gene family PPO-2 consisting of the *Ppo-A2, Ppo-D2,* and *Ppo-B2* genes was identified and mapped to the long arm of homeologous group 2 chromosomes with Ppo-A2 and Ppo-D2 localized to within 10cM of their PPO-1 paralogous counterparts (Beecher and Skinner 2011; Beecher et al. 2012). This is in agreement with prior genetic studies demonstrating the locations of major PPO genes are localized to the homeologous group 2 chromosomes (Jimenez and Dubcovsky 1999; Chang et al. 2007; Sun et al. 2005; He et al. 2007; Demeke et al. 2001; Zhang et al. 2005). Beecher and Skinner (2011) reported that *Ppo-A2* and *Ppo-D2* of paralogous gene family PPO-2 were shown to account for over 72% of transcripts in developing kernels in the cultivar 'Alpowa', while transcripts of *Ppo-B2* were not detected, indicating that paralogous family PPO-2 may be essential in controlling kernel PPO gene expression. Nilthong et al. (2012) described wheat lines discovered through a screen of national Small Grains Collection as possessing a putative null genotype at the *Ppo-A1* locus. Recently null PPO alleles *Ppo-A1i* and *Ppo-D1c* were characterized and markers PPOA2d1074 and PPOD1CAP were developed to identify null alleles conditioning very low kernel PPO levels. In this very low PPO genetic background, allelic variation at both Ppo-A1 and Ppo-D1 loci was associated with kernel PPO activity. Allelic variation at the Ppo-A1 locus was shown to have greatest effect on kernel PPO activity compared to allelic variation at the *Ppo-D1* locus. This is in accordance with prior works (Sun et al. 2005; Raman et al. 2005; He et al. 2007; Wang et al. 2009; Martin et al. 2011; Nilthong et al. 2012). However, it should be noted that allelic variation at *Ppo-A2* had minimal effect on kernel PPO activity and that expression of *Ppo-D2* and *Ppo-B2* was negligible

relative to the paralogous group PPO-1 gene family suggesting that the paralogous group PPO-2 gene family has a minimal effect in this genetic background. Although the mutations resulting in null PPO alleles at the Ppo-A1 and Ppo-D1 loci conferred very low levels of kernel PPO activity as measured by (L-3-[dihydroxyphenyl] alanine) (L-DOPA) solution, their effect upon end product quality has yet to be determined.

The goals of this study were (1) characterize the effect of the null PPO alleles *Ppo-A1i* and *Ppo-D1c* on the color and texture profile of Chinese raw (white salted) noodles prepared from refined white and whole wheat flour, (2) Assess the impact of the newly characterized alleles on kernel and flour characteristics; (3) examine the relationship between noodle color over time for lines segregating for the null PPO alleles *Ppo-A1i* and *Ppo-D1c.*

Materials and Methods

Evaluation of Genetic Materials

Hard white spring line 07OR1074 derived from mating Australian white-seeded spring wheat genotypes PI 117635/ 'Seaspray'(PI134049) (Onto 2011), was crossed with hard white spring wheat parents 'White Choteau' ('Choteau' PI 633974/5* 'Clear White' PI365044), and 'White Vida' ('Vida' PI 642366/5* 'Clear White' PI365044) (Talbert et al. 2013) to develop populations to assess the newly characterized null PPO alleles on Chinese raw noodle color profile. Initial crosses were grown in the greenhouse in 2011. Plants from the resulting F_1 generation were advanced to produce segregating F_2 populations. The F_2 plants were grown in the field in 3m 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 null and low PPO alleles at the Ppo-D1, Ppo-A1, and Ppo-A2 loci using sequence tagged site (STS) markers PPOA2d1074, PPO18 and PB5, CAT3.4 combo, and cleavage amplified polymorphic sequence (CAPS) marker PPOD1CAP (Sun et al. 2005) One hundred forty-four 07OR1074 / White Choteau F_3:F_4 and one hundred forty-three 07OR1074 / White Vida lines were planted in single 3m rows at a seeding rate of twenty seeds per row in a randomized block design with two replications and four check entries at the Arthur H. Post Field Research Center in near Bozeman MT in 2013 under irrigated conditions consisting of 2-4 cm of water applied once one week prior to flowering and one week after flowering. The check entries were 'Choteau' (PI 633974), ('Vida' PI 642366), 'Glenn' (PI 639273), and 'Mountrail' (PI 607540). Plants from each row was bundled and the grain threshed at maturity. Lines homozygous for the 07OR1074 *Ppo-A1i* and *Ppo-D1c* parental alleles and sister lines containing parental alleles at the Ppo-A1 and Ppo-D1 locus from White Choteau and White Vida were selected for end product quality testing within each population. Due to the lack of available seed after harvesting, 150 g of seed from two individual recombinant inbred lines within each genotypic class were combined according to grain protein content to create sample composites prior to milling.

Evaluation of Kernel Parameters

Kernel weight and diameter were determined from 100 kernels using the Single Kernel Characterization System (SKCS) (Perten Instruments North America Inc., Springfield, IL) from each field grown replication. Grain protein was measured using near-infared spectroscopy for whole grain using the infratec 1241 Grain Analyzer (Foss North America, Eden Praire, NM). Kernel weight and diameter were determined from 100 kernels using the Single Kernel Characterization system (SKCS) (Perten Instruments North America Inc., Springfield, IL) from each field grown replication. Kernel PPO activity for all parents and breeding lines was measured according to the methods described in Anderson and Morris (2001). Kernel protein, weight, diameter, and PPO activity reported here is the average for each milling composite consisting of two individual recombinant inbred lines.

Preparation of Refined
White and Whole Wheat Flour

Wheat was milled on a Brabender Automat Quadrumat Junior Mill (South Hackensack, NJ) after a single stage temper to 15.0% moisture with 203-µm sieve openings. Bran and flour were weighed individually and flour yield was calculated as the proportion of flour to total products. Bran fractions were further milled on a Blentec® Kitchen Mill™ (West Orem, UT) to reduce particle size and sifted using 425µm US standard sifting screen (Seedburo Equipment, Chicago, IL) on a rotating sifter (Ro-Tap RX-29, Leval Lab, Quebec, Canada). Less than 10% of the bran larger than 425µm remained following sifting. The correct proportion of bran fractions less than 424µm

were added back to white flour based on the individual sample flour yield calculations to create whole wheat flour. Flour protein was determined with an Infratec 1241 Grain Analyzer with flour NIR attachment (Foss North America, Eden Prairie, NM) and expressed at a 14.0% moisture basis. Whole-wheat flour moisture was determined by AACC method 44-15A in which 2-3grams of flour is placed in an aluminum dish and placed in a mechanical convection oven at 130 C^0 for 1 hour, then allowed to cool in a desiccator & weighed to calculate % moisture. Flour and bran PPO activity was measured from a 400mg sample with 4 replicates from parent lines and each composite. Flour and bran were deposited into 2 ml tubes and filled with 1mL the L-Dopa solution mentioned above Controls were a blank well filled only with 1mL of (L-3-[dihydroxyphenyl] alanine) (L-DOPA) solution described by Anderson and Morris (2001). Tubes were mixed on a Vari-Mix platform rocker (ThermoFisher Scientific, Waltham, MA, U.S.A.) at speed 20 and a rocking angle of 48^0 for 1hr at room temperature. Tubes were then centrifuged at 13,000 min^{-1} for 10 minutes and 500ul of the supernatant was transferred to an Econospin™ silica membrane mini-spin columns (Epoch Life Sciences, Missouri City, TX.) to further remove flour or bran particles. 200ul of flow-through was then transferred to a flat-bottom, 96-well clear microtiter plate (ThermoFisher) and absorbance (A_{475}) was measured on a spectrophotometer (Molecular Devices, Sunnyvale, CA.) The absorbance value of the blank control was subtracted from the absorbance value of each individual sample.

