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GENETICS OF POLYPHENOL OXIDASE (PPO) ACTIVITY IN WHEAT

(*TRITICUM AESTIVUM* L.)

by

Somrudee Onto

A DISSERTATION

Presented to the Faculty of

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In Partial Fulfillment of Requirements

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Major: Agronomy

Under the Supervision of Professor P. Stephen Baenziger

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GENETICS OF POLYPHENOL OXIDASE (PPO) ACTIVITY IN WHEAT

(*TRITICUM AESTIVUM* L.)

Somrudee Onto, Ph.D.

University of Nebraska, 2011

Advisor: P. Stephen Baenziger

The enzyme activity of polyphenol oxidase (PPO) in grain is regarded as a major factor in time-dependent darkening and discoloration of wheat food products during processing or storage. The darkening phenomena of products reduce the quality of products and affect consumer acceptance. Breeding wheat cultivars with low PPO activity is the best way to reduce the undesirable darkening. The low PPO line PI 117635 was crossed to two low PPO wheats from Idaho, IDO580 and IDO377s to determine whether matings between wheats with low levels of grain PPO would result in complementation, and lines with lower or nil PPO would be generated. The $F_{3:4}$ population derived from PI 117635/IDO580 showed no variation in PPO activity. The $F_{3:5}$ and $F_{3:6}$ populations derived from PI 117635/IDO377s grown at Yuma and Oregon fields in 2009 were analyzed for PPO activity and used to determine whether lines with nil PPO activity were generated. Of the 239 lines, 154 lines were identified to have PPO activity that was not significantly different from Ben durum wheat using *t*-test. STS markers, PPO18, PPO29, and STS01 were used to test lines possibly with either low or high PPO levels at *Ppo-A1* and *Ppo-D1* loci. All lines were fixed for the low PPO allele at *Ppo-A1*. However, PPO29 and STS01 showed the opposite results from the *Ppo-D1* allele prediction. To examine inheritance of PPO activity in multiple wheat genetic

backgrounds and to evaluate the relationship between PPO activity and alleles at two PPO loci, 5 populations (PI 117635/Antelope; Fielder/NE03681; Fielder/Antelope; NW07OR1070/Antelope; NW07OR1066/OR2050272H) were developed. STS markers were used to identify the genotypes and a putative third (null) genotype at *Ppo-A1* allele was discovered in NW07OR1066 and NW07OR1070 lines. ANOVA showed all populations had significant genotypic effects on PPO activity. The generations and genotype nested within generation effects were not significant in all populations. The NW07OR1070/Antelope and NW07OR1066/OR2050272H populations had the lowest PPO activities because both populations had a null allele for *Ppo-A1* on chromosome 2A. Of all 5 populations, 3 populations found the reverse results of *Ppo-D1* allele from the previously reported research, indicating the markers for *Ppo-D1* allele give erroneous results in some genetic backgrounds.

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FOREWORD

This dissertation is written for publication in the format required by the Theoretical and Applied Genetics.

LIST OF ABBREVIATIONS

AU – absorbance units

L-DOPA – L-3,4-dihydroxy phenyl alanine

PPO – polyphenol oxidase

OD - Polyphenol oxidase activity

OD/g - Polyphenol oxidase activity on a per gram of kernel

ODT - log transformation of PPO activity

ODT/g - log transformation of PPO activity on a per gram of kernel

CHAPTER 1

Inheritance of Polyphenol Oxidase Activity in Wheat Breeding Lines Derived from Matings of Low Polyphenol Oxidase Parents."

ABSTRACT

Polyphenol oxidase (PPO) in grain plays a major role in time-dependent discoloration of wheat products, especially fresh noodles. Breeding wheat cultivars with low or nil PPO activity is the best approach to reduce the undesirable product darkening. The low PPO line PI 117635 was crossed to two low PPO wheats from Idaho, IDO580 and IDO377s to determine whether matings between wheats with low levels of grain PPO would result in complementation, and lines with lower or nil PPO would be generated. PPO activity was measured by the L-DOPA assay. The population derived from PI 117635/IDO580 displayed no variation in PPO activity among the progeny. The $F_{3:4}$ populations derived from PI 117635/IDO377s and the reciprocal IDO377s/PI 117635 had normal distributions of PPO activity. The ranges of PPO activity for the IDO580/PI 117635, PI 117635/IDO377s, and IDO377s/PI 117635 populations grown in the 2008 Yuma field were 0.025 – 0.141 AU ($\bar{x}$ = 0.097 AU), 0.142 – 0.502 AU ($\bar{x}$ = 0.272 AU), and 0.111 – 0.620 AU ($\bar{x}$ = 0.312 AU), respectively. The range of values indicated that the PI 117635 and IDO377s parents contain alleles associated with higher and lower PPO activity. The 2009 Yuma and Oregon field-grown populations ($F_{3:5}$; $F_{3:6}$) were analyzed for PPO activity and used to determine whether lines with nil PPO activity were generated. The populations tested were from the IDO377s crosses. Of the 239 lines, 154 lines were verified to have PPO activity that was not significantly different from Ben

durum, a low PPO durum cultivar, wheat by a *t*-test. The average kernel PPO activity of the experimental line genotypes with different PCR fragments amplified by STS markers showed that the populations were fixed for the low PPO allele at *Ppo-A1*. Using markers for *Ppo-D1*, it was found that the average PPO activity of lines with the 490-bp PCR fragments from PPO29 was significantly lower than that of lines with 560-bp fragments from STS01. These results disagreed with that predicted for *Ppo-D1* allele from previous reports. Thus, breeders might just ignore *Ppo-D1*, as the markers for *Ppo-D1* allele might predict erroneous phenotypes and genotypes in some wheat backgrounds.

INTRODUCTION

Wheat is among the most widely cultivated of all cereals. Commercial wheat cultivars belong primarily to two polyploid species: hexaploid common wheat (*Triticum aestivum* L., 2n = 6x = 42) containing A, B and D genomes and tetraploid durum wheat (*T. turgidum* subsp. *Durum* (Desf.), 2n = 4x = 28) containing only the A and B genomes (Gooding 2009). In Asia, over 40% of the common wheat flour is used for making noodles (Hou et al. 1998). Two popular types of Asian noodles are white noodles and yellow alkaline noodles. White noodles are made from soft wheat flour with low protein and salt contents. Yellow noodles contain alkaline salt, and are produced from hard wheat flour with high protein content (Nagao 1996). Different types of noodles vary in firmness and texture, but consumer preference requires all noodle types to have a bright and creamy white color (Wang et al. 2009).

Polyphenol oxidase enzyme (PPO, EC 1.14.18.1) is a major cause of the time-dependent discoloration in noodles, chapattis, and other wheat products (Baik et al. 1995; Miskelly 1996; Hatcher et al. 1999; Morris et al. 2000). PPO is a copper enzyme involved in enzymatic browning using phenolic compounds as the primary substrates (Nicolas et al. 2003). PPOs catalyze two reactions: the hydroxylation of *o*-monophenol to *o*-diphenols (E.C. 1.14.18.1; monophenol monooxygenase, tyrosinase, or cresolase) and the dehydrogenation of *o*-diphenols to *o*-quinones (E.C. 1.10.32; diphenol oxygen oxidoreductase, diphenol oxidase, or catecholase) in the presence of molecular oxygen. The quinones react with amines and thiol groups or undergo self-polymerization to produce dark or brown products (Mayer and Harel 1979; Baik et al. 1994; Anderson et al. 2001). Generally, PPO activity is mostly located in the bran and more specifically in the aleurone layer of wheat kernels, which is mostly removed during the milling process (Sullivan 1964). However, residual contamination by the bran layer after milling is still sufficient to cause discoloration of wheat products (Rani et al. 2001). Therefore, developing wheat cultivars having genetically low or nil PPO activity is one of the priorities in wheat breeding programs.

PPO genes have been studied in many plant species such as potato (*Solanum tuberosum*; Hunt et al. 1993), sugarcane (*Saccharum officianrum* L.; Bucheli et al. 1996), and tomato (*Solanum lycopersicum*; Thipyapong et al. 1997) etc. and PPO appears to be encoded by gene families (Sherman et al, 1995). In common wheat, PPO genes are arranged in a multigene family consisting of two distinct phylogenetic groups/clusters with three members each, based upon re-sequencing of clones representing wheat expressed sequence tags (EST) (Jukanti et al. 2004). Each cluster contained three

different forms of PPO, suggesting the presence of at least six PPO genes. Three genes (GenBank Accession Number AY596268, AY596269, and AY596270) are expressed in developing kernels and may influence PPO activity in flour, the other three genes (GenBank Accession Number AY596266, AY596267, and AF507945) express in non-kernel tissues (Demeke and Morris 2002; Jukanti et al. 2004).

Durum wheat cultivars normally have very low or nil PPO activity, whereas common wheat cultivars vary in the amount of PPO activity (Kruger et al. 1994; Baik et al. 1995; Miskelly 1996). Baik et al. (1994) and Park et al. (1997) reported that PPO activity in wheat grain is influenced by both genotype and environment. Jiménze and Dubcovsky (1999), using chromosome substitution lines from three cultivars into Chinese Spring wheat proposed genes located on homoeologous group 2 of wheat chromosomes play a major role in PPO activity. QTL analysis showed that PPO genes may be located on chromosomes 2A, 2B, 2D, 3D and 6B (Jiménez and Dubcovsky 1999; Demeke et al. 2001; Simeone et al. 2002; Raman et al. 2005). In hexaploid wheat, the 2D chromosome was associated with enhanced levels of kernel PPO activity (Anderson and Morris 2001) and in tetraploid wheat, which lacks the D genome, high PPO activity was mapped to the long arm of chromosome 2A (Simeone et al. 2002; Raman et al. 2005). Beecher and Skinner (2011) identified the expression levels for all PPO genes related to wheat quality. Three new sequences were identified and three new genes, *Ppo-A2*, *Ppo-B2* and *Ppo-D2*, were localized to homoeologous group 2 chromosomes. Real-time PCR analysis showed that *Ppo-A1a*, *Ppo-A2b*, *Ppo-D1b* and *Ppo-D2b* in the wheat cultivar 'Alpowa' were expressed to substantial levels in developing wheat kernels, while *Ppo-B2* expression was not detected. The average contributions of *Ppo-A1a* and *Ppo-A2b* from

the A genome were greater than expression *Ppo-D1b* and *Ppo-D2b* from the D genome. This is in agreement with the results of Raman et al. (2005) that a major QTL for controlling the PPO activities was identified on the long arm of chromosome 2A in a DH population derived from Chara (medium-high PPO) and WW2499 (low PPO).

Complementary dominant STS markers, PPO16 and PPO29 were developed based on the DNA sequences of a PPO gene on chromosome 2D. The two functional markers can recognize two haplotypes of *Ppo-D1* gene (He et al. 2007). STS marker PPO18 was designed based on the DNA sequences of a PPO gene on chromosome 2A (Sun et al. 2005). The complete DNA sequence of the PPO gene was constructed with CD908212 and AY596268, designed as *Ppo-A1* and the STS marker PPO18 proved an efficient marker for *Ppo-A1* (He et al. 2007). Based on a wheat grain PPO mRNA sequence (AY15506), a marker designated STS01 marker was found to effectively discriminate two alleles of PPO gene located on chromosome 2D (Wang et al. 2008). STS01 serves as a surrogate marker for PPO16.

Genetic complementation refers to a relationship between two different strains of an organism in which both have homozygous recessive mutations that produce the same phenotype. Complementation occurs when two mutations with the recessive alleles combined together by a cross result in a wild-type phenotype. Non-complementation occurs when crossing the two mutations together results in a mutant phenotype. The complementation test is a simple assay in genetics used to determine if two mutations with similar phenotypes are allelic or non allelic (Yook 2005). In polyploid organisms such as wheat, a different type of complementation can occur when lines carrying recessive alleles at independent loci are mated, and resulting progeny display recessive or

mutant phenotypes. Waxy (amylose-free) wheat is an example of complementation with a polyploidy organism. For example, waxy wheats carry null alleles at the three loci, *wx-A1*, *wx-B1*, and *wx-D1* encoding the granule bound starch synthase (GBSS) (Nakamura et al. 1992; Graybosch 1998). The first waxy wheat was produced by crossing the *wx-D1*single null line 'BaiHuo' and the *wx-A1*/*wx-B1* double null line 'Kanto 107' resulted in progeny that lacked all isoforms of GBSS (Nakamura et al. 1992) and hence lacked endosperm amylose and resulted in the recessive waxy phenotype. The goal of this study was to determine whether matings between wheats with low levels of grain PPO would result in a similar type of complementation, and lines with lower or nil PPO would be generated. Secondly, the relationship between low grain PPO and previously described DNA markers for PPO activity was investigated.

