2013

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Euphytica (2013) 190:65–73
DOI 10.1007/s10681-012-0777-y

Enzyme activity in wheat breeding lines derived from matings of low polyphenol oxidase parents

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Received: 21 February 2012 / Accepted: 8 August 2012 / Published online: 25 August 2012

Abstract Polyphenol oxidase (PPO) in grain plays a major role in time-dependent discoloration of wheat (*Triticum aestivum* L.) products, especially fresh noodles. Breeding wheat cultivars with low or nil PPO activity can reduce undesirable product darkening. The low PPO line PI 117635 was crossed to two low PPO wheats, IDO580 and 'IDO377s', to determine whether matings between wheats with low levels of grain PPO would result in complementation, such that lines with still lower or nil PPO would be generated. Progeny in a population derived from PI 117635/IDO580 displayed no variation in PPO activity. In the $F_{3:4}$ populations derived from PI 117635/IDO377s, and the reciprocal IDO377s/PI 117635, normal distributions of low to high PPO activity were observed. Field-grown populations ($F_{3:5}$; $F_{3:6}$) derived from IDO377s crosses were analyzed for PPO activity and used to determine whether lines with nil PPO activity were generated. Of 239 lines, 154 were verified to have PPO activity that was not significantly different from the low PPO durum (*Triticum turgidum* var *durum*) cultivar 'Ben'. PCR analysis showed that the populations were fixed for a putative low PPO allele at *Ppo-A1*. Using markers for *Ppo-D1*, it was found that the average PPO activity of lines with 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 from previous reports for markers for *Ppo-D1* alleles. Thus, breeders should exercise caution when making selections using markers for alleles at *Ppo-D1*, as known markers might predict erroneous phenotypes and genotypes in some wheat backgrounds.

Keywords *Triticum aestivum* · Grain enzymes · Product discoloration · Complementation

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Introduction

In Asia, over 40 % of common wheat (*Triticum aestivum* L.) flour is used for making noodles (Hou 2001). 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 higher

protein content (Nagao 1996). Different types of noodles vary in firmness and texture, but consumer preference requires most noodle types to have a bright and creamy white color (Wang et al. 2009).

Polyphenol oxidase (PPO, EC 1.14.18.1) is a major cause of 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-containing 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 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 and Morris 2001). Generally, PPO activity is located in the bran and more specifically in the aleurone layer of wheat kernels, both of which are mostly removed during the milling process (Sullivan 1964). Residual contamination by the bran layer after milling, however, is still sufficient to cause product discoloration (Rani et al. 2001). Developing wheat cultivars having genetically low or nil PPO activity is a current priority of many 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*; Thipypong et al. 1997) and PPO often is encoded by gene families (Sherman et al. 1995). In common wheat, three genes, *Ppo-A1*, *Ppo-B1* and *Ppo-D1*, were described as expressing in developing kernels and may influence PPO activity in flour, and three additional genes express in non-kernel tissues (Demeke and Morris 2002; Jukanti et al. 2004). Beecher and Skinner (2011) recently identified three new PPO-encoding genes, *Ppo-A2*, *Ppo-B2* and *Ppo-D2*, localized to the same chromosome arms as known kernel-expressing PPO genes.

Durum (*Triticum turgidum* var *durum*) wheat cultivars typically have very low or nil PPO activity, whereas common wheats vary in PPO activity (Kruger et al. 1994; Baik et al. 1995; Miskelly 1996). 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 (Simone et al. 2002; Raman et al. 2005). Jiménez and Dubcovsky (1999), using chromosome substitution lines from three cultivars into Chinese Spring, proposed that genes located on homoeologous group 2 of wheat chromosomes play a major role in PPO activity. QTL analysis showed that additional genes influencing PPO activity might be located on chromosomes 2A, 2B, 2D, 3D and 6B (Jiménez and Dubcovsky 1999; Demeke et al. 2001; Simone et al. 2002; Raman et al. 2005). Beecher and Skinner (2011) used real-time PCR analysis to demonstrate that *Ppo-A1a*, *Ppo-A2b*, *Ppo-D1b* and *Ppo-D2b* in the wheat cultivar 'Alpowa' were expressed to substantial levels in developing wheat kernels, whereas *Ppo-B1* and *Ppo-B2* expression was not detected. The average contributions of *Ppo-A1a* and *Ppo-A2b* from the A genome were greater than expression of *Ppo-D1b* and *Ppo-D2b* from the D genome. This is in agreement with the results of Raman et al. (2005) who identified a major QTL controlling PPO activities on the long arm of chromosome 2A in a DH population derived from 'Chara' (medium–high PPO)/'WW2499' (low PPO). Baik et al. (1994) and Park et al. (1997) reported that PPO activity in common wheat is influenced by both genotype and environment.