Preparation of Chinese Fresh Noodles

Chinese fresh (white salted) noodles were prepared using 100 g straight grade flour (140 g kg−1 moisture basis, fwb) and 29.2 ml salt (NaCl) water solution (4.29% w/v) added to the flour during a 30 s time period. Doughs were mixed in a Finney Special mixer (100 g Micro Dough pin mixer, head speed 102 rev/min (National Manufacturing Co., TMCO, Inc., Lincoln NE)) for 5 min and 45 s. Flour adhering to the inside of the mixing bowl and pins was brushed down and premixed for 15 s prior to adding the salt–water solution. The salt–water solution was slowly added so that the last drop was added 30 s after the initial drop was dispensed to insure uniform hydration and complete incorporation. After 30 s of additional mixing, the mixer was stopped to clean dough off the pins and to break up any large lumps of dough. This step was repeated after an additional 1 min and 30 s of mixing followed by a final 3 min of mixing. The crumbly dough was pressed by hand into a cohesive rectangular block then placed in a plastic bag to rest for 30 min at room temperature. The dough block was then passed through a laboratory noodle machine (Otake Noodle Machine Manufacturing Co., Ltd., Tokyo Japan) with an initial gap of 3 mm. The dough was then book-folded once then passed through a 5 mm gap three times and placed in a plastic bag for 30 min at room temperature. The sheet was progressively reduced by sheeting the dough through the following gaps; 4 mm, 3 mm, 2 mm, and 1.5 mm. Two pieces of sheeted dough (7.6cm x 7.6 cm) were reserved for color measurements. The final sheeting thickness was 1.2 mm which was checked in five places using a Pocket Dial Gauge 1010MZ (L. S. Starrett Company Athol, MA). The rectangular dough sheet was then cut into 2.5 mm wide

noodles and stored in a plastic bag for 24 h before cooking and texture evaluation. Dough sheet color measurements were taken on each side of the two reserved dough sheets at 0, 12, 24, and 48 h after sheeting with a Minolta CR-310 Chroma Meter (Minolta, Ramsey, NJ). The Minolta Chroma Meter uses the Commission Internationale de l'Eclairage (CIE) color system and was used to measure L* (brightness) a* (green-red) b* (blue-yellow). More positive values of L*, a* and b* indicate increasing white, red and yellow, respectively. Noodles were cooked by adding 50 g of noodles to 500 ml of boiling distilled water for 5 min. After 5 min of boiling, noodles were removed from the heat. Noodles were transferred to a thermal cup, and maintained in a portion of the steep water for 5 min. Noodles were transferred to a basket strainer then rinsed in a water bath at room temperature with 10 s of agitation using chopsticks. The strainer was removed from the water and tapped 10 times to remove excess water from the noodles before texture analysis. Texture profile analysis (springiness, cohesiveness, adhesiveness, hardness and chewiness) was performed immediately on five strands of rinsed noodles using a TA-XT2 Texture Analyzer and Texture Exponent software (Texture Technologies Corp., Scarsdale, NY) then averaged to obtain texture readings. The method for obtaining the texture measurements was the same as that described by Epstein et al. (2002). Whole-wheat Chinese noodles were prepared according to the methods above with the exception that and 31.0 ml salt (NaCl) water solution was added to the whole-wheat flour prior to mixing.

Statistical Analysis

To evaluate differences in kernel, milling, color and texture profiles among the parents, the traits were analyzed via analysis of variance using R (R Core Development Team, 2013). Fisher's least significant difference (LSD) test values were calculated using agricolae package (Mendiburu 2014) in R. As correlation coefficiants did not vary among populations, White Choteau/ 07OR1074 and White Vida / 07OR1074 populations were combined when calculating linear correlations between PPO activity and color traits.

Results

PPO Alleles Characterized in Hard White Spring Wheat Parents

Both cultivars White Choteau and White Vida contained the *Ppo-D1b* allele associated with high PPO activity (GenBank accessions EF070150) as designated by cleavage amplified polymorphic sequence (CAPs) marker PPOD1CAPS. When genotyped with the PPO18 STS marker, White Vida was found to contain the high PPO allele, *Ppo-A1a* while White Choteau was found to contain the low PPO allele *Ppo-A1b* (Table 8). The very low PPO line 07OR1074 contains a null allele at the *Ppo-A1* locus (no PCR fragments for PPO18) and the novel allele *Ppo-D1c*. The nucleic acid sequences of *Ppo-D1b* and *Ppo-D1c* differ by a SNP resulting in a nonsense mutation at position 171 of *Ppo-D1c* effectively preventing translation of a peptide product.

Table 8. 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-D1	*D1b* [b]	*D1b* [b]	*D1c* [d]

[a] low PPO allele as designated by STS marker PPO18 amplifying a 876-bp PCR product.
[b] high PPO allele as designated by by CAPS marker PPOD1CAPS producing a 931-bp PCR product.
[c] null PPO allele as designated by STS marker PPO18 producing no PCR fragment.
[d] null PPO allele as designated by CAPS marker PPOD1CAPS producing 535-bp and 396-bp PCR products.

Characterization of Kernel and Milling Traits

Kernel and milling characteristics were measured and compared between White Choteau, White Vida, and line 07OR1074. All cultivars differed for kernel PPO and Bran PPO content with White Vida exhibiting the greatest kernel PPO activity ($0.351\Delta_{475}$) and bran PPO activity ($1.561\Delta_{475}$). The cultivar White Choteau had greater kernel protein and subsequently greater flour protein content than White Vida or 07OR1074 (Table 9). The cultivars White Choteau and White Vida varied for kernel weight but did not vary significantly from line 07OR1074. Flour yield and flour ash did not vary among the parents. Among kernel and flour traits measured for the White Choteau/07OR1074 and White Vida/07OR1074 populations, the genotypic class with both loci homozygous for the null alleles (*Ppo-A1i* and *Ppo-D1c*) had significantly lower kernel, flour, and bran PPO activity across both populations (Table 10). In the White Choteau/07OR1074 population, lines homozygous for the Ppo-A1b and Ppo-D1b parental alleles displayed a

higher flour ash content ($P = 0.01$). Flour produced in the White Vida/07OR1074 population from lines homozygous for the null PPO alleles *Ppo-A1i* and *Ppo-D1c* possessed a higher protein content ($P = 0.04$) than lines homozygous for the high PPO alleles *Ppo-A1a* and *Ppo-D1b*. Kernel weight and flour yield did not vary among offspring in either population.

Characterization of Noodle Color Profiles

In measuring the noodle color profile for raw Chinese white salted noodles made from refined white flour, the parental line 07OR1074 produced noodles that were brighter (greater L^*) at 24 and 48 h and more yellow (greater b^*) at 0, 24, and 48 hours compared to both White Choteau and White Vida (Table 11). Additionally, 07OR104 gave noodles that had less change in L^* and an increase in a^* and b^* with time (0-24h). No significant differences were observed in the change in noodle color profile from 24-48 h between the low PPO parent 07OR1074 and White Choteau and White Vida. Lines fixed for the null PPO alleles Ppo-A1i and Ppo-D1c gave noodles with greater L*, a*, and b* at 24 and 48 hours ($P < 0.001$) with lesser change in L* (0-24h) and greater change in a* and b* (0-24h) ($P < 0.001$) compared to lines fixed for the high PPO parental alleles found in White Choteau and White Vida (Table 12). Furthermore, when examining noodle color profile for raw Chinese noodles made from whole wheat flour, line 07OR1074 produced noodles that had greater L* and b* at 0, 24, and 48 hours and less change in L* (0-24h) compared to White Choteau and greater L* and a* at 24 and 48 hours, greater b* at 0 and 48 hours and less change in L* (0-24h) compared to the cultivar White Vida (Table 13). Whole

Table 9. Comparison of means for kernel and flour traits of spring wheat parents 07OR1074, White Choteau, and White Vida.