MATERIALS AND METHODS

Plant materials

Hard white spring wheat line PI 117635 (low PPO) was crossed to two low PPO spring wheats from Idaho, IDO580 and IDO377s. PI 117635 is an Australian white spring wheat, originating in the 1930's, derived from the cross: Kenya/Florence//Dundee. IDO580 (Souza et al. 2005) is a semi-dwarf wheat with nil levels of grain PPO. IDO580 was derived from the cross, 'Cadoux' (PI 591905)//'Maya 74'/M2. M2 is a synthetic hexaploid wheat developed at CIMMYT by crossing 'Ruff' [*T. turgidum* subsp. *Durum* (Desf.)] with the CIMMYT *Aegilops tauschii* (Coss.) germplasm # 112, previously found to have very low levels of seed PPO activity. IDO377s (PI 591045), a hard white spring

wheat cultivar (Souza et al. 1997), has been grown in Pacific Northwest. IDO377s was derived from the cross 'Chova'/59Ab10293-5, where Chova has the pedigree: 'Gallo'/'Yecora' reselection/3/'Aurora'//'Kalyonsona'/'Bluebird'. The pedigree of 59Ab10293-5 is 'Norin 10'/'Brevor'//'Baart'/'Onas'. Whole grain of IDO377s has low levels of PPO activity. Two-hundred and thirty nine heads were selected from the F_3 plants of these crosses sown at Yuma, AZ in the spring of 2008. Heads were snapped and planted in the field as F_3 - derived F_4 single plant progeny rows in fall of 2008 at Yuma, AZ. $F_{3:5}$ and $F_{3:6}$ generations were planted at Yuma, AZ in 2009 and Corvallis, OR in 2009, respectively, also as single rows. Seven cultivars, 'Ben' (durum wheat), 'Express', 'IDO377s', 'IDO580', 'Jubilee', 'PI 117635', and 'Seaspray' were included as controls. A completely random design was used with checks replicated a minimum of three times, and experimental lines un-replicated at each location. Means for each line were determined by averaging 2 location (2009) data.

Measurement of PPO activity

The procedure for measuring PPO activity in wheat grains followed the method described by Anderson and Morris (2001). Five whole seeds were weighed. A 1.5-ml aliquot of 10 m*M* L-DOPA (3, 4-dihydroxyphenylalanine, Sigma-Aldrich Co., St. Louis, MO) as substrate in 50 m*M* MOPS [3-(*N*-morpholino) propane sulfonic acid, Sigma-Aldrich Co., St. Louis, MO] buffer, pH 6.5, was added to a 2-ml microcentrifuge tube containing five seeds. The tubes were rotated for 1 hr at room temperature to allow the reaction to occur. Absorbance was measured on a 250-µl aliquot of the incubated solution at 475 nm using a Shimadzu BioSpec-1601 spectrophotometer (Shimadzu

Corporation, Columbia, MD). Seeds from the cultivars Arapahoe (red winter wheat with high PPO activity), Anton (white winter wheat with low to moderate PPO activity), and Ben (spring durum wheat with low PPO activity) were included with every run as experimental laboratory controls to check the consistency of each run. Each reaction was repeated twice. The L-DOPA solution was made fresh daily.

DNA isolation and STS analysis

Genomic DNA was isolated from young leaf tissues using a CTAB (cetyltrimethyl ammonium bromide) method modified from Doyle and Doyle (1987). Sequence tagged site (STS) markers PPO18, PPO29, and STS01 were synthesized by Invitrogen Co. (Carlsbad, CA) using primers presented in He et al. (2007) and Wang et al. (2004). For STS analysis, PCR reactions were performed in a total volume of 25-µl containing 100-ng of genomic DNA, 10-mM Tris-HCl, 1.5-mM $MgCl_2$, 50-mM KCl buffer, 0.2-mM of each dNTP, 0.4-µM of each oligonucleotide primer, 0.028 unit of *Taq* DNA polymerase (Roach, Mannheim, Germany) in a Bio-Rad DNA Engine Peltier Thermal Cycler. The thermocycling program was 95°C for 5 min, followed by 40 cycles of touchdown PCR at 95°C for 1 min, 56-50°C for 1:30 min for PPO18 or 62-57°C for 1 min for STS01, 72°C for 2 min, with a final extension of 72°C for 8 min. PPO29 PCR amplification was performed at 95°C for 5 min, followed by 36 cycles of 94°C for 1 min, 69.6°C for 1 min, 72°C for 1 min, with final extension of 72°C for 8 min. Amplified PCR fragments were separated on a 1.5% agarose gel and stained with ethidium bromide and visualized using UV light.

Statistical analysis

All statistical computations were made using the SAS computer packages version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used to test for significant difference among environment, genotype, and genotype nested within environment using appropriate error terms. Transgressive segregation among lines and parents was determined using a Least Significant Difference procedure with $\alpha = 0.05$. Pairwise comparisions (*t*- test) were used to compare the mean of each line with Ben at $\alpha = 0.05$ to find the lines that were not significantly different from Ben. The PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA) was used for the above statistical analyses. Genotypes were designated BA (*Ppo-A1b/ Ppo-D1b*); BH (*Ppo-A1b/ Ppo-D1a -D1b*); BB (*Ppo-A1b/ Ppo-D1a*); Ben; Express; IDO377s; IDO580; Jubilee; PI 117635l; and Seaspray. PROC UNIVARIATE was used for tested normality. To determine the relationship between kernel PPO activity (Optical density = OD) and kernel PPO activity per gram seed weight (OD/g), Pearson correlations were calculated using PROC CORR. Both actual and log transformed data were analyzed.

RESULTS AND DISCUSSION

A log transformation was used to normalize the data designated as ODT. Both the actual and transformed data showed the same results. Therefore, the tables in this study include both actual and transformed values, and, since the results were the same, only the actual values will be discussed.

The laboratory control cultivars, Arapahoe (high PPO activity), Anton (moderate to low PPO activity) and Ben (low PPO activity), gave average PPO activities (mean ± SE) of 1.032 ± 0.018 AU, 0.376 ± 0.015 AU and 0.075 ± 0.003 AU, respectively (n = 27) (Table 1). While some daily fluctuation was observed in average values for the controls, the relative ranking of the three laboratory controls never changed (Fig. 1).

The 2008 Yuma field-grown populations derived from the crosses PI 117635/IDO580 and PI 117635/IDO377s were analyzed for PPO activity. For the PI 117635/IDO580 cross, the assay indicated there was no variation in PPO level (0.097 ± 0.004 AU; Fig. 2) among the progeny, even though they have different markers for a PPO gene on chromosome 2D (Fig. 2). However, the mean 2008 PPO activity for the lines derived from the cross PI 117635/IDO377s and the reciprocal cross IDO377s/PI 117635 were 0.272 ± 0.008 AU (n = 124) and 0.312 ± 0.011 AU (n = 115), respectively. Observed ranges of PPO activity were 0.142 – 0.502 AU for PI 117635/IDO377s and 0.111– 0.620 AU for IDO377s/PI 117635. The range of values indicated that both parents contain alleles associated with higher and lower PPO activity, even though both parents themselves were first selected due to low PPO activity. The PI 117635/IDO377s population had slightly more lines with low PPO activities and the distribution was not normal (P = 0.02; Fig 3A), whereas the reciprocal IDO377s/PI 117635 population had a normal distribution (P = 0.65; Fig 3B).

The 2009 Yuma (Yuma09) and Oregon (OR09) field-grown populations derived from the cross PI 117635/ IDO377s, two parents and five check cultivars (Ben, Express, IDO580, Jubilee and Seaspray) were analyzed with the PPO L-DOPA assay and the STS

markers to determine whether lines with nil PPO (defined as PPO levels equal to Ben durum wheat) were generated, and, if so, did the trait relate to previously identified DNA markers. Two complementary dominant STS markers, STS01 and PPO29 were tested for PPO genes located on chromosome 2D. In previous reports, STS01 marker amplified a 560-bp PCR fragment in most cultivars with low PPO activity, which corresponded to the *Ppo-D1a* allele (Wang et al. 2008). PPO29 reportedly amplified a 490-bp PCR fragment in cultivars with high PPO activity, indicating the presence of *Ppo-D1b* allele (He et al. 2007). In previous papers, the codominant marker for alleles at *Ppo-A1*, PPO18, amplified two fragments, a 685-bp PCR fragment in the cultivars with high PPO activity (*Ppo-A1a* allele) and a 876-bp PCR fragment in the cultivars with low PPO activity (*Ppo-A1b* allele) (Sun et al. 2005; He et al. 2007). Of the 239 lines derived from the cross PI 117635/ IDO377s, 119 lines amplified a 490-bp PCR fragment (A pattern) with PPO29 marker and STS01 amplified a 560-bp PCR fragment (B pattern) in 98 lines. The remaining 22 lines amplified both 490-bp and 560-bp PCR fragments (H pattern) with PPO29 and STS01 markers (heterozygous lines). Based on Mendel's law, expected proportions of $F_{3:5}$ genotypes are 3A: 2H: 3B. A chi-square test was used for analyze the genotypic ratio of marker segregation in F_3 -derived F_5 materials. Because the chi-square probability was 34.26, this probability was over the critical value of 0.05 ($\lambda^2_{0.05,\,2} = 5.99$), indicating the segregation was far from the expected 3A: 2H: 3B ratio. The deviation from expected arose from a deficiency of H individuals. The A and B classes fit the expected 1: 1 ratio ($\lambda^2 = 2.03$; $P = 0.154$). PPO18 marker amplified a 865-bp fragment from all of 239 lines, indicating all lines were fixed for the putative low PPO allele at *Ppo-A1*. From the marker screening, the genotypes of progeny were assigned to three

classes: *Ppo-A1b/ Ppo-D1b* designated as BA; *Ppo-A1b/ Ppo-D1a -D1b* designated as BH; *Ppo-A1b/ Ppo-D1a* designated as BB.

From the Yuma and Oregon 2009 samples, L-DOPA assay activity was calculated on both a raw (OD) and on a per gram of kernels basis (OD/g) to determine whether seed weight had an effect on the observed values. Analysis of variance showed highly significant differences among environment, genotype (PPO alleles) and genotype nested within environment interaction (Table 2), suggesting a differential effect of environment on expression of grain PPO activity, confirming previously observed environmental effects on levels of wheat PPO activity (Park et al. 1997; Mc Caig et al. 1999; Demeke et al. 2001). Identical results (Table 2) were obtained using OD and OD/g. Pearson correlation coefficients were calculated separately for each environment and for the combined two environments, in order to determine possible relations between OD and OD/g. R-values of Yuma09, OR09 and the combined environments were 0.93, 0.98 and 0.95, respectively, indicating OD was highly correlated with OD/g in all cases (P-value < 0.0001). Weighing seed before assaying PPO activity is, therefore not necessary, and this result would allow breeding programs to evaluate more materials with less labor.