DNA marker studies have attributed most of the variation in wheat PPO activity to alleles at *Ppo-A1* and *Ppo-D1*. Intra-locus sequenced-tagged site (STS) marker PPO18 was designed based on DNA sequences in *Ppo-A1* (Sun et al. 2005). PPO18 proved an efficient marker for *Ppo-A1* (He et al. 2007), amplifying two fragments, a 685 bp PCR fragment in cultivars with high PPO activity (*Ppo-A1a* allele) and a 876 bp PCR fragment in cultivars with low PPO activity (*Ppo-A1b* allele) (Sun et al. 2005; He et al. 2007). Complementary dominant STS markers, PPO29 and STS01were developed based on the DNA sequences of the *Ppo-D1* PPO gene on chromosome 2D (He et al. 2007; Wang et al. 2009). PPO29 reportedly amplified a 490 bp PCR fragment in cultivars with high PPO activity, indicating the presence of the *Ppo-D1b* allele (He et al. 2007). STS01 amplified a 560 bp PCR fragment which corresponded to the *Ppo-D1a* allele in most cultivars with low PPO activity (Wang et al. 2008).

In polyploid organisms such as wheat, a type of complementation can occur when lines carrying recessive alleles at independent loci are crossed, and resulting progeny display recessive or mutant phenotypes. Waxy (amylose-free) wheat is an example. Waxy wheats carry null alleles at loci, *wx-A1*, *wx-B1*, and *wx-D1*, encoding isoforms of 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' resulting in progeny that lacked all isoforms of GBSS (Nakamura et al. 1992) and also lacked endosperm starch amylose. The goal of the present study was to determine whether crosses between cultivars with low phenotypic levels of grain PPO could produce a similar type of complementation, allowing lines with lower or nil PPO to 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 accession PI 117635 (low PPO) was crossed to two low PPO spring wheats IDO580 and 'IDO377s'from Idaho. For purposes of this study, lines were considered "low" when PPO values (see below) were consistently lower than the medium–low hard white winter wheat control cultivar 'Anton' (Graybosch et al. 2011). 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 germplasm line with low 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' durum with CIMMYT *Triticum tauschii* (Coss.) Schmalh. 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 cultivated in the Pacific Northwest region of the USA. IDO377s was derived from the cross 'Chova'/ 59Ab10293-5. Chova has the pedigree: 'Gallo'/'Yecora' reselection/3/'Aurora'//'Kalyansona'/'Bluebird'. The pedigree of 59Ab10293-5 is 'Norin 10'/'Brevor'// 'Baart'/'Onas'.

Five hundred heads were selected at random from the F_3 populations of these crosses and sown at Yuma, AZ in the spring of 2008. Heads were snapped, threshed individually, and seed planted in the field as F_3- derived F_4 single plant progeny rows in fall of 2008 at Yuma, AZ. The $F_{3:5}$ generation was harvested from Yuma, AZ in early spring 2009 and the $F_{3:6}$ generation was immediately planted at Corvallis, OR, also as single rows. Seven cultivars, 'Ben' (durum wheat), and the common wheats 'Express', IDO377s, 'DO580, 'Jubilee', PI 117635, and 'Seaspray' were included as controls with the 2009 experiments. An augmented 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 data from the two locations (F_4 and F_5 generations).

Yuma, AZ (32.69 Lat. -114.62 Long.) in southwestern USA is characterized by a desert climate, with an average annual precipitation of 7.5 cm. Wheat production always requires irrigation. Corvallis, OR (44.56 Lat. -123.26 Long.) is in the Pacific Northwest, with a mild climate and average annual precipitation of over 100 cm. Wheat production is rainfed.