Parents	n	Kernel PPO[†] activity Δ_{475}	Kernel [b] Protein	Kernel [a] Weight mg	Kernel [a] Diameter mm	Flour [c] Yield (%)	Flour [b] Protein (%)	Flour [b] Ash (%)	Flour PPO activity Δ_{475}	Bran PPO activity Δ_{475}
White Choteau	3	0.281 *a*	16.4 *a*	38.6 *a*	3.13 *a*	68.7 *a*	15.5 *a*	0.431 *a*	0.205 *a*	1.102 *a*
White Vida	3	0.351 *b*	15.5 *b*	36.8 *b*	3.08 *a*	71.1 *a*	13.4 *b*	0.432 *a*	0.216 *a*	1.561 *b*
07OR1074	3	0.043 *c*	15.4 *b*	37.4 *ab*	3.23 *b*	68.4 *a*	13.7 *b*	0.414 *a*	0.095 *b*	0.494 *c*
LSD (0.05)		0.01	0.73	1.11	0.10	3.22	0.35	0.042	0.069	0.210

[†]Means with the same letter (*a*, *b*, *c*) are not significantly different at (P =0.05)

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

[b] Kernel protein, flour protein, and ash concentration was determined by near-infrared spectroscopy

[c] Flour yield was calculated as the proportion of flour to total products.

Table 10. Comparison of means for kernel and flour traits of spring wheat populations fixed for null PPO alleles *Ppo-A1i* and *Ppo-D1c* and high PPO alleles *Ppo-A1a/b* and *Ppo-D1b.*

Population	n	Kernel PPO activity Δ_{475}	Kernel [b] Protein	Kernel [a] Weight mg	Kernel [a] Diameter mm	Flour [c] Yield (%)	Flour [b] Protein (%)	Flour [b] Ash (%)	Flour PPO activity Δ_{475}	Bran PPO activity Δ_{475}
White Choteau/ 07OR1074										
Ppo-A1i, Ppo-D1c	16	0.054	15.6	37.8	3.11	68.86	14.5	0.409	0.136	0.639
Ppo-A1b ,Ppo-D1b	17	0.213	15.7	38.1	3.09	68.47	14.6	0.425	0.172	1.045
P value		0.003	0.934	0.614	0.524	0.278	0.530	0.015	0.021	<0.0001
White Vida/ 07OR1074										
Ppo-A1i, Ppo-D1c	16	0.077	15.1	38.1	3.06	69.2	14.4	0.415	0.160	0.077
Ppo-A1a ,Ppo-D1b	17	0.373	15.5	37.9	3.09	69.8	13.9	0.416	0.248	1.157
P value		<0.0001	0.236	0.726	0.274	0.129	0.045	0.995	0.006	0.001

[a] Kernel weight and size was determined using the single kernel characterization system (SKCS) on three reps of 100 seeds each.

[b] Kernel protein, flour protein, and ash concentration was determined by near-infrared spectroscopy

[c] Flour yield was calculated as the proportion of flour to total products.

wheat noodles produced from offspring homozygous for the null PPO alleles *Ppo-A1i* and *Ppo-D1c* exhibited increased L*, a* and b* at 24 and 48 hours with less change in L* and increase in a* and b* from over a 24 hour period compared to their high PPO parental allele counterparts (Table 14). Figure 3 plots the relationship between noodle color profiles over time for both refined white and whole wheat noodles from the White Choteau/07OR1074 population. An increase in noodle b* from 0- 12 hours followed by a steady decline is observed in both refined white noodles and whole wheat noodles among all offspring. A similar trend is seen in a* values but instead a* values seem to stay the same after 12 hours in whole wheat noodles and after 24 hours in noodles made from refined white flour. A sharp decrease in L* is observed in noodles made from refined white flour and whole wheat flour in both genotypic classes. However the decrease in noodle L* from 0-24 hours is less in noodles produced from refined white flour than those produced from whole wheat flour. Similar trends are observed when examining the relationship between noodle color profiles over time for both whole wheat and refined white noodles from White Vida/07OR1074 population (Figure 4). Just as the White Chouteau/07OR1074 population showed, an increase in noodle b* from 0-12 hours followed by a steady decline is observed in both refined white noodles and whole wheat noodles. However, b*, a* and L* values hold steady after 24 hours among the offspring containing the null PPO alleles *Ppo-A1i* and *Ppo-D1c* compared to their sister line counterparts.

Table 11. Comparison of Chinese (white salted) noodle color profile of spring wheat parents 07OR1074, White Choteau, and White Vida.

Parents	n	Brightness [a†]				Redness [a]				Yellowness [a]			
		L* 0h	L* 24h	L* 48h	L* (0-24h)	a* 0h	a* 24h	a* 48h	a* (0-24h)	b* 0h	b* 24h	b* 48h	b* (0-24h)
White Choteau	3	80.8 *a*	68.1 *a*	65.7 *a*	12.7 *a*	1.6 *a*	2.3 *a*	2.1 *a*	-0.7 *a*	17.2 *a*	19.8 *a*	19.3 *a*	-2.6 *a*
White Vida	3	81.6 *ab*	66.4 *a*	65.5 *a*	15.2 *b*	1.4 *ab*	1.6 *b*	1.6 *a*	-0.3 *a*	17.9 *a*	21.8 *a*	19.8 *a*	-3.9 *ab*
07OR1074	3	82.6 *b*	73.1 *b*	71.9 *b*	9.5 *c*	0.9 *b*	2.7 *a*	2.3 *a*	-1.3 *b*	19.5 *b*	24.6 *b*	23.9 *b*	-5.1 *b*
LSD (0.05)		1.65	3.18	3.58	1.83	0.59	0.64	0.70	0.60	0.89	2.13	2.68	2.27

[†]Means with the same letter (*a*, *b*, *c*) are not significantly different at (*P* =0.05)

[a] Dough sheet color measurements measured with a Minolta CR-310 Chroma Meter after sheeting to a thickness of 1.2mm.

Table 12. Comparison of Chinese (white salted) noodle color profile of spring wheat populations fixed for null PPO alleles *Ppo-A1i* and *Ppo-D1c* and high PPO alleles *Ppo-A1a/b* and *Ppo-D1b.*

		Brightness [a]				Redness [a]				Yellowness [a]			
Population	n	L*0h	L*24h	L*48h	L* (0-24h)	a*0h	a*24h	a*48h	a* (0-24h)	b*0h	b*24h	b*48h	b* (0-24h)
White Choteau/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	81.3	71.8	70.3	9.4	1.5	3.2	3.1	-1.5	19.3	24.7	24.3	-5.5
Ppo-A1b ,Ppo-D1b	17	81.6	68.3	67.3	13.3	1.5	2.3	2.1	-0.8	17.4	21.1	19.9	-3.7
P value		0.302	<0.001	0.001	<0.001	0.581	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
White Vida/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	81.5	71.4	70.2	10.2	1.6	3.1	2.8	-1.4	18.7	24.3	23.3	-5.7
Ppo-A1a ,Ppo-D1b	17	81.5	67.6	66.5	13.9	1.4	1.9	1.9	-0.5	17.8	21.2	20.6	-3.31
P value		0.917	<0.001	0.001	<0.001	0.021	<0.001	<0.001	<0.001	0.011	<0.001	<0.001	<0.001

[a] Dough sheet color measurements measured with a Minolta CR-310 Chroma Meter after sheeting to a thickness of 1.2mm.

Table 13. Comparison of whole wheat noodle color profile of spring wheat parents 07OR1074, White Choteau, and White Vida.