PPO activity of lines derived from PI 117635/ IDO377s, and the reciprocal cross, was assayed from two environments, Yuma09 and OR09. The Yuma09 and OR09 population means for PPO activity were 0.183 ± 0.004 AU and 0.099 ± 0.003 AU (n = 239), respectively, lower than the values observed for initial 2008 observations. The minimum and maximum PPO activities were 0.091 and 0.373 AU for Yuma09 and 0.025 and 0.247 AU for OR09. The average PPO activity for the two environments was 0.141

± 0.004 AU. The minimum and maximum PPO activities of lines from the PI 117635/ IDO377s population were 0.065 and 0.258 AU. The overall average PPO activity for the parents was 0.079 ± 0.012 AU for PI 117635 (n = 9) and 0.132 ± 0.009 AU for IDO377s (n = 8). The check cultivars, Ben, Express, IDO580, Jubilee and Seaspray gave average PPO activities of 0.052 ± 0.004 AU (n = 8), 0.249 ± 0.061 AU (n = 8), 0.059 ± 0.010 AU (n = 7), 0.157 ± 0.030 AU (n = 8) and, 0.180 ± 0.034 AU (n = 6), respectively. The PPO activity distribution of the PI 117635/ IDO377s population was normal (P = 0.41) whereas the reciprocal IDO377s/ PI 117635 population was not normal (P = 0.01) (Fig. 4A and 4B) and some progenies exhibited higher PPO activity than those of either parent (transgressive segregation). Transgressive segregation mostly results from the complementary gene action (the appearance of combinations of alleles from both parents affecting a trait in the same direction) (Rieseberg et al. 2003). The LSD test at $\alpha = 0.05$ confirmed the presence of transgressive segregation within the population. Therefore, PPO activity is controlled by multiple loci for the PI 117635/ IDO377s population and intermatings of such low PPO lines might generate segregates with nil levels of PPO activity. Of the 239 lines, 154 lines were proven to have PPO activity that was not significantly different from Ben durum wheat by *t*-tests statistical analysis ($LSD_{\alpha} = 0.05$). It is clear now that variation exists among common wheats to derive materials with PPO activity as low as Ben. IDO580 was as low as Ben, but it was derived from a synthetic population, meaning it has genes from *Triticum tauschii*, and the materials derived from IDO377s/PI 117635 only have genes from common wheats.

The average kernel PPO activity of the experimental line genotypes with different PCR fragments amplified by STS markers from Yuma09 and OR09 were 0.11, 0.15 and

0.17 AU for *Ppo-A1b/ Ppo-D1b* (BA), *Ppo-A1b/ Ppo-D1a -D1b* (BH) and *Ppo-A1b/ Ppo-D1a* (BB) genotypes, respectively (Table 3). Among the 239 wheat lines measured with the L-DOPA assay, the results showed that 119 lines (49.79%) amplified an 876-bp PCR fragment with PPO18 marker and a 490-bp fragment with PPO29. However, 98 lines (41%) also showed high levels of PPO activity and amplified 876-bp and 560-bp fragments with PPO18 and STS01, respectively. Statistical analysis indicated that the average PPO activity of lines with 490-bp fragments was significantly lower than that of the lines with 560-bp PCR fragments. This result does not agree with earlier reports for STS01 and PPO29 markers, in which STS01 was a dominant marker for low PPO activity, and would amplify a 560-bp fragment in most low PPO cultivars (Wang et al. 2008), while PPO29 would amplify a 490-bp PCR fragment in most high PPO cultivars (He et al. 2007). The *Ppo-A1b/ Ppo-D1b* (BA) genotype gave the lowest PPO activity and it was significantly lower than *Ppo-A1b/ Ppo-D1a* (BB) and *Ppo-A1b/ Ppo-D1a -D1b* (BH) genotypes. All lines produced the 876-bp fragment from PPO18, indicating all lines were fixed for the low allele at *Ppo-A1* while the results of *Ppo-D1* alleles were opposite from the prediction. However, all genotypes showed low levels of PPO activity when compared with the check cultivars. Previous studies found that a major locus for grain PPO activity was located on the long arm of chromosome 2A in a doubled haploid population derived from Chara/WW2449 using QTL approach (Raman et al. 2005). Subsequently, Beecher and Skinner (2011) identified and quantified expression levels for all PPO genes relevant to wheat quality and found that the A genome contributed greater grain PPO expression than the D genome and the A genome contributed more than 80% of the PPO gene transcripts present in the developing seeds. This is in agreement with

Martin et al. (2011) who found both *Ppo-A1* and *Ppo-D1* loci affected the levels of grain PPO activity (P<0.01) but the effect for *Ppo-A1* was larger than that for *Ppo-D1*. However, the expression of PPO gene located on chromosome 2B was not detected in developing kernels of 'Alpowa' (Beecher and Skinner 2011).

In conclusion, crosses of low to low PPO lines did produce progeny with nil levels of PPO activity. Our study showed that the populations were fixed for the low allele at *Ppo-A1*, yet the PI 117635/ IDO580 population showed no segregation for PPO, and, in the PI 117635/ IDO377s, the results were contrary to those previously predicted for *Ppo-D1* allele. These results indicate breeders might ignore *Ppo-D1*, as most of the activity comes from *Ppo-A1*, and the markers for *Ppo-D1* allele might predict erroneous phenotypes and genotypes in some backgrounds. In addition, both genotypic and genotypic x environment effects modulate the expression of PPO activity.

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Table 1. Means and standard error for polyphenol oxidase activity of experimental controls (n = 27)

Experimental controls	PPO activity (means ± SE)
Arapahoe	1.032 ± 0.018
Anton	0.376 ± 0.015
Ben	0.075 ± 0.004

Table 2. Mean squares from analysis of variance of kernel PPO activity within PI 117635/ IDO377s population grown in Yuma and Oregon field in 2009

Source of variation	df	Mean square			
		PPO activity (OD)[a]	PPO activity (OD/g)[b]	PPO activity (ODT)[c]	PPO activity (ODT/g)[d]
Environment	1	0.2291*	2.7395*	0.1660*	0.8003*
Genotype	9	0.0718*	2.6917*	0.0538*	0.9186*
Environment x genotype	9	0.0131*	0.2458*	0.0084*	0.0430*
Error	510	0.0021	0.0593	0.0015	0.0181

[a]Polyphenol oxidase activity
[b]Polyphenol oxidase activity on a per gram of kernel
[c]log transformation of PPO activity
[d]log transformation of PPO activity on a per gram of kernel
*significant at p = 0.05

Table 3. Average kernel PPO activities of the genotypes with different PCR fragments amplified by PPO18, PPO29 and STS01 markers within PI 117635/IDO377s population

Genotypes	N	OD	OD/g	ODT	ODT/g
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
BA	238	0.113 ± 0.004	0.536 ± 0.017	0.106 ± 0.003	0.416 ± 0.010
BH	44	0.151 ± 0.009	0.753 ± 0.038	0.140 ± 0.008	0.551 ± 0.022
BB	194	0.174 ± 0.005	0.938 ± 0.020	0.159 ± 0.004	0.652 ± 0.010
Ben	8	0.053 ± 0.004	0.254 ± 0.014	0.051 ± 0.004	0.226 ± 0.011
Express	8	0.249 ± 0.061	1.264 ± 0.275	0.214 ± 0.047	0.770 ± 0.113
IDO377s	8	0.132 ± 0.009	0.872 ± 0.056	0.124 ± 0.008	0.624 ± 0.030
IDO580	7	0.059 ± 0.010	0.316 ± 0.046	0.057 ± 0.010	0.271 ± 0.036
Jubilee	8	0.157 ± 0.030	0.810 ± 0.106	0.144 ± 0.026	0.581 ± 0.059
PI 117635	9	0.079 ± 0.012	0.342 ± 0.033	0.073 ± 0.011	0.292 ± 0.024
Seaspray	6	0.180 ± 0.034	0.872 ± 0.103	0.163 ± 0.029	0.620 ± 0.055

BA = *Ppo-A1b/ Ppo-D1b*
BH = *Ppo-A1b/ Ppo-D1a-D1b*
BB = *Ppo-A1b/ Ppo-D1a*

Figure 1. Average PPO activity of experimental controls; Arapahoe, Anton and Ben

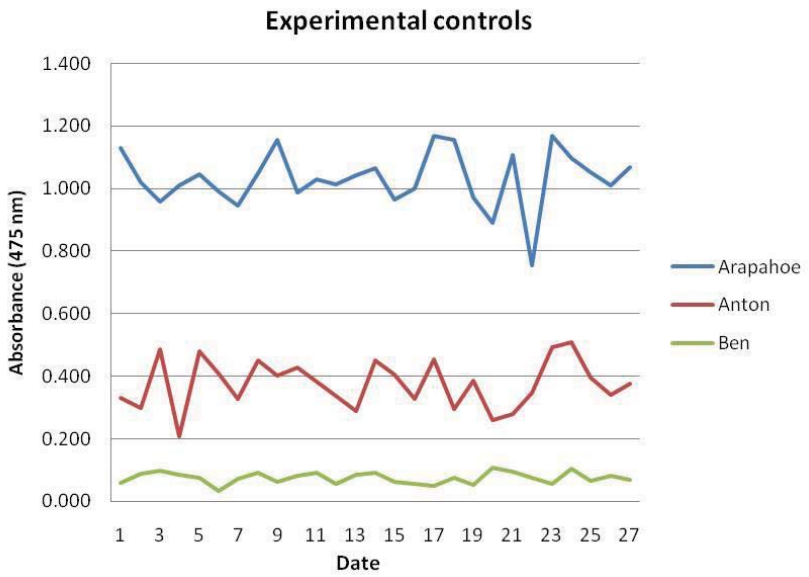


Figure 2. Distribution of mean PPO activity from IDO580/ PI 117635 populations grown in Yuma field in 2008

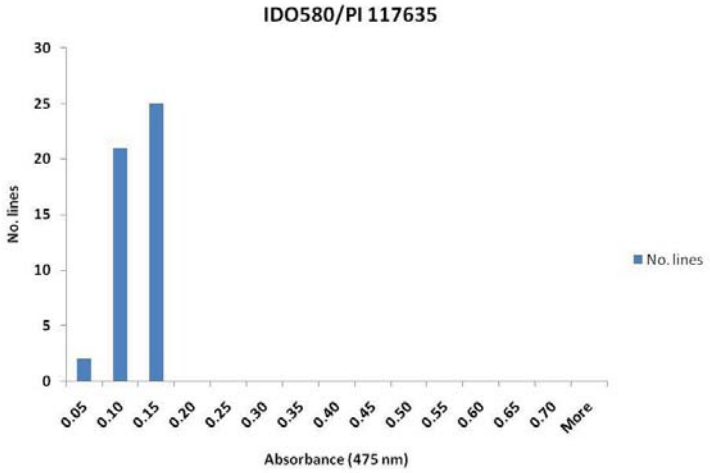


Figure 3. Distribution of mean PPO activity from PI 117635/ IDO377s (A) and IDO377s/ PI 117635 (B) populations grown in Yuma field in 2008