Measurement of PPO activity

PPO activity in wheat grains was measured as per Anderson and Morris (2001). Five whole seeds were weighed. A 1.5-ml aliquot of 10 mM L-DOPA (3, 4-dihydroxyphenylalanine, Sigma-Aldrich Co., St. Louis, MO) as substrate in 50 mM 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 the five seeds. The tubes were placed on a Labquake Rotisserie Shaker from Barnstead/Thermolyne (Dubuque, IA) and rotated at 8 rpm for 1 h 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 with low PPO activity) were included with every run as experimental laboratory

controls to measure consistency of the protocol. Each reaction was run in duplicate. The L-DOPA solution was freshly made each day.

DNA isolation and STS analysis

Genomic DNA was isolated from young leaf tissues of a minimum of eight plants 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). PCR were performed in total volumes 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 1.5 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, and final extension of 72 °C for 8 min. Amplified PCR fragments were separated on 1.5 % agarose gels, stained with ethidium bromide, and visualized using UV light.

Statistical analysis

All statistical computations were made using SAS computer packages version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Analysis of variance (Proc GLM) was used to test for significant differences among environments, genotypes, and genotypes nested within environments using appropriate error terms for an augmented design with replicated check cultivars. Transgressive segregation among lines and parents was determined using a Least Significant Difference procedure with $\alpha = 0.05$. Pairwise comparisions (*t*-test) were used at $\alpha = 0.05$ to compare PPO levels of lines with Ben. Based on PCR results, 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 testing normality. Chi-squared analysis was used to test segregation ratios of DNA markers. 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. A log transformation was used to normalize the data and 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.

Results and discussion

Average PPO activities of the laboratory control cultivars were: Arapahoe, 1.032 ± 0.018 AU; Anton, 0.376 ± 0.015 AU and Ben, 0.075 ± 0.003 AU ($n = 27$). Daily fluctuation was proportional to mean values, with little fluctuation observed in Ben (Fig. 1). Relative rankings of control samples never changed.

The 2008 Yuma field-grown F_4 populations derived from crosses PI 117635/IDO580 and PI 117635/IDO377s were analyzed for PPO activity. In the PI 117635/IDO580 cross, little variation in PPO level (population mean = 0.097 ± 0.004 AU; Fig. 2) occurred among the progeny, even though marker data indicated segregation of different alleles at *Ppo-D1* (marker data not shown). A wider range in PPO activity was observed in the populations derived from PI 117635 and IDO377s. The observed ranges in PPO activity were 0.142–0.502 AU for PI 117635/IDO377s

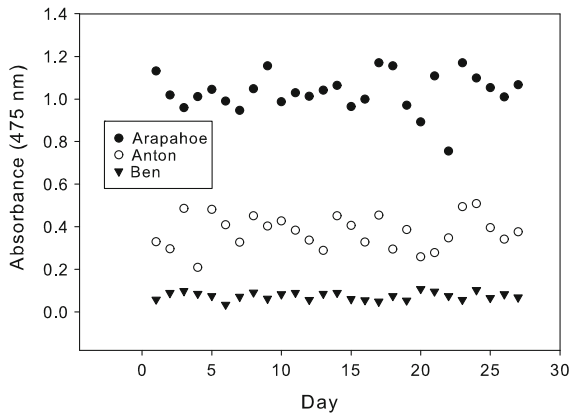

Fig. 1 Observed daily PPO activity of laboratory experimental controls

and 0.111–0.620 AU for IDO377s/PI 117635. Mean 2008 F_4 PPO activity for the lines derived from PI 117635/IDO377s was 0.272 ± 0.008 AU ($n = 124$) and the reciprocal cross IDO377s/PI 117635, 0.312 ± 0.011 AU ($n = 115$). The range of values indicated that both parents contain alleles associated with higher and lower PPO activity, even though both parents were first selected for low PPO activity. The PI 117635/IDO377s population had slightly more lines with low (AU <0.30) 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).