Population	n	Brightness†				Redness				Yellowness			
		L* 0h	L* 24h	L* 48h	L* (0-24h)	a* 0h	a* 24h	a* 48h	a* (0-24h)	b* 0h	b* 24h	b* 48h	b* (0-24h)
White Choteau	3	67.6 *a*	53.9 *a*	52.1 *a*	13.8 *a*	4.4 *a*	5.9 *ab*	6.1 *a*	-1.6 *a*	18.2 *a*	18.9 *a*	18.5 *a*	-0.69 *a*
White Vida	3	68.8 *ab*	52.1 *a*	49.2 *a*	13.4 *a*	3.8 *b*	5.7 *a*	5.4 *b*	-1.9 *b*	17.9 *a*	18.8 *a*	16.4 *b*	-0.84 *a*
07OR1074	3	70.6 *b*	60.1 *b*	59.3 *b*	10.5 *b*	4.1 *ab*	6.3 *b*	6.2 *a*	-2.2 *c*	19.5 *b*	20.8 *b*	20.6 *c*	-1.24 *a*
LSD (0.05)		2.45	3.38	5.52	2.85	0.35	0.33	0.57	0.215	0.639	1.12	1.81	1.24

†Means with the same letter (*a*, *b*, *c*) are not significantly different at (P =0.05)

Table 14. Comparison of means for whole wheat noodle color profile of spring wheat populations fixed for null PPO alleles *Ppo-A1i* and *Ppo-D1c* and high PPO alleles *Ppo-A1a/b* and *Ppo-D1b.*

		Brightness[†]				Redness				Yellowness			
Population	n	L* 0h	L* 24h	L* 48h	L* (0-24h)	a* 0h	a* 24h	a* 48h	a* (0-24h)	b* 0h	b* 24h	b* 48h	b* (0-24h)
White Choteau/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	68.0	56.7	55.0	11.3	4.7	7.0	6.7	-2.3	19.8	21.2	20.2	-1.4
Ppo-A1b ,Ppo-D1b	17	68.0	53.2	50.5	14.9	4.3	6.2	6.0	-1.9	18.6	19.4	18.1	-0.9
P value		0.921	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.03
White Vida/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	69.0	58.2	57.1	10.9	4.3	6.5	6.4	-2.2	19.4	21.1	20.8	-1.7
Ppo-A1a ,Ppo-D1b	17	68.4	52.9	50.9	15.4	4.1	6.0	5.8	-1.9	18.8	19.3	17.8	-0.46
P value		0.129	<0.001	0.001	<0.001	0.069	0.014	<0.001	0.108	0.008	<0.001	<0.001	<0.001

[a] Dough sheet color measurements measured with a Minolta CR-310 Chroma Meter after sheeting to a thickness of 1.2mm.

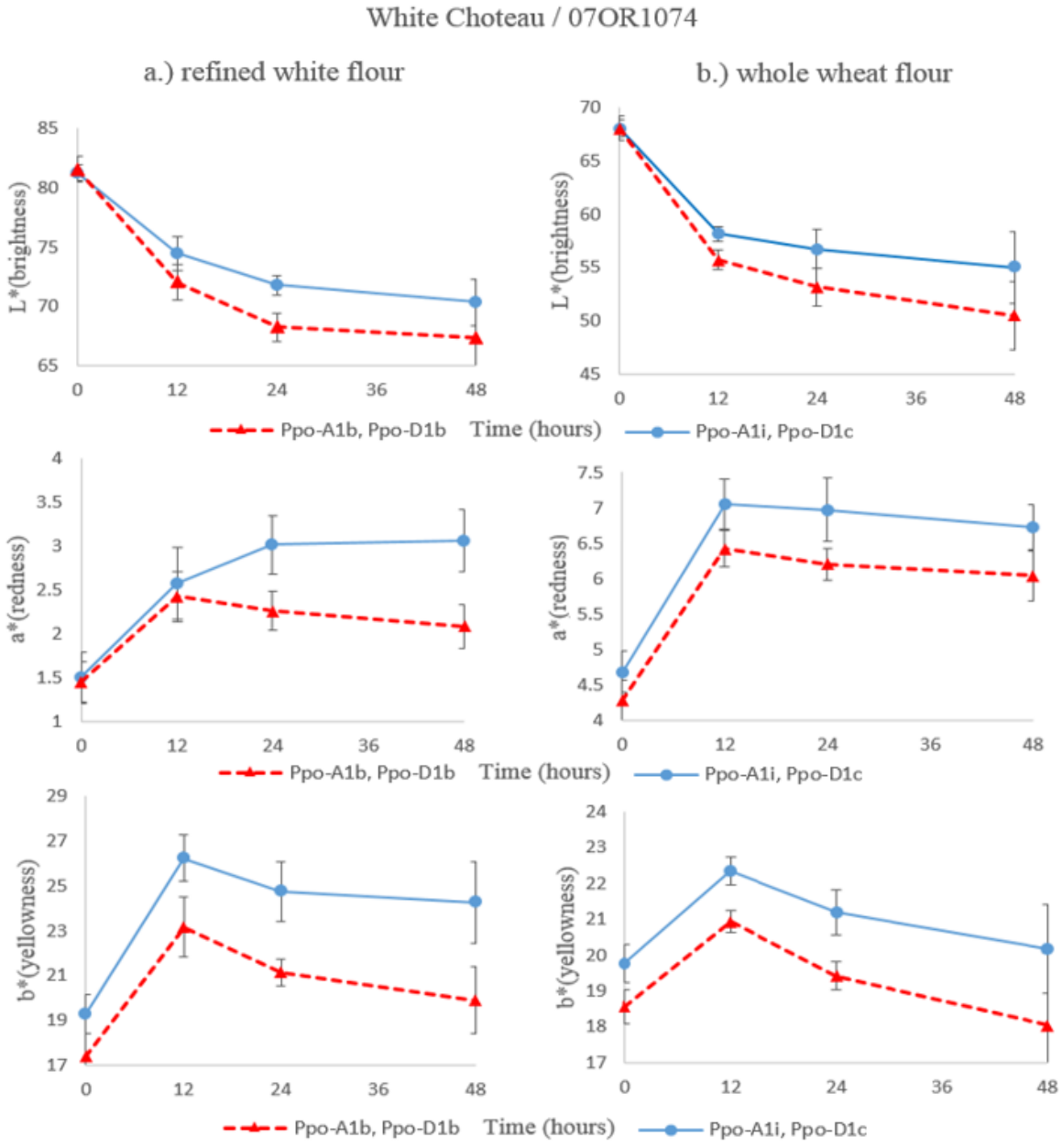

Figure 3. Comparison of noodle color components of Chinese fresh (white salted) made from a) refined white flour and b) whole wheat flour from White Choteau / 07OR1074 breeding population fixed for the mutant null PPO alleles Ppo-A1i and Ppo-D1c and wild-type alleles. Values are the average of nine replicates measured over a 48 hour time period. Error bars indicate one standard deviation above and below the mean value.