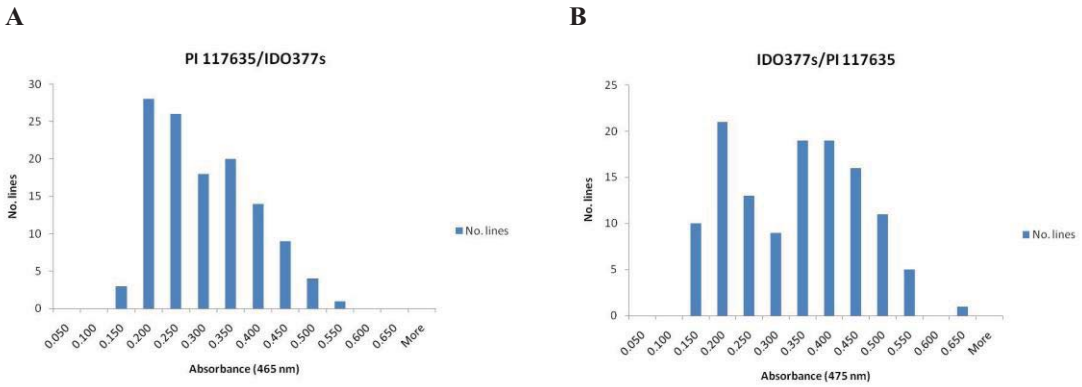
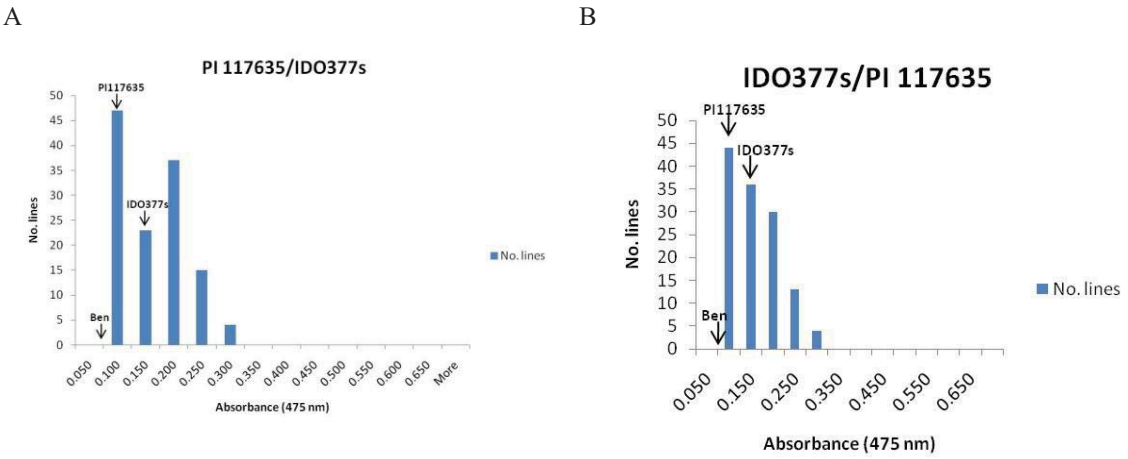


Figure 4. Distribution of mean kernel PPO activity from PI 117635/ IDO377s (A) and IDO377s/ PI 117635 (B) populations grown in Yuma and Oregon field in 2009. Arrows indicate the mean values of parents of the PI 117635, IDO377s, and Ben.



Chapter 2

Inheritance of grain polyphenol oxidase (PPO) activity in multiple wheat (*Triticum aestivum* L.) genetic backgrounds

ABSTRACT

Polyphenol oxidase (PPO) is involved in the time-dependent discoloration of wheat (*Triticum aestivum* L.) products. Five crosses (PI 117635/Antelope; Fielder/NE03681; Fielder/Antelope; NW07OR1070/Antelope; NW07OR1066/OR2050272H) were selected to study the genetic inheritance of PPO activity. STS markers, PPO18, PPO29 and STS01 were used to identify lines possibly with either low or high PPO alleles at the *Ppo-A1* and *Ppo-D1* loci. However, a putative third (null) genotype (no PCR fragments for PPO18) was discovered in NW07OR1066 and NW07OR1070 derived lines. ANOVA showed all populations had significant genotypic effects on PPO activity ($P < 0.0001$). The generations and genotype nested within generation effects were not significant in any population. Of all 5 populations, the NW07OR1070/Antelope and NW07OR1066/OR2050272H populations had the lowest PPO activities as both populations had a null allele for *Ppo-A1* on chromosome 2A. Results demonstrated both *Ppo-A1* and *Ppo-D1* loci affect the kernel PPO activity but the *Ppo-A1* has the major effect. The PI 117635/Antelope, NW07OR1070/Antelope, and NW07OR1066/OR2050272H populations showed the reverse results of *Ppo-D1* alleles from the marker prediction, suggesting the markers for *Ppo-D1* allele might give erroneous results in some genetic backgrounds.

INTRODUCTION

Polyphenol oxidase (PPO, E.C. 1.14.18.1), a nuclear-encoded copper containing enzyme, is ubiquitously distributed in plants (Robb 1984; Flurkey 1989; Steffens et al. 1994; Lee and Whitaker 1995). PPO catalyzes the formation of quinones from phenols in the presence of molecular oxygen. The quinones react with amines and thiol groups or undergo self-polymerization to produce dark or brown products (Mayer and Herel 1979). In wheat (*Triticum aestivum* L.), PPO is a factor in time-dependent discoloration of noodles, chapattis, and other wheat products (Baik et al. 1995; Kruger et al. 1994; Feillet et al. 2000; Singh and Sheoran 1972). Significant PPO activity has been detected in the endosperm of immature seeds. As the kernel ripens, the level of PPO activity in the endosperm decreases, whereas the level of PPO activity in the outer layers and embryo increases (Kruger 1976; March and Galliard 1986), predominately in the aleurone layer which is mostly removed during the milling process (Bertrand and Muttermilch 1907; Sullivan 1946). However, residual contamination by the bran layer after milling appears to be sufficient to cause discoloration during food processing and storage (Rani et al. 2001).

Levels of PPO activity in wheat grain are very different among different genotypes, and PPO activity is also influenced by environment (Baik et al. 1994; Park et al. 1997). PPO activity can be measured on flour or whole seed using either oxygen consumed (Marsh and Galliard 1986) or the production of colored products (spectrophotometric method). Enzyme substrates have included phenol (Wrigley 1976), catechol (Milner and Gould 1951), L-tyrosine (Bernier and Hows 1994; McCaig et al.

1999) and L-3,4-dihydroxyphenyl alanine (L-DOPA) (Anderson and Morris 2001). Whole seed assays have been recognized as efficient methods of characterizing wheat germplasm for PPO variation (Raman et al. 2005).

PPOs are often encoded by mutigene families in higher plant species such as potato (*Solanum tuberosum* L.) (Thygesen et al. 1995), sugarcane (*Saccharum officinarum* L.) (Bucheli et al. 1996), and tomato (*Lycopersicon esculentum* Mill.) (Thipyapong et al. 1997). Previous studies showed that genes controlling PPO activity are located on wheat homoeologous group 2 (Udall 1996; Jimenez and Dubcovsky 1999; Anderson and Morris 2001; Demeke et al. 2001; Mares and Campbell 2001), group 3 (Udall 1996; Demeke et al. 2001), group 5 (Udall 1996), and 6B chromosomes (Demeke et al. 2001) of bread wheat. QTLs influencing PPO activity have been localized on 5B and 7D chromosomes and on homoeologous group 6 using nullisomics and tetrasomic lines of Chinese Spring using a maize (*Zea mays* L.) PPO gene probe (Li et al. 1999). A study by Jimenze and Dubcovsky (1999) showed that genes located in homoeologous group 2 of the wheat chromosome play a major role in PPO activity. Subsequently, Raman et al. (2005) identified a major locus controlling PPO activities on the long arm of chromosome 2A (2AL) in a doubled-haploid population derived from Chara/WW2449, explaining 82-84% of the genetic variation, using a QTL mapping approach. These loci contain the genes actually encoding the enzymes.

Demeke and Morris (2002) cloned the first partial sequence of a wheat PPO gene (Genbank accession number AF507945) using a pair of primers designed from conserved copper-binding regions of other plant PPO genes. Subsequently, Jukanti et al. (2004)

cloned several additional partial sequences of wheat PPO genes by assembling expressed sequence tags (ESTs). These PPO genes were classified into two clusters on the basis of similarity of the sequences. One cluster contained three PPO genes (Genbank accession numbers AY596268, AY596269, and AY596270) believed to be expressed in developing kernels and perhaps influencing PPO activity in wheat flour. The other three genes (Genbank accession numbers AY596266, AY596267, and AF507945) expressed in non-kernel tissues. Sun et al. (2005) developed a STS marker PPO18 derived from a PPO gene located on chromosome 2AL (Genbank accession number AY596268) which was considered to be an efficient molecular marker for wheat kernel PPO activity. PPO18 amplified a 685-bp and an 876-bp PCR fragment from cultivars with high and low PPO activity, respectively. The 191-bp variation was found in the first intron of a PPO gene (AY596268) located on the chromosome 2AL between cultivars with high PPO activity and low PPO activity (Sun et al. 2005). The additional 191-bp insertion sequence might influence the splicing of premature mRNA, which could interfere with the expression of PPO gene. Therefore, variations in PPO genes do have an effect on PPO activity in wheat (Sun et al. 2005).

He et al. (2007) characterized a complete genomic DNA sequences of two PPO genes designated *Ppo-A1* and *Ppo-D1*, one each located on chromosome 2A and 2D, respectively, and developed two complementary dominant STS markers, PPO16 and PPO29 based on the PPO gene haplotypes located on chromosome 2D, for the *Ppo-D1* gene. PPO16 amplified a 713-bp fragment in cultivars with low PPO activity and a 490-bp fragment in cultivars with high PPO activity. Based on a wheat grain PPO mRNA sequence (AY15506), a STS marker (STS01) was developed which could effectively

discriminate two alleles of the *Ppo-D1* gene (Wang et al. 2008). Comparison of STS01with the STS marker PPO29 showed that STS01 was the complementary maker of PPO29. STS01 amplified a 560-bp PCR fragment in cultivars with mostly low PPO activity (Wang et al. 2008).

Use of molecular markers associated with PPO activity has the potential to improve selection efficiency for low PPO activity. In a previous study, we crossed the low PPO line, PI 117635, with the low PPO wheat from Idaho, IDO377s. The average kernel PPO activity of the progeny genotypes with different PCR fragments amplified by STS markers (PPO18, PPO29, and STS01) showed the population was fixed for the low allele at *Ppo-A1* whereas PPO29 and STS01 showed the opposite results from the *Ppo-D1* allele prediction. On the basis of the opposite results in the previous study, we developed five new populations (1) to further examine inheritance of PPO activity in multiple wheat genetic backgrounds and (2) to further evaluate the relationship between PPO activity and alleles at two PPO loci.

MATERIALS AND METHODS

Plant materials

Eight U.S. white winter wheats grown under greenhouse and field conditions were used to determine whether PPO activity levels are consistent between the two environments. Each reaction was repeated three times. To develop populations to study the genetics of polyphenol oxidase, twenty-seven white wheat genotypes including

Australian, U.S. spring and winter wheats were checked with markers. These lines were used as parents to develop populations. Based on the results using the three markers, 120 crosses were produced in a greenhouse in spring 2009. Five crosses were selected based on the parental genotypes and seed number. All F_1 seeds of each cross were planted in the greenhouse in fall 2009. The heads of each F_1 plant were harvested and threshed as F_2 seeds. One-hundred and forty four F_2 seeds were randomly planted per population in the greenhouse in spring 2010 and used to evaluate the correlation between PPO levels and PPO alleles at the *Ppo-A1* and *Ppo-D1* loci. One to three seeds per F_2 head were randomly planted as F_3 generations. Each plant was harvested and threshed as $F_{3:4}$ seeds.

Measurement of PPO activity

Polyphenol oxidase activity in whole wheat grains was determined for all lines in the F_2 and F_3 generations using the L-DOPA (3, 4-dihydroxyphenylalanine, Sigma-Aldrich Co., St. Louis, MO) assay reported by Anderson and Morris (2001). A 1.5-ml aliquot of 10 m*M* L-DOPA as substrate in 50 m*M* MOPS [3-(*N*-morpholino) propane sulfonic acid, Sigma-Aldrich Co., St. Louis, MO] buffer, pH 6.5, was added to five seeds in a 2-ml microcentrifuge tube. Seed samples were weighed before addition of substrate. The tubes were rotated for 1 hr at room temperature to allow the reaction to occur. Absorbance was measured on a 250-µl aliquot of the incubated solution at 475 nm using a Shimadzu BioSpec-1601 spectrophotometer (Shimadzu Corporation, Columbia, MD). Seeds from the cultivars Arapahoe (red winter wheat with high PPO activity), Anton (white winter wheat with low to moderate PPO activity), and Ben [spring durum (*Triticum turgidum* L.) wheat with low PPO activity] were included as experimental

controls to check the consistency of each run. Each reaction was repeated two times. The L-DOPA solution was made fresh daily. L-DOPA assay was not calculated on a per gram of kernels basis (OD/g) because in a previous experiment (chapter 1) OD and OD/g gave identical results.