Of the 239 F_3-derived lines from the crosses between PI 117635 and IDO377s, 119 lines amplified a 490 bp PCR fragment (A pattern) with the PPO29 marker. 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 markers PPO29 and STS01 and were considered heterozygous. The expected proportions of $F_{3:5}$ genotypes are 3A: 2H: 3B. The Chi-squared value was 34.26, over the critical value ($\chi^2 0.05$, 2 df = 5.99), indicating that the segregation deviated from the expected 3A:2H:3B ratio. This deviation arose from a deficiency of H individuals. The A and B classes fit the expected 1:1 ratio ($\chi^2 = 2.03$; $P = 0.154$). The PPO18 marker amplified the same 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. PI 117635 was identified as BA (*Ppo-A1b/Ppo-D1b*) and IDO377s confirmed as BB (*Ppo-A1b/Ppo-D1a*).

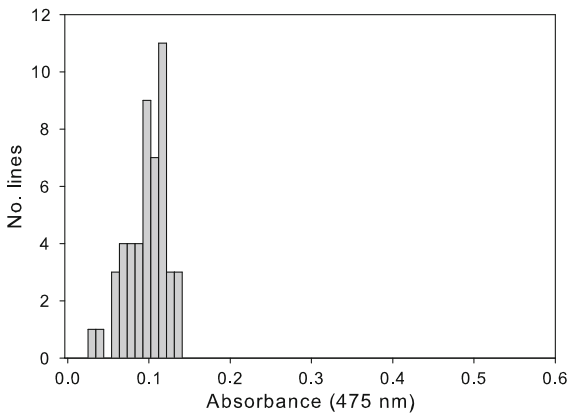

Fig. 2 Distribution of mean PPO activity in the population derived from IDO580/PI 117635 grown in Yuma AZ in 2008

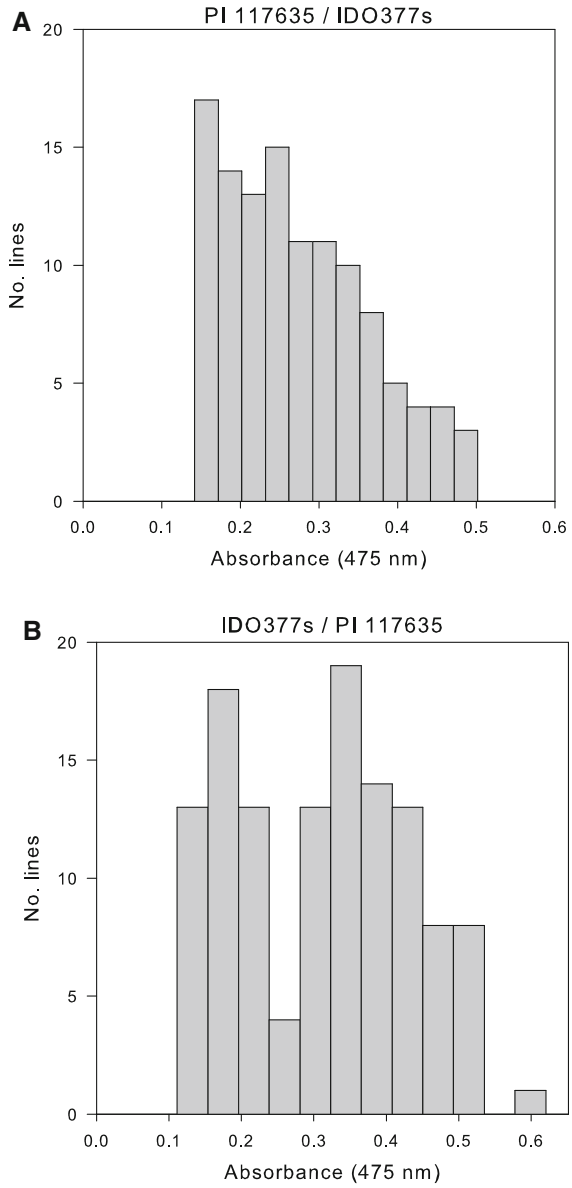

Fig. 3 Distribution of mean PPO activity in the populations derived from PI 117635/IDO377s (**a**) and IDO377s/PI 117635 (**b**) grown in Yuma AZ in 2008

Analysis of variance of Yuma09 and OR09 grown materials (F_4 and F_5 generations) showed highly significant differences among environments, genotypes (PPO alleles) and environment by genotype interaction (Table 1), suggesting a differential effect of environment on expression of grain PPO activity,