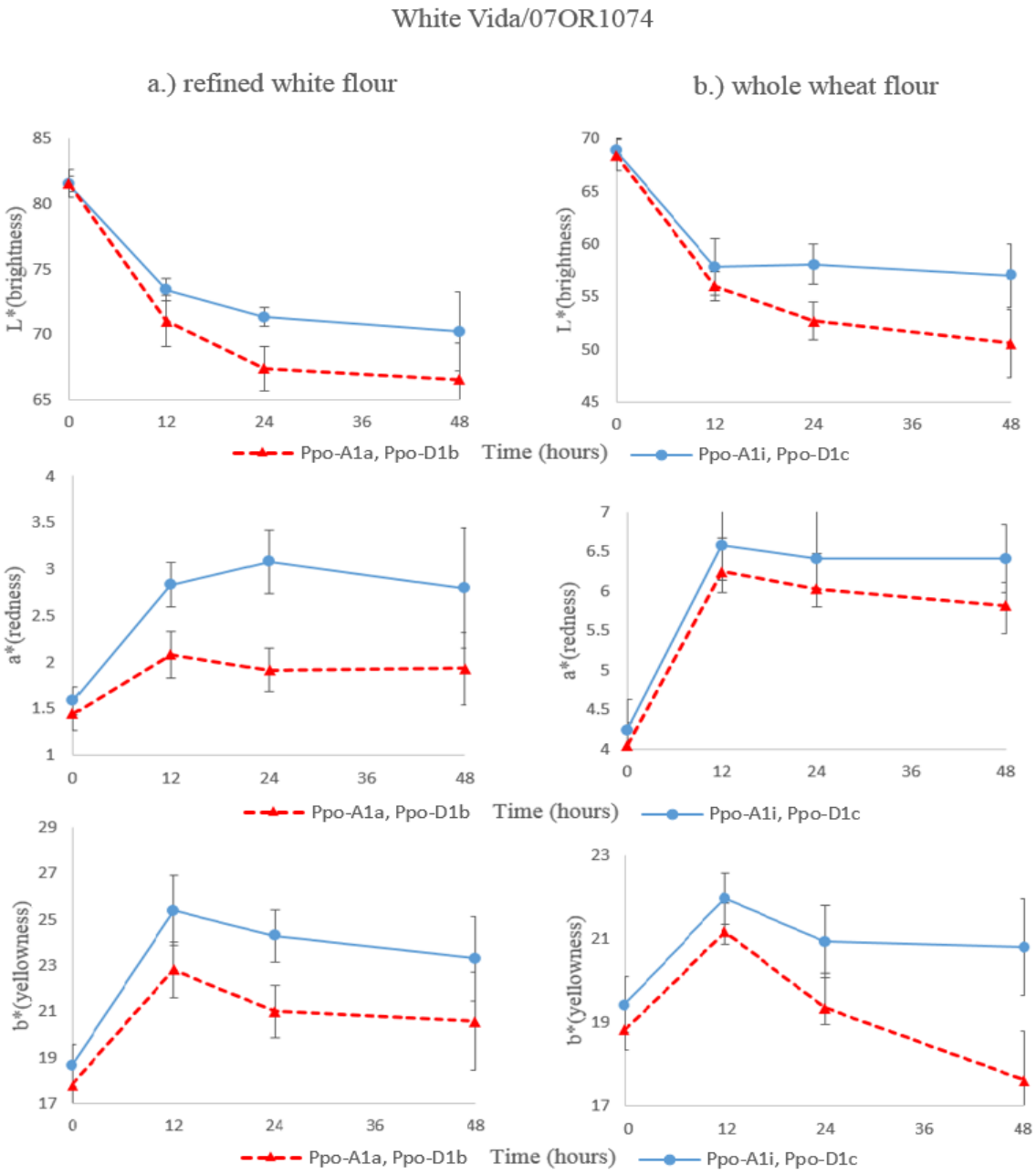

Figure 4. Comparison of noodle color components of Chinese fresh (white salted) made from a) refined white flour and b) whole wheat flour from White Vida / 07OR1074 breeding population fixed for the mutant null PPO alleles Ppo-A1i and Ppo-D1c and wild-type alleles. Values are the average of nine replicates measured over a 48 hour time period. Error bars indicate one standard deviation above and below the mean value.

Associations among Noodle and Kernel Traits

Linear correlations were calculated between noodle color and important kernel and flour traits (Table 15, 16). Kernel, bran, and flour PPO activity were all negatively correlated with L*, a*, and b* at 24 and 48 hours and change in L*, a*, and b* (0-24h) for both whole wheat and refined white noodles. Kernel protein and flour protein had a strong negative correlation with L* and a* at 0h ($r > 0.60$, $P < 0.001$) in both whole wheat noodles and noodles made from refined white flour. As flour protein increased, noodles were darker (lower L*) and more red (higher a*) at 0 h. However as time increased correlations between flour protein and L* and a* became weaker ($r < 0.10$) and not significant at ($P = 0.5$). Flour ash was negatively related b* at 0h ($r = 0.35$, $P = 0.05$) for noodles produced from refined white flour.

Characterization of Noodle Texture Components

Line 07OR1074 varied for noodle springiness, cohesiveness and chewiness for Chinese white salted noodles made from refined white flour in comparison to both White Choteau and White Vida (Table 17). Additionally, White Choteau and 07OR1074 varied hardness with White Choteau producing noodles that were more firm. White Choteau and White Vida varied only for cohesiveness and hardness with White Choteau producing firmer and less cohesive noodles made from refined white flour. Chinese noodles made with refined white flour from lines homozygous for *Ppo-A1i* and *Ppo-D1c* alleles in White Choteau/ 07OR1074 population were less cohesive, adhesive, firm, and chewy ($P < .05$) compared to lines homozygous for the PPO alleles *Ppo-A1b* and *Ppo-D1b*.

Table 15. Linear correlations between kernel, flour, and Chinese raw (white salted) noodle color components for spring wheat populations White Choteau/07OR1074 and White Vida/ 07OR1074.

Trait	Kernel PPO activity Δ_{475}	Flour PPO activity Δ_{475}	Bran PPO activity Δ_{475}	Kernel [a] Protein	Flour [a] Protein	Flour [a] Ash
L* 0 h	0.12	-0.100	0.168	-0.630***	-0.704***	0.026
L* 24 h	-0.738***	-0.445***	-0.526***	-0.150	-0.123	-0.131
L* 48 h	-0.494	-0.332**	-0.351**	-0.102	-0.069	0.123
L* (0-24 h)	0.814***	0.422***	0.613***	-0.098	-0.155	0.147
a* 0 h	-0.184	0.056	-0.201	0.615***	0.706***	-0.086
a* 24 h	-0.826***	-0.382**	-0.569***	0.361**	0.462**	-0.045
a* 48 h	-0.668***	-0.256*	-0.387**	0.187	0.218	0.004
a* (0-24h)	0.866***	0.463***	0.564***	-0.141	-0.217	0.014
b* 0 h	-0.473***	-0.236	-0.379**	0.180	0.175	-0.349**
b* 24 h	-0.738***	-0.424***	-0.547***	0.024	0.018	-0.195
b* 48 h	-0.544***	-0.291*	-0.331**	-0.093	-0.120	-0.100
b* (0-24 h)	0.700***	0.433***	0.494***	0.117	0.122	-0.005

[a] Kernel protein, flour protein, and ash concentration was determined by near-infrared spectroscopy.* P <0.05, **P <0.01, and ** P < 0.001 (Student's *t*-test).

Table 16. Linear correlations between kernel, flour, and whole wheat Chinese raw (white salted) noodle color components for spring wheat populations White Choteau/07OR1074 and White Vida/ 07OR1074.

	Kernel PPO activity Δ_{475}	Flour PPO activity Δ_{475}	Bran PPO activity Δ_{475}	Kernel [a] Protein	Flour [a] Protein	Flour [a] Ash
L* 0 h	-0.056	-0.162	-0.020	-0.491***	-0.513***	-0.027
L* 24 h	-0.684***	-0.360**	-0.424***	-0.067	-0.026	-0.100
L* 48 h	-0.540***	-0.242	-0.325**	-0.148	-0.107	-0.085
L* (0-24 h)	0.703***	0.312*	0.442***	-0.142	-0.194	0.094
a* 0 h	-0.419***	-0.201	-0.499***	0.488***	0.493***	-0.192
a* 24 h	-0.501***	-0.303*	-0.512***	0.272*	0.284*	-0.180
a* 48 h	-0.669***	-0.363**	-0.541***	0.204	0.263*	-0.177
a* (0-24h)	0.320**	0.244*	0.257*	0.082	0.071	0.074
b* 0 h	-0.487***	-0.133	-0.476***	0.297*	0.234	-0.193
b* 24 h	-0.666***	-0.383**	-0.551***	0.061	0.031	-0.202
b* 48 h	-0.621***	-0.267*	-0.342**	-0.090	-0.065	-0.095
b* (0-24 h)	0.424***	0.366**	0.289*	0.172	0.157	0.091

[a] Kernel protein, flour protein, and ash concentration was determined by near-infrared spectroscopy.* P <0.05, **P <0.01, and ** P < 0.001 (Student's *t*-test).

In contrast, no significant differences ($P = 0.05$) were detected in texture components between lines homozygous for null PPO genotypic class versus the high PPO allele genotypic class in White Vida/ 07OR1074 population (Table 17). Whole wheat noodles produced from White Choteau and White Vida did not vary for any texture trait measured (Table 18). Line 07OR1074 varied for noodle cohesiveness and chewiness compared to both parental lines White Choteau and White Vida. Furthermore, parental lines 07OR1074 and White Choteau varied for noodle firmness. Whole wheat noodles produced from lines homozygous for *Ppo-A1i* and *Ppo-D1c* alleles in White Choteau/ 07OR1074 population were less springy, cohesive, firm, and chewy ($P < .05$) compared to lines homozygous for the PPO alleles *Ppo-A1b* and *Ppo-D1b*. In contrast, no significant differences ($P = 0.05$) were detected in texture components between lines homozygous for null PPO genotypic class versus the high PPO allele genotypic class in White Vida/ 07OR1074 population.