DNA isolation and STS analysis

Genomic DNA from each line including parents was extracted from young leaf tissues using a CTAB (cetyltrimethyl ammonium bromide) method described by Doyle and Doyle 1987. Sequence tagged site (STS) markers PPO18, PPO29, and STS01 were synthesized by Invitrogen Co. (Carlsbad, CA) using primers presented in He et al. (2007) and Wang et al. (2008). PPO18 marker has been reported to amplify a 685-bp PCR fragment from the cultivars with high PPO activity, indicating *Ppo-A1a* and an 876-bp fragment from the cultivars with low PPO activity, indicating the *Ppo-A1b* allele. PPO29 marker reported to amplify a putative 490-bp fragment from cultivars with high PPO activity, designated *Ppo-D1b* and STS01 amplified a putative 560-bp fragment in those with low PPO activity, designated *Ppo-D1a* (He et al. 2007; Wang et al. 2008).

PCR reactions were performed in a total volume of 25-µl containing 100-ng of genomic DNA, 10-mM Tris-HCl, 1.5-mM $MgCl_2$, 50-mM KCl buffer, 0.2-mM of each dNTP, 0.4-µM of each oligonucleotide primer, 0.028 unit of *Taq* DNA polymerase (Roach, Mannheim, Germany) in a Bio-Rad DNA Engine Peltier Thermal Cycler. The thermocycling program was 95°C for 5 min, followed by 40 cycles of touchdown PCR at 95°C for 1 min, 56-50°C for 1:30 min for PPO18 or 62-57°C for 1 min for STS01, 72°C for 2 min, with a final extension of 72°C for 8 min. PPO29 PCR amplification was

performed at 95°C for 5 min, followed by 36 cycles of 94°C for 1 min, 69.6°C for 1 min, 72°C for 1 min, with final extension of 72°C for 8 min. Amplified PCR fragments were separated on a 1.5% agarose gel and stained with ethidium bromide and visualized using UV light.

Statistical analysis

All statistical computations were made using the SAS computer packages version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used to test for significant differences among generation, genotype, and genotype nested within generation using PROC GLM. Chi-square tests were used to analyze the genotypic ratio of marker segregation using PROC FREQ. PROC UNIVARIATE was used for testing normality and data were transformed by taking a square-root. PROC MIXED was used to test population means against each other.

RESULTS

Comparison of greenhouse and field PPO activities

Eight white wheat genotypes (Table 1) were used to determine if PPO activity can be accurately measured on wheat grown under greenhouse conditions. The results showed that the PPO activity of greenhouse grown materials ranged from 0.170 to 0.704 absorbance units (AU) at 475 nm, whereas the field grown materials ranged from 0.355 to 1.034 AU. Statistical analysis showed a highly significant difference of average PPO activity among wheats grown under greenhouse and field conditions. The means for PPO

activities from the field were approximately two times higher than greenhouse grown materials (Table 1). However, the ranking of genotypes remained unchanged, and the low PPO genotype Anton was significantly lower than all other genotypes in both tests. As a result, experiments on greenhouse-grown samples were used for PPO measurement. The daily laboratory control cultivars, Arapahoe (high PPO activity, hard winter wheat), Anton (moderate to low PPO activity, hard white winter wheat) and Ben durum wheat (low PPO activity) gave average PPO activity 0.903 ± 0.018 AU (means ± SD), 0.422 ± 0.015 AU and 0.065 ± 0.003 AU, respectively.

Genetic effects of PPO alleles on grain PPO activity

PCR-based molecular markers, PPO18, PPO29, and STS01 were used to identify lines possibly with either low or high PPO levels. These lines were used as parents to develop populations for genetic studies. Twenty-seven cultivars were checked with markers and results showed in Table 2. However, in these materials, a putative third (null) genotype (no PCR fragments for PPO18) was discovered in NW07OR1040, NW07OR1062, NW07OR1066, NW07OR1070, NW07OR1071, NW07OR1073, and NW07OR1074. All these lines were derived from the same parents (PI 117635/SeaSpray). Five crosses, PI 117635/Antelope, Fielder/NW03681, Fielder/Antelope, NW07OR1070/Antelope, and NW07OR1066/OR2050272H were created in a greenhouse in spring 2009 (Table 3) and planted in the greenhouse as F_1 generation in fall 2009. STS markers were used to confirm the hybrid nature of each F_1 plant. PPO18 produced 685-bp (A pattern) and 876-bp (B pattern) PCR fragments, identifying *Ppo-A1a* and *Ppo-A1b*, respectively. Each F_2 and F_3 line was classified as

homozygous for either *Ppo-A1a* (A pattern) or *Ppo-A1b* (B pattern) or mixed (*Ppo-A1ab*; H pattern), meaning the line was a heterozygous plant. Complementary STS marker PPO29 and STS01 amplified a 490-bp product (A pattern), indicating *Ppo-D1b* and a 560-bp fragment (B pattern), indicating *Ppo-D1a*, respectively. Similarly, each line was classified as homozygous for either *Ppo-D1b* (A pattern) or *Ppo-D1a* (B pattern) or mixed (*Ppo-D1ab*; H pattern). As shown in Table 4, the F_1 genotypes of all five crosses consisted of both *Ppo-A1* and *Ppo-D1* alleles from the parents. However, of the 5 crosses, two crosses did not show PCR fragments from PPO18, designated null genotype (N pattern), because the NW07OR1070 and NW07OR1066 parents had no PCR product from PPO18.

PI 117635/Antelope

ANOVA found highly significant differences among genotypes whereas generations and genotype nested within generation were not significant (Table 5). The PI 117635 parent had a lower mean PPO activity than the second parent, Antelope, for both generations. The parents PI 117635 and Antelope had 0.073±0.002 AU and 0.606±0.042 AU when grown with the F_2 generation, and 0.126±0.008 AU and 0.466±0.157 AU when grown with the F_3 generation, respectively. This population had a normal distribution of PPO activity for the F_2 generation whereas the F_3 generation was normally distributed only for the transformed data (Fig. 1A). The population means for PPO activities were 0.388±0.016 AU for the lines in the F_2 generation and 0.368±0.021 AU for the lines in the F_3 generation. The range of PPO activity for the whole population was 0.043 to 0.797 for the lines in the F_2 generation and 0.030 to 1.186 for the line in the F_3 generation. The

lines plus parents for both two generations were assayed using STS markers and classified into 9 genotypes; AA, AH, AB, HA, HH, HB, BA, BH, and BB. Based on Mendel's law, expected proportions of F_2 genotypes are 1AA: 2AH: 1AB: 2HA: 4HH: 2HB: 1BA: 2BH: 1BB and 9AA: 6AH: 9AB: 6HA: 4HH: 6HB: 9BA: 6BH: 9BB ratio for F_3 generation. A chi-square test was used to analyze the genotypic ratio of marker segregation in F_2 and F_3 materials. The chi-square probability was 8.733 for F_2 and 7.494 for F_3 generations, which were less than the critical value of 0.05 ($\lambda^2_{0.05, 8} = 15.507$), hence the segregation fit the expected ratio (Table 6 - 7). Average PPO activity of each genotype for F_2 and F_3 generations is shown in Table 8 and 9, respectively. For both generations, BB genotype was expected to have the lowest PPO activity and AA genotype was expected to have the highest PPO activity. Results confirmed this expectation.

Fielder/NW03681

The genotypic and generation effects were highly significant based on ANOVA. However, genotype nested within generation effect was non-significant (Table 5). This population had a normal distribution of PPO activity for the F_2 generation. For F_3 generation, the distribution was not a normal distribution but it was a normal distribution when data were transformed (Fig. 1B). The average PPO activity for the parents was 0.584±0.012 AU when grown with the F_2 generation and 0.464±0.098 AU when grown with the F_3 generation for Fielder; and 0.726±0.000 AU when grown with the F_2 generation and 0.361±0.015 AU with the F_3 generation for NW03681. The minimum and maximum PPO activities for the lines in the F_2 generation were 0.085 and 1.113 AU, and

0.090 and 1.438 AU for the lines in the F_3 generation. Chi-square test was used to analyze the genotypic ratio of marker segregation in the F_2 and F_3 generations. The chi-square probabilities were 14.386 and 8.834 for the F_2 and F_3 generations (Table 6-7), respectively, which were non-significant. Their genotypic ratios fit the expected genetic hypothesis (see above). The average PPO activity for each genotype is shown in Table 8 and 9. The BB genotype had the lowest PPO activity in both the F_2 and F_3 generations which was agreement with the marker expectation.

Fielder/Antelope

The Fielder/Antelope population had a normal distribution for PPO activity for the F_2 generation whereas the distribution was not normal for the F_3 generations (Fig. 1C). ANOVA revealed significant differences among genotypes but there were not significant generations and genotype nested within generation interaction (Table 5). The parents Fielder and Antelope had similar PPO activities when grown with the F_2 and F_3 generations (0.584±0.012 and 0.606±0.042 AU when grown with the F_2 generation and 0.464±0.098 and 0.466±0.157 AU when grown with the F_3 generation, respectively). The mean PPO activities were 0.534±0.017 AU for the lines in the F_2 generation and 0.650±0.030 AU for the lines in the F_3 generation. The PPO activity for the lines in the F_2 generation ranged from 0.136 to 0.945 AU and from 0.085 to 1.793 AU for the lines in the F_3 generation. These results demonstrate low PPO lines can be derived from matings of cultivars with high PPO activities. The chi-square probability was 17.677 for the F_2 and 19.555 for the F_3 generation (Table 6-7), these probabilities were higher than the critical value of 0.05 ($\lambda^2_{0.05,\,8}$ = 15.507), meaning that the observed chi-square was

significantly different than expected. Therefore, the observed numbers were not consistent with those expected under our genetic hypothesis (see above). The average PPO activity of each genotype is shown in the Tables 8 and 9. Both the F_2 and F_3 generations, the BB genotype had the lowest PPO activity and the AA genotype had the highest PPO activity. These observations were agreement with the expectation based on previous marker work.

NW07OR1070/Antelope

ANOVA identified a highly significant difference among genotypes whereas generations and genotype nested within generation did not show significance (Table 5). The NW07OR1070 parent had a lower average PPO activity than the Antelope parent. The NW07OR1070/Antelope population had a normal distribution of PPO activity for the F_2 generation. For the F3 generation, the distribution was normal when using the transformed data (Fig. 1D). The PPO activity for the population ranged from 0.024 to 0.745 AU for the lines in the F_2 generation and 0.031-1.431 AU for the lines in the F_3 population. The mean PPO activities were 0.355±0.016 AU for the lines in the F_2 generation and 0.342±0.027 AU for the lines in the F_3 generation. This population had lower PPO activity than PI 117635/Antelope, Fielder/NW03681, and Fielder/Antelope populations for both generations. The genotypic classes were identified with STS markers and classified into six genotypes; 1AA: 2AH: 1AB: 2HA: 4HH: 2HB: 1BA: 2BH: 1BB for the F_2 generation and 9AA: 6AH: 9AB: 6HA: 4HH: 6HB: 9BA: 6BH: 9BB for the F_3 generation. Chi-square test was analyzed and showed that the chi-square values were 5.287 and 10.841 for the F_2 and F_3 generations (Table 6-7), respectively.

These values were less than the critical value of 0.05 ($\lambda^2_{0.05, 5}$ = 11.070), meaning that the observed genetic ratio was not significantly different from the expected genetic ratio. Therefore, the genotypic ratio of marker segregation in the F_2 and F_3 generations was consistent with those expected. The mean PPO activity of each genotype is shown in Table 8 and 9 for the F_2 and F_3 generations, respectively. In both generations, the NA and NB classes had significantly lower PPO activities than the AA and AB classes. PPO activity of NA and NB classes was not significantly different; likewise, activity of AA and AB classes also was not significantly different. Results of this population suggest *Ppo-A1* exerts greater control of PPO activity, and that the putative null allele at this locus further reduces PPO activity.