Table 1 Mean squares from analysis of variance of kernel PPO activity within the PI 117635/IDO377s population grown in Yuma and Oregon 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]
Environments	1	0.2291*	2.7395*	0.1660*	0.8003*
Genotypes	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

* Significant at $P = 0.05$

[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

confirming previously observed environmental effects (Park et al. 1997; McCaig et al. 1999; Demeke et al. 2001). Identical results (Table 1) were obtained using OD and OD/g. Pearson correlation coefficients were calculated separately for each environment and for combined environments, in order to determine possible relations between OD and OD/g. R values for this correlation using Yuma09, OR09 and the combined environments were 0.93, 0.98 and 0.95, indicating OD was highly correlated with OD/g in all cases ($P < 0.0001$). Weighing seed before assaying PPO activity is therefore not necessary, allowing breeding programs to evaluate more materials with less labor.

The respective Yuma09 and OR09 experimental means for PPO activity were 0.183 ± 0.004 AU and 0.099 ± 0.003 AU ($n = 239$), lower than the values observed for initial 2008 observations (Table 2). The minimum and maximum PPO activities of experimental lines were 0.091 and 0.373 AU for Yuma09 and 0.025 and 0.247 AU for OR09. The mean PPO activities of the parents were 0.079 ± 0.012 AU for PI 117635 and 0.132 ± 0.009 AU for IDO377s, whereas that of Ben durum was 0.053 ± 0.004 (Table 2). The PPO activity distribution of the PI 117635/IDO377s population was normal ($P = 0.41$) whereas the reciprocal IDO377s/PI 117635 population was not ($P = 0.01$) (Fig. 4a, b). In both populations, some progenies exhibited significantly higher PPO activities than those of either parent (Table 2) confirming the presence of transgressive segregation within the population. Transgressive segregation arises from complementary gene action (the occurrence of combinations of alleles from both parents affecting a trait in the same direction) (Rieseberg et al. 2003). Of the inbred lines, 154 had PPO activities not significantly different from that of Ben durum when assessed by *t*-tests ($LSD_{\alpha} = 0.05$). It is now clear that variation exists among common wheat lines to derive materials with PPO activities as low as Ben. PPO activity of IDO580 was as low as Ben; however, it was derived from a synthetic population, with genes from *T. tauschii*. The materials derived from IDO377s/PI

Table 2 Average kernel PPO activities of genotypes with different PCR fragments amplified by markers PPO18, PPO29 and STS01 within PI 117635/IDO377s population

Genotype	N	(OD) Mean ± SE	(OD/g) Mean ± SE	(ODT) Mean ± SE	(ODT/g) 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 (BB)	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 (BA)	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*