Table 17. Comparison of Chinese (white salted) noodle color profile of spring wheat parents 07OR1074, White Choteau, and White Vida and spring wheat populations fixed for null PPO alleles *Ppo-A1i* and *Ppo-D1c* and high PPO alleles *Ppo-A1a/b* and *Ppo-D1b.*

Parents	n	Springiness[†]	Cohesiveness	Adhesiveness	Hardness	Chewiness
White Choteau	3	0.936 *a*	0.561 *a*	-32.3 *a*	976.2 *a*	477.9 *a*
White Vida	3	0.936 *a*	0.609 *b*	-30.3 *a*	839.9 *b*	513.1 *a*
07OR1074	3	0.893 *b*	0.516 *c*	-29.3 *a*	887.3 *b*	409.2 *b*
LSD (0.05)		0.02	0.039	7.84	50.11	44.203
White Choteau/ 07OR1074						
Ppo-A1i, Ppo-D1c	16	0.918	0.532	-27.2	895.8	437.3
Ppo-A1b ,Ppo-D1b	17	0.930	0.555	-30.4	962.1	498.4
P value		.0696	<0.001	0.028	<0.001	<0.001

Table 17 Continued

White Vida/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	0.927	0.561	-27.3	860.6	448.2
Ppo-A1a ,Ppo-D1b	17	0.927	0.572	-29.1	854.3	453.0
P value		0.945	0.114	0.209	0.740	0.707

†Means with the same letter (*a, b, c*) are not significantly different at (*P* =0.05).

Table 18. Comparison of Chinese (white salted) whole wheat noodle color profile of spring wheat parents 07OR1074, White Choteau, and White Vida and spring wheat populations fixed for null PPO alleles *Ppo-A1i* and *Ppo-D1c* and high PPO alleles *Ppo-A1a/b* and *Ppo-D1b.*

Parents	n	Springiness†	Cohesiveness	Adhesiveness	Hardness	Chewiness
White Choteau	3	0.925 *a*	0.488 *a*	-30.8 *a*	1197.4 a	549.5 *a*
White Vida	3	0.936 *a*	0.504 *a*	-26.5 *a*	1110 ab	519.7 *a*
07OR1074	3	0.906 *a*	0.435 *b*	-30.9 *a*	988.7 b	388.7 *b*
LSD (0.05)		0.069	0.034	6.037	143.41	114.9
White Choteau/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	0.885	0.447	-32.5	1086.7	430.5
Ppo-A1b ,Ppo-D1b	17	0.915	0.470	-32.5	1170.2	505.4
P value		0.001	<0.001	0.995	<0.001	<0.001
White Vida/ 07OR1074 *Ppo-A1i, Ppo-D1c*	16	.887	0.467	-29.5	1023.7	424.8
Ppo-A1a ,Ppo-D1b	17	.9036	0.474	-28.9	1056.8	453.5
P value		0.074	0.179	0.661	0.199	0.079

†Means with the same letter (*a, b, c*) are not significantly different at (*P* =0.05).

Discussion

Enzymatic discoloration brought upon by the formation of melanin pigments by the oxidation of phenolic compounds via PPO has hindered improvements in both whole grain foods and products produced from refined white flour. As PPO and its phenolic

substrates are localized to the bran, the majority of the enzyme is removed during flour milling (Rani et al. 2006; Okot-Kotber et al. 2001). However, the minor amounts that make it into flour are sufficient to discolor refined flour products such as yellow alkaline and white-salted noodles which have high moisture content and are stored for prolonged periods of time. Complications of kernel PPO activity is especially problematic in whole grain products where PPO and its phenolic substrates are prevalent in whole wheat flour. Increasing the amount of fiber fortification in food products traditionally made from refined white flour in order to address the potential health benefits associated with increasing dietary fiber intake poses several challenges to quality improvement efforts. Firstly, bran and germ particles act as physical barriers that can interfere with formation of a complex protein network that gives wheat dough its elasticity. This ultimately effects noodle texture parameters reducing springiness, cohesiveness, and firmness of the noodle product (Zhang and Moore 1997). Secondly, phenolic acids localized in the aleurone layer of the bran are associated with bitterness and astringency in the final product (Zhou et al. 1999). Finally, the abundance of PPO enzyme in the bran layer can oxidize sufficient phenolic acids present causing the formation of dark melanin pigments. The subsequent impact of these quality complications depend on the amount of bran added. The addition of 30-60g kg^{-1} of rice bran to wheat flour had little to no impact on taste, texture, or appearance of noodles, however the addition of 90 g kg^{-1} of rice bran significantly impacted the appearance, taste, texture, and acceptability of noodles when compared to noodles produced from refined white flour (Kim et al. 1997). Therefore, improvements in texture, taste, and color is essential in order to increase consumer

acceptability of whole grain products. As consumers are particularly influenced by noodle appearance before purchasing the product, color is considered a primary trait as noodles are seen and must appear desirable prior to consumption (Hou 2010). As kernel PPO has been shown to influence noodle color (Martin et al. 2011; Fortmann & Joiner 1971; Morris et al. 2000; Mayer 1979) efforts to effectively reduce or eliminate kernel PPO activity are essential to improve quality of noodles made from refined white flour and further consumer acceptability of whole grain noodles. Parental line 07OR1074, which carries null PPO mutations not previously used in wheat breeding programs (Table 8), had substantially lower mean PPO activity (0.043 Δ475) compared to White Choteau (0.281 Δ475) and White Vida (0.351 Δ475) (Table 9). However the relative importance of these null PPO mutations on noodle color profiles and texture components was unknown. The objective of this study was to determine the impact of these null PPO alleles on kernel and flour characteristics, and on the color and texture profile of Chinese raw (white salted) noodles prepared from refined white flour and whole wheat flour color. The null PPO alleles were not associated with any reduction in seed size or weight in the White Choteau/ 07OR1074 and White Vida/ 07OR1074 populations. Among flour and milling traits, the null PPO loci were associated with lower flour ash content in the White Choteau/ 07OR1074 population and reduced flour protein in the White Vida/ 07OR1074 population. Flour ash content was slightly lower in the 07OR1074 parent compared to the cultivar White Choteau. PPO activity in the flour is for the most part due to bran contamination which is echoed by the ash content of the flour (Rani et al. 2001). Thereby increasing ash content in the flour would likely result in an increase in flour PPO

activity. Martin et al. (2011) showed that lines that were homozygous for low PPO alleles, *Ppo-A1b* and *Ppo-D1a* exhibited a higher flour ash content and flour yield but smaller seed size. They attributed the increase in ash content as less efficient milling of smaller kernels. As flour yield did not vary for the parents or across genotypic classes in each population the differences in ash content may be reflected by the slight differences in kernel size between the parents, with White Choteau having a slightly higher kernel weight compared to 07OR1074. The larger kernels could result in more endosperm contamination during the milling process. The differences in flour PPO activity between lines homozygous for the null PPO alleles was associated with a lower ash content in the White Choteau / 07OR1074 population however differences in ash content is unlikely to responsible for differences seen in flour PPO activity as flour PPO levels were drastically different across genotypic classes in the White Vida/ 07OR1074 population while having essentially equal flour ash contents. The cultivar White Choteau had greater kernel protein and subsequently greater flour protein content than White Vida or 07OR1074 (Table 9) however, genotypic classes homozygous for the null PPO alleles was not associated with a reduction in kernel protein content.