NW07OR1066/OR2050272H

The genotypic effect was highly significant based on ANOVA (Table 5) whereas generations and genotype nested within generation again did not show significance. This population did not have a normal distribution for both generations but the distribution was normal when transformed data were used (Fig. 1E). The average PPO activity for the parents was 0.068±0.035 AU when grown with the F_2 generation and 0.079±0.022 AU when grown with the F_3 generation for NW07OR1066; and 0.262±0.017 AU when grown with the F_2 generation and 0.299±0.139 AU when grown with the F_3 generation for OR2050272H. The minimum and maximum PPO activities for NW07OR1066/OR2050272H population were 0.035 and 0.465 AU for the F_2 generation and 0.024 and 0.466 AU for the F_3 generation. The overall mean PPO activity was 0.154±0.006 AU for the lines in the F_2 generation and 0.148±0.0.009 AU for the lines in

the F_3 generation. This population had the lowest PPO activity of all five populations. Based on the STS markers, six genotypes were classified and the genotypic ratio tested using a chi-square test. The chi-square value for the F_2 generation was 12.137 which was over 11.070 ($\lambda^2_{0.05, 5}$), hence the observed ratio was significantly different from the 3BA: 6BH: 3BB: 1NA: 2NH: 1NB expected ratio. However the chi-square value of the F_3 generation was 9.185, less than 11.070, thus the observed ratio was not significant different from 15BA: 10BH: 15BB: 9NA: 6NH: 9NB expected ratio, meaning the genotypic ratio fit the expected Mendelian ratio in the F_3 generation, but not fit in the F_2 generation. The mean PPO activity of each genotype is shown in Table 8 and 9. Both F_2 and F_3 generations, the NB genotype was expected to have the lowest PPO activity as in the NW07OR1070/Antelope population and the BA genotype was expected to have the highest PPO activity. However, the observations showed the NA genotype had the lowest PPO activity and the BB genotype had the highest PPO activity in both generations. This indicates potential recombination events present in some lineages led to a lack of concordance between previously identified markers for low PPO activity from *Ppo-D1* and enzyme activity.

DISCUSSION

All populations showed significant genotypic effect on kernel PPO activity ($P < 0.001$), meaning both *Ppo-A1* and *Ppo-D1* loci affect the kernel PPO activity. The differences between the *Ppo-A1a* and *Ppo-A1b* loci were substantially larger when *Ppo-D1* was fixed for either the *a* or *b* allele. For example, the AA (*Ppo-A1a/Ppo-D1b*)

genotype had higher PPO activity than BA (*Ppo-A1b/Ppo-D1b*) genotype for populations 1-3 (Table 8-9). In addition, the NW07OR1070/Antelope and NW07OR1066/OR2050272H populations had no PCR fragments at *Ppo-A1* allele amplified by PPO18 marker, designated the null genotype. Of all 5 populations, the NW07OR1070/Antelope and NW07OR1066/OR2050272H populations had the lowest PPO activities. However, the average PPO activity of the NW07OR1066/OR2050272H population was significantly lower than the NW07OR1070/Antelope population ($P < 0.0001$) although both populations had a null allele on chromosome 2A. The reason for this result may be that the NW07OR1066/OR2050272H population carried the *Ppo-A1b* (low PPO activity) and null alleles but the NW07OR1070/Antelope population had the *Ppo-Ala* (high PPO activity) and null alleles. The lines with a null allele on chromosome 2A showed much lower PPO activity than other lines in the NW07OR1070/Antelope and NW07OR1066/OR2050272H populations. Chang et al. (2007) studied the relationship between variation in PPO genes and PPO activity of immature wheat seeds in 216 common wheat cultivars and found that *TaPPO-A1* and *TaPPO-D1* had high polymorphisms related to PPO activity. Five cultivars (Gaiyuerui, 9114, ZM2851, ZM2855, and Xiaobingmai33) with a null allele at *TaPPO-D1* were observed and showed very low PPO activity of whole grains. Primers were used to confirm that the *TaPPO-D1* allele was present in these 5 cultivars. No PCR fragment was detected indicating that *TaPPO-D1* is not present in these five cultivars and provided additional evidence that the gene has an important function in contributing to PPO activity. The results of Chang et al. (2007) support our results that the null alleles at *Ppo-A1* affect the PPO activity by decreasing it to a very low or zero level.

The difference between *Ppo-D1a* and *PpoD1b* alleles was not significant when *Ppo-A1* allele was fixed. Because of these results, *Ppo-1A* allele had a major influence on PPO activity in wheat kernels and the effects for the two loci were not additive. This result is in agreement with the results of Martin et al. (2011), who determined a possible effect of allelic variation for *Ppo-A1* and *Ppo-D1* on Chinese raw noodle color profile and kernel and flour characteristics and found that *Ppo-A1* has a larger effect than *Ppo-D1* and the effects of both *Ppo-A1* and *Ppo-D1* loci were not additive. A major QTL for PPO activity was found on the long arm of chromosome 2A in a double haploid population derived from Chara/WW2449 using a QTL mapping approach (Raman et al. 2005). Whole kernel wheat PPO activity was measured in disomic substitution lines derived from three *Triticum dicocoides* accessions in the background of *durum wheat* 'Langdon' using antigenic staining. The results showed that PPO activity was primarily associated with chromosome 2A and to a much lower degree with chromosome 2B (Fuerst et al. 2008).

Beecher and Skinner (2011) identified the new three genes, *Ppo-A2*, *Ppo-B2* and *Ppo-D2* in wheat. The *Ppo-A2* and *Ppo-D2* genes located on the long arm of chromosome 2A and 2D, respectively, the same chromosomes as the *Ppo-A1* and *Ppo-D1* genes. The *Ppo-B2* localized to chromosome 2B. Real time PCR analysis showed that in the wheat cultivar 'Alpowa', *Ppo-A1a*, *Ppo-A2b*, *Ppo-D1b* and *Ppo-D2b* were all expressed to a high degree in developing wheat seeds while the *Ppo-B2* expression was not detected. The average contributions of the A and D genes were determined from the mean expression levels of genes *Ppo-A1a* and *Ppo-A2b* from the A genome and *Ppo-D1b* and *Ppo-D2b* from the D genome. The A genome contributed 89.6% of the PPO gene

transcripts in the developing seeds and over 72% of the PPO transcripts were from *Ppo-2*. The *Ppo-A1* and *Ppo-A2* genes present together contribute far more transcript than those from either the B or D genome.

The functional PPO29 marker for the PPO gene located on chromosome 2D was developed by He et al. (2007). In their report, PPO29 marker amplified a 490-bp PCR fragment in cultivar with high PPO activity. The dominant STS marker, STS01 was developed which amplified a fragment of 560-bp in most cultivars with low PPO activity. The STS01 marker was located on chromosome 2DL and complementary to PPO29 (Wang et al. 2008). In both Fielder/NW03681 and Fielder/Antelope populations, lines amplifying a 685-bp PCR fragment with PPO18 and a 490-bp fragment from PPO29 (AA genotype) showed higher average PPO activity in seed than lines producing a 685-bp fragment from PPO18 and a 560-bp fragment from STS01 (AB genotype). These data are in agreement with the previous data described for PPO29 and STS01. However, in the PI 117635/Antelope population, the *Ppo-A1b/Ppo-D1b* (BA) genotype had the lowest mean PPO activity and it was lower than *Ppo-A1b/Ppo-D1a* (BB) genotype. Both NW07OR1070/Antelope and NW07OR1066/OR2050272H populations showed the *null/Ppo-D1b* (NA) genotype had the lowest average PPO activity and lower than *null/Ppo-D1a* (NB) genotype. These data suggest that PPO29 marker in some populations confers lower PPO activity than STS01 marker. Therefore, the PI 117635/Antelope, NW07OR1070/Antelope, and NW07OR1066/OR2050272H populations had the opposite results of PPO29 and STS01 from the previously reported research. This conclusion was consistent with the results of our previous study in a PI 117635/IDO377s population which had results that contradicted that predicted for *Ppo-*

D1 allele. The pedigrees of NW07OR1070 and NW07OR1066 cultivars are PI 117635/Seaspray. These four populations (PI 117635/IDO377s, PI 117635/Antelope, NW07OR1070/Antelope, and NW07OR1066/OR2050272H) may have had the same opposite result from the *Ppo-D1* allele prediction because they all inherited the same allele and markers from PI 117635.

In conclusion, wheat grown under the greenhouse conditions can be used to study PPO activity. Both *Ppo-A1* and *Ppo-D1* alleles affect kernel PPO activity, but the *Ppo-A1* has the large effect on PPO activity in wheats. Very low PPO activity was detected in lines with a null allele at *Ppo-A1* indicating that *Ppo-A1* may not be present in these genetic backgrounds and decreases the PPO activity to a very low level. The PI 117635/Antelope, NW07OR1070/Antelope, and NW07OR1066/OR2050272H populations showed the reverse results of *Ppo-D1* marker allele from the prediction, indicating the markers for *Ppo-D1* allele give erroneous results in some genetic backgrounds. All populations showed significant genotypic effects, whereas the generations and genotype nested within generation were not significant. This result suggests it is possible to screen in early generations which is good news for breeders.

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Table 1. Average PPO activity of wheat genotypes grown in the greenhouse and field conditions

Cultivars	PPO activity (mean ± SD)	
	Greenhouse	Field
Antelope	0.479 ± 0.006	0.894±0.047
Anton	0.170 ± 0.023	0.355±0.032
Arrowsmith	0.464 ± 0.043	0.855±0.096
Alice	0.526 ± 0.036	0.802±0.072
NuDakota	0.412 ± 0.036	0.735±0.087
BC98331-03-2w	0.371 ± 0.047	0.693±0.087
HV9W02-267w	0.704 ± 0.097	0.932±0.064
NW04Y2188	0.373 ± 0.046	1.034±0.158

Table 2. Genotypes used in the study

Cultivars	2A		2D		Cultivars	2A		2D	
	PPO18 685-bp	PPO18 876-bp	PPO29 490-bp	STS01 560-bp		PPO18 685-bp	PPO18 876-bp	PPO29 490-bp	STS01 560-bp
Antelope	+	-	-	+	OR2060092H	+	-	-	+
Anton	-	+	-	+	OR2060099H	+	-	-	+
Arrowsmith	+	-	+	-	OR2060101H	+	-	-	+
Cook	+	-	-	+	OR2060108H	-	+	-	+
Fielder	-	+	+	-	PI 117635	-	+	+	-
HV9W02-267W	+	-	+	-	Tincurrin	-	+	-	+
IDO377s	-	+	-	+	NW07OR1040	-	-	+	-
IDO580	-	+	-	+	NW07OR1062	-	-	+	-
NW03681	+	-	-	+	NW07OR1066	-	-	+	-
OR2050042H	-	+	-	+	NW07OR1070	-	-	+	-
OR2050186H	+	-	+	-	NW07OR1071	-	-	+	-
OR2050272H	-	+	-	+	NW07OR1073	-	-	+	-
OR2060051H	-	+	-	+	NW07OR1074	-	-	+	-
OR2060074H	+	-	-	+					

Table 3. Genotypes of each selected population

Populations	Type	Pedigrees	Genotypes			
			2A		2D	
			PPO18	PPO18	PPO29	STS01
			685-bp	876-bp	490-bp	560-bp
Population 1	Female	PI 117635	-	+	+	-
	Male	Antelope	+	-	-	+
Population 2	Female	Fielder	-	+	+	-
	Male	NW03681	+	-	-	+
Population 3	Female	Fielder	-	+	+	-
	Male	Antelope	+	-	-	+
Population 4	Female	NW07OR1070	-	-	+	-
	Male	Antelope	+	-	-	+
Population 5	Female	NW07OR1066	-	-	+	-
	Male	OR2050272H	-	+	-	+

+ Presence of PCR fragment

- Absence of PCR fragment

Table 4 F_1 genotypes screening with PPO18, PPO29, and STS01 markers

Populations	Type	Pedigree	Parents				F_1 generation			
			2A		2D		2A		2D	
			PPO18	PPO18	PPO29	STS01	PPO18	PPO18	PPO29	STS01
			685-bp	876-bp	490-bp	560-bp	685-bp	876-bp	490-bp	560-bp
Populations 1	Female	PI 117635	-	+	+	-	+	+	+	+
	Male	Antelope	+	-	-	+				
Populations 2	Female	Fielder	-	+	+	-	+	+	+	+
	Male	NW03681	+	-	-	+				
Populations 3	Female	Fielder	-	+	+	-	+	+	+	+
	Male	Antelope	+	-	-	+				
Populations 4	Female	NW07OR1070	-	-	+	-	+	-	+	+
	Male	Antelope	+	-	-	+				
Populations 5	Female	NW07OR1066	-	-	+	-	-	+	+	+
	Male	OR2050272H	-	+	-	+				