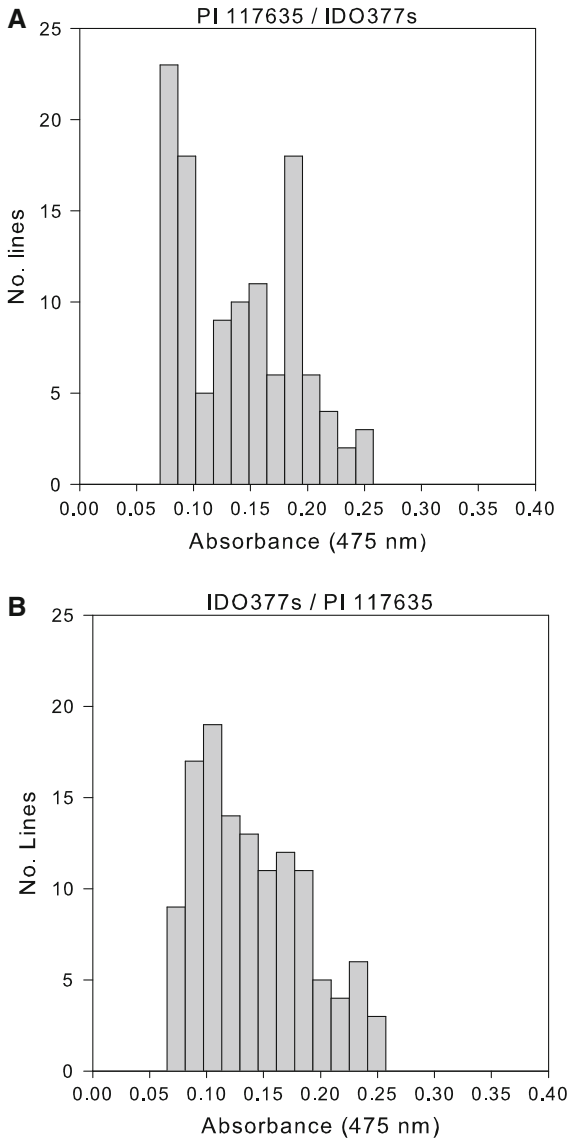

Fig. 4 Distribution of mean kernel PPO activity in populations derived from PI 117635/IDO377s (**a**) and IDO377s/PI 117635 (**b**) grown in Yuma, AZ and Corvallis, OR in 2009. Mean values: Ben durum, 0.0.053; IDO377s, 0.132; PI 117635, 0.079

117635 only have genes from common wheats. Such sources of low PPO within the common wheat gene pool might ultimately prove more useful in breeding programs.

The average kernel PPO activities of the tested genotypes were: *Ppo-A1b/Ppo-D1b* (BA) 0.11 AU; *Ppo-A1b/Ppo-D1a-D1b* (BH) 0.15 AU; and *Ppo-A1b/Ppo-D1a* (BB) 0.17 AU (Table 2). The average PPO activity of lines amplifying the 490 bp PPO29 fragment was significantly lower than that of the lines with positive STS01 reactions (560 bp PCR fragments). However, this result does not agree with earlier reports for the STS01 and PPO29 markers, in which STS01 was a dominant marker for low PPO activity, and amplified a 560 bp fragment in most low PPO cultivars (Wang et al. 2008), whereas PPO29 amplified 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 ($P < 0.05$) than the *Ppo-A1b/Ppo-D1a* (BB) and *Ppo-A1b/Ppo-D1a-D1b* (BH) genotypes. All lines produced a 876 bp fragment from PPO18, indicating fixation of the low allele at *Ppo-A1*. All tested genotypes, however showed low levels of PPO activity when compared with the check cultivars.

Chromosome 2A was previously implicated as the site of major genes affecting kernel PPO activity (Raman et al. 2007). 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; the A genome contributed more than 80 % of the PPO gene transcripts present in developing seeds. This is in agreement with Martin et al. (2011) who found both the *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*. It is possible that in the presence of the low *Ppo-A1b* allele, as in the present study, the allelic status at *Ppo-D1* is irrelevant, especially considering the findings of Beecher and Skinner (2011) that this locus contributes little to overall PPO activity. Supporting this view is the lack of PPO variation observed for the progeny of PI 117635/IDO580, even though there was segregation for *Ppo-D1* allelic markers. Additional genes encoding active PPO enzymes might also have influenced the results. While most previous work concentrated on the effects of alleles at *Ppo-A1* and *Ppo-D1* Beecher et al. (2012) demonstrated the presence of additional PPO-encoding loci designated *Ppo-A2*, *Ppo-B2*, and *Ppo-D2*. In a segregating population derived from the cross 'Louise × Penawawa', variation in kernel PPO activity was significantly associated with markers closely linked to both *Ppo-B2*, and *Ppo-D2*. As these loci were not investigated in the present study, it is possible that segregation for functional alleles at one or more of these

newly described loci confounded the effects of the *Ppo-D1* alleles studied herein.

In conclusion, crosses between common wheat low PPO lines produced progeny with levels of PPO activity not significantly different from Ben durum. Our study showed that the populations were fixed for the low allele at *Ppo-A1b*, yet the PI 117635/IDO580 population showed no variation in PPO activity in the presence of segregation for *Ppo-D1* alleles, and, in the PI 117635/IDO377s populations, the results were contrary to those previously predicted for *Ppo-D1* alleles. This last observation suggests that a rare recombination event might have occurred within the *Ppo-D1* locus, between the sites of primer binding and the sequences coding the active site of the enzyme. These results indicate that breeders could ignore variation at *Ppo-D1*, as most of the activity arises from *Ppo-A1*. Furthermore, markers for *Ppo-D1* alleles might predict erroneous phenotypes and genotypes in some backgrounds and lineages. In addition, both genotypic and genotype × environment effects modulate the expression of PPO activity.

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