Color is a complex trait of which can the perception can be influenced by a multitude of factors present in the environment such as the source of light and compounds present in the flour and noodles such as flavonoids and phenolic compounds (Miskelly 1996). The most common color components reported for noodles are brightness (L*), redness (a*), and yellowness (b*). Both redness and yellowness of some noodles and pasta can be attributed to free unsaturated fatty acids, tocopherols, and

carotenoids present in the noodle and are influenced by PPO, lipoxygenase, and peroxidase enzymes (Feillet et al. 2000) as well as the Maillard reaction during processing. Noodle brightness and color stability is highly influenced by kernel PPO activity. The deterioration in noodle brightness over a 24 hour period has been shown to be strongly correlated as well as the decrease in b* values over the same amount of time (Hatcher and Kruger 1993; Martin et al. 2011) Martin et al. (2011) first described the relative importance of allelic variation of both Ppo-A1 and Ppo-D1 loci on noodle color in which lines fixed for the low PPO alleles *Ppo-A1b* and *Ppo-D1a* had brighter, more yellow, and more red than the high PPO alleles *Ppo-A1a* and *Ppo-D1b* at 24 hours. The results of this experiment is in agreement with prior reports in that Chinese white salted noodles produced with refined white flour from the low PPO parent 07OR1074 gave noodles that were brighter (greater L*), more red (greater a*), and more yellow (higher b*) at 24 and 48 h with less darkening over time (L* 0-24) but more change in a* and b* over time (0-24h) (Table 11, 13). Interestingly, whole wheat noodles produced from parent 07OR1074 were also brighter at 0 and 24 hours and darkened less with time (0-24h) but were only slightly more red (a*) and more yellow (b*) than White Vida and were not statistically different (P = 0.05) from White Choteau which exhibits moderate kernel PPO activity. These results seem to suggest that a higher concentration of secondary phenolic compounds in the bran of whole wheat noodles is likely to influence noodle a* more so than PPO levels. Linear correlations calculated between PPO activity of kernel tissues and color components of refined white and whole wheat noodles show that PPO activity is strongly negatively correlated to L*, a*, and b* at 24 and 48 hours

(Table 15, 16). Although the deterioration of noodle L* has shown to be highly correlated with PPO content (Kruger et al. 1995), the results show that a reduction in kernel PPO content is strongly associated with an increase in kernel a* and b*. As the oxidation of phenolic compounds and subsequent production of melanin pigments is a time dependent process, color components L*, a*, and b* were measured over a 48 hour period. For both genotypic classes in both populations the rate of darkening and stability of the redness and the yellowness components of noodle color appear to remain constant after 24 hours. From 0 – 12 hours, L* decreases rapidly while a* and b* promptly increase in both refined white and whole wheat noodles. Lipoxygenase, believed to be a bleaching agent, could be largely responsible for the decline in red and yellow pigments (Figure 3, 4) due to the oxidation of unsaturated fatty acids that subsequently produce fatty acid radicals that react and further oxidize chlorophyll, xanthophyll, and carotene compounds (McDonald 1979).

While consumers initially rely on visual cues to judge the quality of fresh noodle products, textural properties determine whether noodles products are pleasing upon mastication. Noodle cohesiveness, adhesiveness, springiness, hardness, and chewiness are objective sensory parameters that attempt to quantify and relate the physical properties of noodles with consumer's preference. The null PPO alleles *Ppo-A1i* and *Ppo-D1c* produced refined white and whole wheat noodles that were less cohesive, adhesive, firm, and chewy in the White Choteau/ 07OR1074 population (Table 17, 18). However, no differences in noodle texture profiles were observed across genotypic classes in the White Vida/ 07OR1074 population. Previous studies have shown that low

PPO alleles are associated with more cohesive noodles (Martin et al. 2011). Cohesiveness is a measure of extent noodle structure disrupted by compression force. Although differences in noodle cohesiveness, adhesiveness, firmness, and chewiness were detected by texture profile analysis in individuals that possessed the null PPO alleles Ppo-A1i and Ppo-D1c in the White Choteau / 07OR1074 population, the lack of differences in texture parameters occurring in the White Vida/ 07OR1074 population across genotypic classes infers that null PPO alleles do not affect noodle texture parameters nor are genes affecting noodle texture in linkage to PPO genes on chromosomes 2A and 2D. Hardness is the maximum force derived when cutting through a set of noodles. The addition of bran or fiber rich fractions to produce whole wheat noodles has been shown to decrease noodle firmness by disrupting the formation of the gluten protein matrix by bran and germ particles (Bruinsma et al. 1978). However, whole wheat noodles produced from lines White Choteau, White Vida, and 07OR1074 exhibited increased firmness (Table 18). Izydorczyk et al. (2005) observed that the addition of fiber rich fractions disrupted the protein-starch matrix as evidence of scanning electron microscopy but that the addition of bran and germ fractions appeared to form a semisolid network which likely contributed to the increase in noodle firmness. It is not known whether the differences observed here in noodle texture profile analysis would be discernable by consumers.

In conclusion, facilitating selection for null PPO genes by marker assisted selection has the capacity to improve end use quality traits in bread wheat. Among kernel and flour traits measured for the White Choteau/07OR1074 and White Vida/07OR1074 populations, the genotypic class with both loci homozygous for the null alleles (*Ppo-A1i*

and *Ppo-D1c*) had significantly lower kernel, flour, and bran PPO activity across both populations. Additionally, our results show that when fixed for the null PPO alleles *Ppo-A1i* and *Ppo-D1c*, Chinese white salted noodles made from both refined white and whole wheat flour produced noodles that were brighter (L*), more red (a*), and more yellow (b*) at 24 and 48 hours. Furthermore, the null PPO alleles gave noodles that had less change in L^* and an increase in a^* and b^* with time (0-24h). Differences were detected in texture profile analysis for lines homozygous for the null PPO alleles in the White Choteau/ 07OR1074 population, however the differences were observed in the White Vida/ 07OR1074 population, and therefore it is unlikely that differences in noodle texture could be attributed to genes linked to the *Ppo-A1* and *Ppo-D1* genes or allelic variation at the Ppo-A1 and Ppo-D1 loci.

Acknowledgments

We thank the Montana Wheat and Barley Committee for their financial support.

REFERENCES CITED

Anderson J, Lubberstedt T (2003) Functional markers in plants. Trends Plant Sci. 18:554-560.

Anderson JV, Morris CF (2001) An improved whole-seed assay for screening wheat germplasm for polyphenol oxidase activity. Crop Sci. 41:1697-1705.

Baik BK, Czuchajowska Z, Pomeranz Y (1995) Discoloration of dough for oriental noodles. Cereal Chem. 72(2):198-205.

Beecher BS, Carter AH, See DR (2012) Genetic mapping of new seed-expressed polyphenol oxidase genes in wheat (Triticum aestivum L.). Theor Appl Genet. 124:1463-1473.

Beecher BS, Skinner DZ (2011) Molecular cloning and expression analysis of multiple polyphenol oxidase genes in developing wheat (Triticum aestivum) kernels. J Cereal Sci. 19:291-296.

Chang C, Zhang HP, Xu J, You MS, Li BY, Liu GT (2007) Variation in two PPO genes associated with polyphenol oxidase activity in seeds of common wheat. Euphytica. 154:181-193.

Demeke T, Morris CF, Campbell KG, King GE, Anderson JA (2001) Wheat polyphenol oxidase: distribution and genetic mapping in three inbred line populations. Crop Sci. 44:1750-1757.

Elias EM, Miller JD (1998) Registration of 'Mountrail' durum wheat. Crop Sci. 40: 1499-1500.

Epstein J, Morris CF, Huber KC (2002) Instrumental texture of white salted noodles prepared from recombinant inbred lines of wheat differing in the three granule bound starch synthase (waxy) genes. J. Cereal Sci. 35: 51-63.

Feillet P, Autran JC, Icard-Verniere C (2000) Mini review pasta brownness: an assessment. Journal of Cereal Science. 32: 215-233.

Flurkey WH (1986) Polyphenol oxidase in higher plants. Plant physiol. 81: 614-618

Fuerst PE, Xu SS, Beecher B (2008) Genetic characterization of kernel polyphenol oxidases in wheat and related species. Journal of Cereal Science. 48: 359-368.

Fortmann KL, Joiner RR (1971) Wheat pigments and flour color. In: Pomeranz, Y. (Ed.) Wheat: Chemistry and Technology, American Association of Cereal Chemists. St. Paul, MO.

Hatcher DW, Kruger JE (1993) Distribution of polyphenol oxidase of flour millstreams of Canadian common wheat classes milled to three extraction rates. Cereal Chem. 70: 51-55.

He XY, He ZH, Zhang LP, Sun DJ, Morris CF, Fuerst EP, Xia XC (2007) Allelic variation of polyphenol oxidase (PPO) genes located on chromosome 2A and 2D and development of functional markers for the PPO genes in common wheat. Theor Appl Genet. 115:47-58.

Holderbaum DF, Kon T, Kudo T, Guerra MP (2010) Enzymatic browning, polyphenol oxidase activity, and polyphenols in four apple cultivars: dynamics during fruit development. HortScience. 45(8):1150-1154.