+ Presence of PCR fragment

- Absence of PCR fragment

Table 5. Mean squares from analysis of variance of PPO activity

Source of variance	*df*	Mean square	Pr > F
Pop 1: PI 117635/Antelope			
Generation	1	0.0003	0.9051^{NS}
Genotype	8	0.7564	<0.0001*
Generation x genotype	8	0.0170	0.5014^{NS}
Error	235	0.0185	
Pop 2: Fielder/NW03681			
Generation	1	1.5787	<0.0001*
Genotype	8	0.4248	<0.0001*
Generation x genotype	8	0.0807	0.1974^{NS}
Error	200	0.0575	
Pop 3: Fielder/Antelope			
Generation	1	0.4036	0.0126^{NS}
Genotype	8	0.5190	<0.0001*
Generation x genotype	8	0.0742	0.3234^{NS}
Error	250	0.0639	
Pop 4: NW07OR1070/Antelope			
Generation	1	0.0005	0.9109^{NS}
Genotype	5	0.7978	<0.0001*
Generation x genotype	5	0.0481	0.2642^{NS}
Error	212	0.0369	
Pop 5: NW07OR1066/OR2050272H			
Generation	1	0.0067	0.2064^{NS}
Genotype	5	0.1010	<0.0001*
Generation x genotype	5	0.0025	0.7015^{NS}
Error	231	0.0042	

Table 6 Chi-square test for F_2 generation

Source	n	Observed ratio	λ^2 test	Pr 〉F
Parent: PI117635/Antelope				
2A: 1A: 2H: 1B	135	29: 72: 34	0.9704	0.6156^{NS}
2D: 1A: 2H: 1B		38: 55: 42	4.8667	0.0877^{NS}
2A2D: 1AA: 2AH: 1AB: 2HA: 4HH: 2HB: 1BA: 2BH: 1BB		7: 13: 9: 23: 25: 24: 8: 17: 9	8.7333	0.3653^{NS}
Parent: Fielder/NW03681				
2A: 1A: 2H: 1B	114	29: 60: 25	0.5965	0.7421^{NS}
2D: 1A: 2H: 1B		29: 49: 36	3.1053	0.2117^{NS}
2A2D: 1AA: 2AH: 1AB: 2HA: 4HH: 2HB: 1BA: 2BH: 1BB		9: 12: 8: 12: 35: 15: 8: 4: 13	14.3860	0.0722^{NS}
Parent: Fielder/Antelope				
2A: 1A: 2H: 1B	136	40: 76: 20	7.7647	0.0206^{*}
2D: 1A: 2H: 1B		43: 56: 37	4.7647	0.0923^{NS}
2A2D: 1AA: 2AH: 1AB: 2HA: 4HH: 2HB: 1BA: 2BH: 1BB		12: 16: 12: 28: 31: 17: 3: 9: 8	17.6765	0.0238^{*}
Parent: NW07OR1070/Antelope				
2A: 3A :1N	116	81: 35	1.6552	0.1983^{NS}
2D: 1A: 2H: 1B		32: 54: 30	0.6207	0.7332^{NS}
2A2D: 3AA: 1NA: 6AH: 2NH: 3AB: 1NB		19: 13: 40: 14: 22: 8	5.2874	0.3818^{NS}
Parent: NW07OR1066/OR2050272H				
2A: 3B :1N	139	91: 48	6.7362	0.0094^{*}
2D: 1A: 2H: 1B		36: 62: 41	1.9784	0.3719^{NS}
2A2D: 3BA: 1NA: 6BH: 2NH: 3BB: 1NB		21: 14: 38: 24: 32: 10	12.1367	0.0330^{*}

NS: not significant at $P < 0.05$

* = signigicant at $P < 0.05$

AA = *Ppo-A1a/ Ppo-D1b* AH = *Ppo-A1a/ Ppo-D1ab* AB = *Ppo-A1a/ Ppo-D1a*

HA = *Ppo-A1ab/ Ppo-D1b* HH = *Ppo-A1ab/ Ppo-D1ab* HB = *Ppo-A1ab/ Ppo-D1a*

BA = *Ppo-A1b/ Ppo-D1b* BH = *Ppo-A1b/ Ppo-D1ab* BB = *Ppo-A1b/ Ppo-D1a*

NA = *null allele/ Ppo-D1b* NH = *null allele/ Ppo-D1ab* NB = *null allele/ Ppo-D1a*

Table 7 Chi-square test for F_3 generation

Source	n	Observed ratio	λ^2 test	Pr 〉 F
Parent: PI117635/Antelope				
2A: 3A: 2H: 3B	125	44: 33: 48	0.3013	0.8601^{NS}
2D: 3A: 2H: 3B		48: 35: 42	0.9840	0.6114^{NS}
2A2D: 9AA: 6AH: 9AB: 6HA: 4HH: 6HB: 9BA: 6BH: 9BB		19: 10: 14: 16: 6: 11: 13: 18: 17	7.4942	0.4844^{NS}
Parent: Fielder/NW03681				
2A: 3A: 2H: 3B	106	41: 30: 35	1.0692	0.5859^{NS}
2D: 3A: 2H: 3B		35: 29: 42	0.9308	0.6279^{NS}
2A2D: 9AA: 6AH: 9AB: 6HA: 4HH: 6HB: 9BA: 6BH: 9BB		18: 10: 13: 5: 11: 14: 12: 8: 15	8.8344	0.3565^{NS}
Parent: Fielder/Antelope				
2A: 3A: 2H: 3B	139	63: 39: 37	7.1775	0.0276^{*}
2D: 3A: 2H: 3B		51: 29: 59	1.8825	0.3901^{NS}
2A2D: 9AA: 6AH: 9AB: 6HA: 4HH: 6HB: 9BA: 6BH: 9BB		20: 11: 32: 20: 5: 14: 11: 13: 13	19.5548	0.0122^{*}
Parent: NW07OR1070/Antelope				
2A: 5A :3N	114	66: 48	1.0316	0.3098^{NS}
2D: 3A: 2H: 3B		38: 39: 37	5.1696	0.0754^{NS}
2A2D: 15AA: 9NA: 10AH: 6NH: 15AB: 9NB		17: 21: 27: 12: 22: 15	10.8405	0.0546^{NS}
Parent: NW07OR1066/OR2050272H				
2A: 5B :3N	135	85: 50	0.0123	0.9115^{NS}
2D: 3A: 2H: 3B		43: 40: 52	2.3432	0.3099^{NS}
2A2D: 15BA: 9NA: 10BH: 6NH: 15BB: 9NB		20: 23: 28: 12: 37: 15	9.1845	0.1019^{NS}

NS: not significant at $P < 0.05$

* = signigicant at $P < 0.05$

AA = *Ppo-A1a/ Ppo-D1b* AH = *Ppo-A1a/ Ppo-D1ab* AB = *Ppo-A1a/ Ppo-D1a*

HA = *Ppo-A1ab/ Ppo-D1b* HH = *Ppo-A1ab/ Ppo-D1ab* HB = *Ppo-A1ab/ Ppo-D1a*

BA = *Ppo-A1b/ Ppo-D1b* BH = *Ppo-A1b/ Ppo-D1ab* BB = *Ppo-A1b/ Ppo-D1a*

NA = *null allele/ Ppo-D1b* NH = *null allele/ Ppo-D1ab* NB = *null allele/ Ppo-D1a*

Table 8 Genotypes and PPO activity of all F_2 populations and parents

2A		2D		Genotypes	PPO activity of F_3 seeds (mean±sd)				
PPO18 685-bp	**PPO18 876-bp**	**PPO29 490-bp**	**STS01 560-bp**		**Population 1** PI 117635/Antelope	**Population 2** Fielder/NW03681	**Population 3** Fielder/Antelope	**Population 4** NW07OR1070/Antelope	**Population 5** NW07OR1066/OR2050272H
+	-	+	-	AA	0.522±0.081	0.530±0.048	0.640±0.052	0.412±0.031	-
+	-	+	+	AH	0.599±0.040	0.631±0.076	0.622±0.055	0.414±0.024	-
+	-	-	+	AB	0.567±0.058	0.345±0.072	0.597±0.055	0.438±0.017	-
-	+	+	-	BA	0.140±0.017	0.312±0.065	0.361±0.224	-	0.107±0.020
-	+	+	+	BH	0.186±0.013	0.182±0.036	0.427±0.029	-	0.161±0.007
-	+	+	-	BB	0.158±0.029	0.227±0.034	0.277±0.012	-	0.203±0.011
+	+	+	-	HA	0.392±0.024	0.421±0.043	0.548±0.034	-	-
+	+	+	+	HH	0.424±0.024	0.486±0.037	0.541±0.033	-	-
+	+	-	+	HB	0.423±0.026	0.392±0.048	0.488±0.033	-	-
-	-	+	-	NA	-	-	-	0.158±0.038	0.073±0.006
-	-	+	+	NH	-	-	-	0.234±0.042	0.150±0.009
-	-	-	+	NB	-	-	-	0.223±0.037	0.186±0.016
+	-	-	+	Antelope	0.606±0.042				
-	+	+	-	Fielder	0.584±0.012				
+	-	-	+	NW03681	0.726±0.000				
-	-	+	-	NW07OR1066	0.068±0.035				
-	-	+	-	NW07OR1070	0.043±0.005				
-	+	-	+	OR2050272H	0.262±0.017				
-	+	+	-	PI 117635	0.073±0.002				

+ Presence of PCR fragment

- Absence of PCR fragment

Table 9 Genotypes and PPO activity of all F_3 populations and parents

2A		2D		Genotypes	PPO activity of F_4 seeds (mean±sd)				
PPO18 685-bp	**PPO18 876-bp**	**PPO29 490-bp**	**STS01 560-bp**		**Population 1** PI 117635/Antelope	**Population 2** Fielder/NW03681	**Population 3** Fielder/Antelope	**Population 4** NW07OR1070/Antelope	**Population 5** NW07OR1066/OR2050272H
+	-	+	-	AA	0.569±0.054	0.751±0.081	0.916±0.092	0.499±0.090	-
+	-	+	+	AH	0.546±0.057	0.640±0.076	0.673±0.073	0.494±0.039	-
+	-	-	+	AB	0.599±0.049	0.761±0.079	0.756±0.060	0.466±0.060	-
-	+	+	-	BA	0.103±0.014	0.556±0.067	0.468±0.062	-	0.102±0.015
-	+	+	+	BH	0.155±0.019	0.465±0.078	0.462±0.069	-	0.159±0.015
-	+	+	-	BB	0.205±0.019	0.271±0.040	0.332±0.050	-	0.209±0.016
+	+	+	-	HA	0.325±0.029	0.587±0.147	0.762±0.090	-	-
+	+	+	+	HH	0.403±0.069	0.692±0.090	0.461±0.086	-	-
+	+	-	+	HB	0.488±0.046	0.511±0.100	0.537±0.059	-	-
-	-	+	-	NA	-	-	-	0.133±0.032	0.067±0.008
-	-	+	+	NH	-	-	-	0.123±0.020	0.105±0.022
-	-	-	+	NB	-	-	-	0.183±0.064	0.169±0.026
+	-	-	+	Antelope	0.466±0.157				
-	+	+	-	Fielder	0.464±0.098				
+	-	-	+	NW03681	0.361±0.015				
-	-	+	-	NW07OR1066	0.079±0.022				
-	-	+	-	NW07OR1070	0.151±0.094				
-	+	-	+	OR2050272H	0.299±0.139				
-	+	+	-	PI 117635	0.126±0.008				

+ Presence of PCR fragment

- Absence of PCR fragment

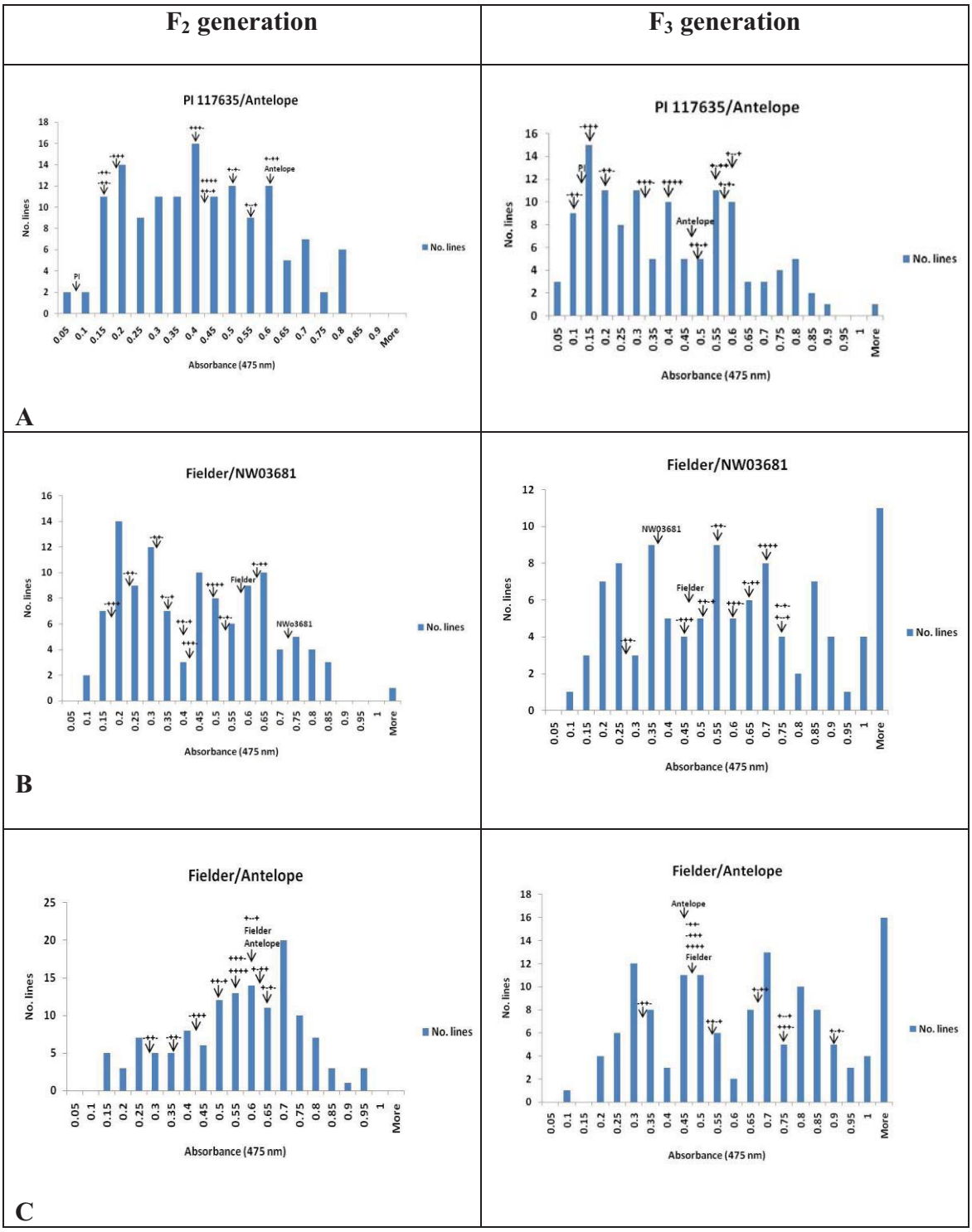


Fig.1. Distribution of PPO activity of each genotype including parents in F_2 and F_3 generations for PI 117635/Antelope (A), Fielder/NW03681 (B), Fielder/Antelope (C), NW07OR1070/Antelope (D), and NW07OR1066/OR2050272H (E)

+-+- = *Ppo-A1a/ Ppo-D1b*	+-++ = *Ppo-A1a/ Ppo-D1ab*	+--+ = *Ppo-A1a/ Ppo-D1a*
+++- = *Ppo-A1ab/ Ppo-D1b*	++++ = *Ppo-A1ab/ Ppo-D1ab*	++-+ = *Ppo-A1ab/ Ppo-D1a*
-++- = *Ppo-A1b/ Ppo-D1b*	-+++ = *Ppo-A1b/ Ppo-D1ab*	-+-+ = *Ppo-A1b/ Ppo-D1a*
--+- = *null allele/ Ppo-D1b*	--++ = *null allele/ Ppo-D1ab*	---+ = *null allele/ Ppo-D1a*

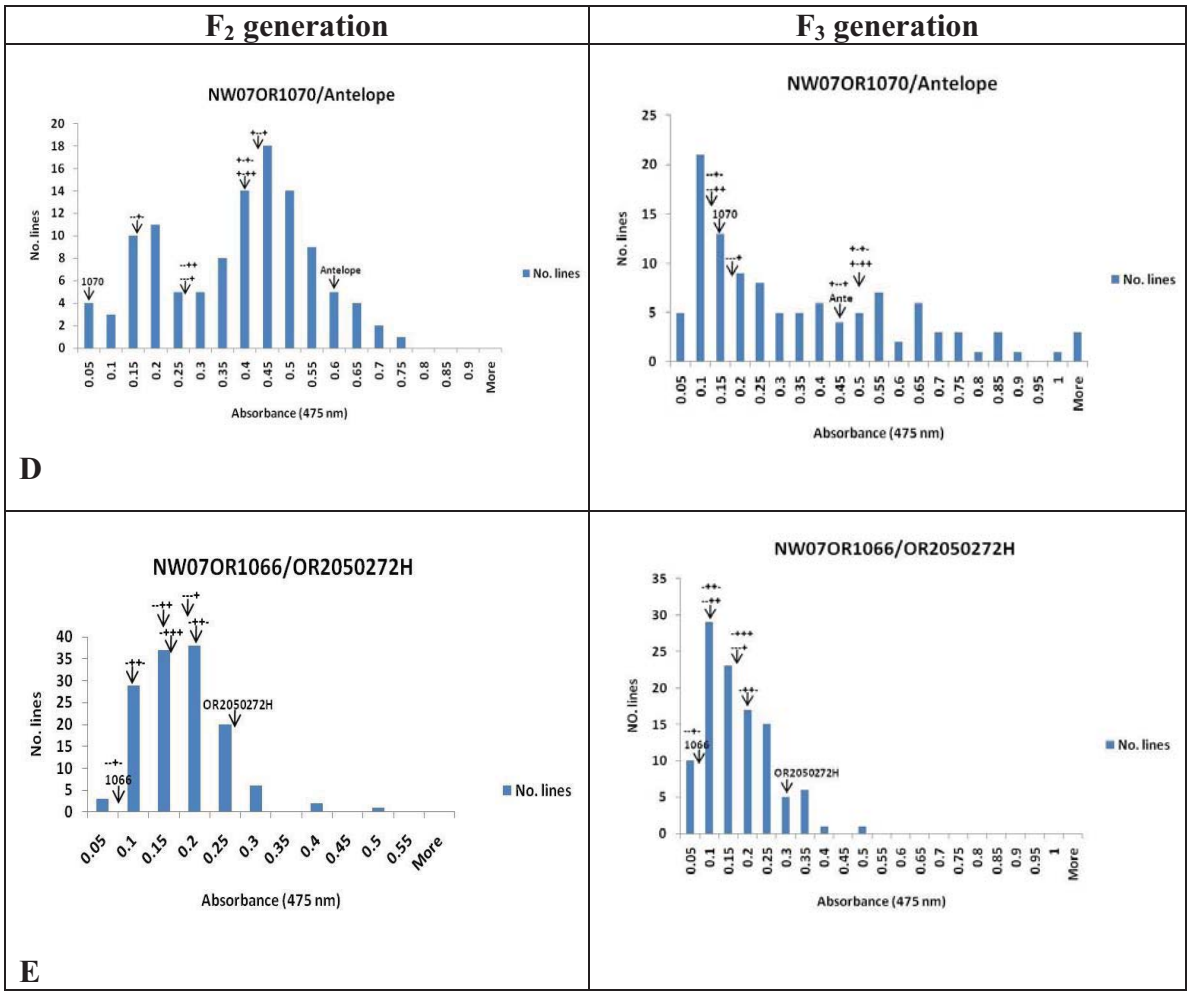
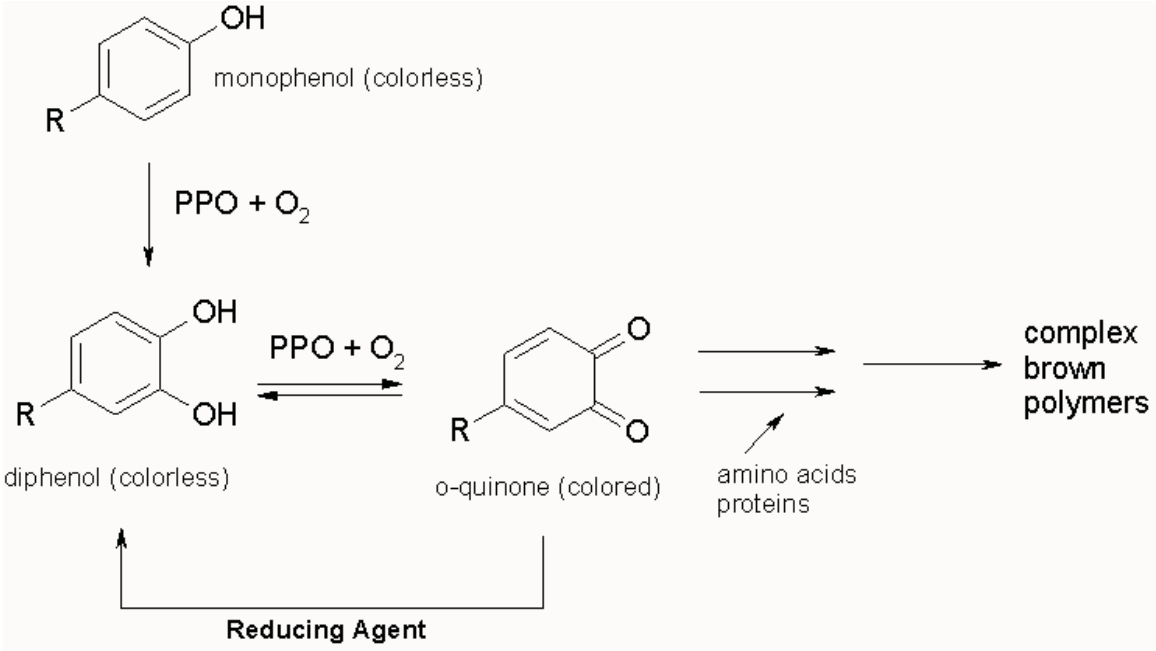


Fig.1. Distribution of PPO activity of each genotype including parents in F_2 and F_3 generations for PI 117635/Antelope (A), Fielder/NW03681 (B), Fielder/Antelope (C), NW07OR1070/Antelope (D), and NW07OR1066/OR2050272H (E) (continue)

+-+- = *Ppo-A1a/ Ppo-D1b*	+-++ = *Ppo-A1a/ Ppo-D1ab*	+--+ = *Ppo-A1a/ Ppo-D1a*
+++- = *Ppo-A1ab/ Ppo-D1b*	++++ = *Ppo-A1ab/ Ppo-D1ab*	++-+ = *Ppo-A1ab/ Ppo-D1a*
-++- = *Ppo-A1b/ Ppo-D1b*	-+++ = *Ppo-A1b/ Ppo-D1ab*	-+-+ = *Ppo-A1b/ Ppo-D1a*
--+- = *null allele/ Ppo-D1b*	--++ = *null allele/ Ppo-D1ab*	---+ = *null allele/ Ppo-D1a*

Appendix 1 Polyphenol oxidase catalyzed reactions (Mayer and Herel 1979)