Hou G (Ed.) (2010). Asian noodles: Science, technology, and processing. John Wiley & Sons.

Jacobs DR, Meyer KA, Kushi LH, Folsom AR (1998) Whole-grain intake may reduce the risk of ischemic heart disease death in postmenopausal women: the Iowa Women's Health Study. The American journal of clinical nutrition. 68(2): 248-257.

Jensen MK, Koh-Banerjee P, Hu FB, Franz M, Sampson L, Grønbæk M, Rimm EB (2004) Intakes of whole grains, bran, and germ and the risk of coronary heart disease in men. The American journal of clinical nutrition. 80(6): 1492-1499.

Jerkovic A, Krigel AM, Bradner JR, Atwell BJ, Roberts TH, Willows RD (2010) Strategic distribution of protective proteins within bran layers of wheat protects the nutrient-rich endosperm. Plant Phys. 152: 1459-1470.

Jimenez M, Dubcovsky J (1999) Chromosome location of genes affecting polyphenol oxidase activity in seeds of common and durum wheat. Plant Breed 118:395-398.

Jukanti AK, Bruckner PL, Fischer AM (2004) Evaluation of wheat polyphenol oxidase genes. Cereal Chem. 81(4): 481-485.

Jukanti AK, Bruckner PL, Fisher AM (2006) Molecular and biochemical characterization of polyphenol oxidases in developing kernels and senescing leaves of wheat (Triticum aestivum). Functional Plant Biology. 33: 685-696.

Kahn V (1975) Polyphenol oxidase activity and browning of three avocado varieties. J Sci Fd Agric. 26: 1319-1324.

Kim, YS, Ha TY, Lee SH, Lee HY (1997) Effect of rice bran dietary fiber on flour rheology and quality of wet noodles. Korean Journal of Food Science and Technology (Korea Republic).

Martin JM, Berg JE, Hofer P, Kephart KD, Nash D, Bruckner PL (2011) Allelic variation of polyphenol oxidase genes impacts on Chinese raw noodle color. Journal of Cereal Science. 54:387-394.

Matheis G, Whitaker JR (1984) Modification of proteins by polyphenol oxidase and peroxidase and their products. Journal of Food Biochemistry 8:137-162.

Mayer AM, Harel E (1979) Polyphenol oxidases in plants. Phytochemistry. 18: 193-215.

McCallum JA, Walker JR (1990) O-diphenol oxidase activity, phenolic content and colour of New Zealand wheats, flours and milling streams. Journal of Cereal Science. 12: 83-96.

Mendiburu and the R Development Core Team (2014) Agricolae: Statistical procedures for agricultural research. R package version 1.2-0.

Mergoum M, Frohberg RC, Olson T, Friesen TL, Rasmussen JB, Stack RW (2006) Registration of 'Glenn' wheat. Crop Sci. 46: 473-474.

Meyer KA, Kushi LH, Jacobs DR, Slavin J, Sellers TA, Folsom AR (2000) Carbohydrates, dietary fiber, and incident type 2 diabetes in older women Am J Clin Nutr. 71: 921–930.

Miskelly DM (1996) The use of alkali for noodle processing. Pasta and noodle technology. 227-273.

Morris CF, Jeffers HC, Engle DA (2000) Effect of processing, formula and measurement variables on alkaline noodle color- toward an optimized laboratory system. Cereal Chem. 77(1):77-85.

Nicot N, Hausman JF, Hoffman L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of Experimental Botany. 56 (421):2907-2914.

Nilthong S, Graybosch RA, Baenziger PS, (2012) Inheritance of grain polyphenol oxidase (PPO) activity in multiple wheat (Triticum aestivum L.) genetic backgrounds. Theor Appl Genet. 125: 1705-1715.

Nilthong S, Graybosch RA, Baenziger (2013) Enzyme activity in wheat breeding lines derived from matings of low polyphenol oxidase parents. Euphytica. 190(1):65-73.

Onto S (2011) Genetics of polyphenol oxidase (PPO) activity in wheat (Triticum aestivum L.). Theses, dissertations, and student research in agronomy and horticulture. Paper 35 http://digitalcommons.unk.edu/agronhortdiss/35.

Okot-kotber M, Liavoga A, Yong KJ, Bagorogoza K, (2001) Activity and inhibition of polyphenol oxidase in extracts of bran and other milling fractions from a variety of wheat cultivars. Cereal Chem.78: 514-520.

Park WJ, Shelton DR, Peterson CJ, Martin TJ, Kachman SD, Wehling RL (1997) variation in polyphenol oxidase activity and quality characteristics among hard white wheat and hard red winter wheat samples. Cereal Chem. 74:7-11.

Pinheiro J, Bates D, DebRoy S, Sarkar D and the R Development Core Team (2013) nlme: Linear and nonlinear mixed effects models. R package version 3.1-113.

Raman R, Raman H, Johnstone K, Lisle C, Smith A, Martin P, Allen H (2005) Genetic and in silico comparative mapping of the polyphenol oxidase gene in bread wheat (Triticum aestivum L.) Functional and Integrated Genomics. 5: 185-200.

Rani KU, Prasada Rao UJS, Leelavathi K, Haridas Rao P (2001) Distribution of enzymes in wheat flour mill streams. J. Cereal Sci. 34: 233- 242.

Riede CR, Anderson JA (1996) Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Science. 36: 905-909.

Salmeron J, JE Manson, MJ Stampfer, GA Colditz, AL Wing, WC Willett (1997) Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. JAMA. 277: 472–477.

Slavin JL (2000) Mechanisms for the impact of whole grain foods on cancer risk. Journal of the American College of Nutrition. 19: 300-307.

Sun DJ, He ZH, Xia XC, ZHANG LP, Morris CF, Appels R, Ma WJ, Wang H (2005) A novel STS marker for polyphenol oxidase activity in bread wheat. (2005) Molecular Breeding. 16:209-218.

Swan CG, Meyer FD, Hogg AC, Martin JM, Giroux JM (2006) Puroindoline b limits biding of puroindoline a to starch and grain softness. Crop Sci. 46:1656-1665.

Talbert LE, Hofer P, Nash D, Martin JM, Lanning SP, Sherman JD, Giroux MJ (2013) Hard white versus hard red wheats: taste tests and milling and baking properties. Cereal Chem. 90(3):249-255.

Tran LT, Taylor JS, Constabel CP (2012) The polyphenol oxidase gene family in land plants: Lineage-specific duplication and expansion. BMC Genomics. 13:395.

Ventura E, Davis J, Byrd-Williams C, Alexander K, McClain A, Lane CJ, Spruijt-Metz D, Weigensberg M, Goran M. (2009) Reduction in risk factors for type 2 diabetes mellitus in response to a low-sugar, high-fiber dietary intervention in overweight Latino adolescents. Archives of pediatrics & adolescent medicine. 163(4): 320-327.

Wang XB, MA CX, Si HQ, Qiao YQ, Chang C, He XF, Xia YX (2009) Gene markers for grain polyphenol oxidase activity in common wheat. Mol Breed. 23: 163-170.

Watanabe N, Takeuchi A, Nakayama A (2004) Inheritance and chromosomal location of the homeologous genes affecting phenol colour reaction of kernels in durum wheat. Euphytica. 139: 87-93.

Whitaker L, Lee CY (1995) Recent advances in chemistry of enzymatic browning: Enzymatic browning and its prevention. ACS Symposium Series 600, American Chemical Society: Washington, DC

Zhang LP, Ge XX, He ZH, Wang DS, Yan J, Xia XC, Sutherland MW (2005) Mapping QTLs for polyphenol oxidase activity in a DH population from common wheat. Acta Agronomica Sinica. 31: 7-10.

Zhang D, Moore WR (1997) Effect of wheat bran particle size on dough rheological properties. Journal of the Science of Food and Agriculture. 74(4):490-496.

Zhou M, Robards K, Glennie-Holmes M, Helliwell S, (1999) Analysis of volatile compounds and their contribution to flavour in cereals. Journal of Agricultural and Food Chemistry. 47: 3941-3953.