Genetic characterization of kernel polyphenol oxidases in wheat and related species

E. Patrick Fuerst a,b, Steven S. Xu c, Brian Beecher b, *

a Department of Crop and Soil Sciences, Washington State University, Pullman, WA 99164-6394, USA
b USDA-ARS, Western Wheat Quality Laboratory, E202 Food Quality Building, Pullman, WA 99164-6394, USA
c USDA-ARS, Northern Crop Science Laboratory, Fargo, ND 58105, USA

Received 13 September 2007; revised 19 October 2007; accepted 25 October 2007

Abstract

Polyphenol oxidase (PPO) activity causes undesirable darkening of raw Asian noodles and other wheat products. In this study we investigate the genetic origins and diversity of wheat kernel PPO. PPO was characterized via activity assays, antigenic staining, and Southern blots in Triticum aestivum, Triticum dicoccoides, Triticum durum, Triticum dicoccum, Triticum monococcum, Triticum urartu, Aegilops speltoides, and Aegilops tauschii. Among these species, PPO activity was well-correlated with antigenic staining intensity toward a wheat kernel-type PPO antibody. High PPO activity was observed in all three T. monococcum accessions (A genome), one Ae. speltoides accession, one T. durum accession, and two hexaploid wheat cultivars. Southern blots suggested the presence of two or more kernel-type PPO genes in diploid progenitors of the hexaploid A, B, and D genomes. Whole-kernel PPO activity was evaluated in disomic substitution lines derived from three T. dicoccoides accessions in the background of T. durum ‘Langdon’. PPO activity was primarily associated with chromosome 2A and to a much lower degree with chromosome 2B. DNA sequence comparisons showed that the intron associated with the high PPO allele on chromosome 2AL of hexaploid wheat had 94% nucleotide identity with the homoeologous intron found in T. monococcum, a species with high kernel PPO activity. This implies that the ancestral PPO allele on the A genome is one of the high activity, and the low PPO allele found in hexaploid wheat represents a relatively recent genetic alteration. Results confirm the presence of multiple kernel-type PPO genes in the diploid and tetraploid progenitors and relatives of hexaploid wheat. However, it is likely that relatively few of the many kernel-type PPO genes present in wheat contribute substantially to kernel PPO activity. A single genetic locus on homoeologous group 2 chromosomes may be the primary cause of high PPO activity in wheat kernels.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Triticum aestivum; T. durum; T. monococcum; T. dicoccoides; T. urartu; T. dicoccum; Aegilops speltoides; Ae. tauschii; Chromosome substitution; Noodle discoloration

Abbreviations: Ae. speltoides, Aegilops speltoides Tausch; Ae. tauschii, Aegilops tauschii Coss.; L-DOPA, 3,4-dihydroxy-L-phenylalanine; MOPS, 3(N-morpholino) propanesulfonic acid; PPO, polyphenol oxidase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; T. aestivum, Triticum aestivum L. subsp. aestivum; T. dicoccoides, Triticum turgidum L. subsp. dicoccoides (Körn. ex Asch. & Graebn.) Thell.; T. dicoccum, Triticum turgidum L. subsp. dicoccum (Schrank) Thell.; T. durum, Triticum turgidum L. subsp. durum (Desf.) Husn.; T. monococcum, Triticum monococcum L. subsp. monococcum; T. urartu, Triticum urartu Tumanian ex. Gandilyan; Tris, Tris(hydroxymethyl)aminomethane.

* Mention of trademark or proprietary products does not constitute a guarantee or warranty of a product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable. This article is in the U.S. Public Domain and is not copyrightable.

* Corresponding author. Tel.: +1 509 335 4062; fax: +1 509 335 8573.
E-mail address: bbeecher@wsu.edu (B. Beecher).
1. Introduction

PPO is a major cause of browning reactions in fresh and processed fruits and vegetables, as well as certain products made from cereal grains (Whitaker and Lee, 1995). The brown or grey colors formed in raw yellowish alkaline (Canterese) and white salted (Udon) Asian wheat noodles are unacceptable to Asian consumers (Mares and Panozzo, 1999; Morris et al., 2000, 2002), and development of improved cultivars is imperative for exports to be competitive in the Asian market. Rapid whole-kernel PPO assays have substantially accelerated development of wheat cultivars with low PPO activity (AACC, 2000; Anderson and Morris, 2001). However, further improvement is possible if the specific alleles for low PPO are identified and combined during cultivar development. PPO has been shown to be localized to plastids of green tissue while potential phenolic substrates are localized in the vacuole. Consequently, browning reactions are initiated when tissue is disrupted, as in mechanical damage to plant tissue or processing of fresh foods (Whitaker and Lee, 1995). PPO activity is localized in the bran of wheat, and PPO activity in flour is due to contamination with trace quantities of bran (Hatcher and Kruger, 1993; Rani et al., 2001). The specific phenolic substrates that cause PPO-mediated darkening in wheat are unknown.

Our understanding of the wheat PPO multigene family is expanding rapidly. The first wheat PPO gene sequence reported was the 1509 bp genomic sequence AF507945 (Demeke and Morris, 2002) and additional sequences were reported by Anderson and Morris (2003a). Wheat PPO gene sequences were subsequently classified in two clusters, each having three closely related genes based on EST and genomic sequence homologies (Jukanti et al., 2004). The “kernel” cluster represented genes known to be expressed in wheat kernels, whereas the “non-kernel” cluster represented genes not expressed in kernels (Anderson et al., 2006; Jukanti et al., 2004). Massa et al. (2007) characterized the diversity of kernel-type genes by sequencing a ~500 bp polymorphic coding region in wheat and wild relatives. They obtained a total of 21 distinct PPO sequences of ~600-900 bp. Among these, there were at least four paralogous PPO groups, each of which contained multiple orthologs. We will refer to these as “kernel-type” PPO genes because they are related to genes expressed in the kernels, but tissue-specific expression has not been determined in most cases.

Numerous studies have independently shown that high PPO is inherited as a single gene and associated with homeologous chromosomes 2A, 2B, and/or 2D (Anderson et al., 2006; Chang et al., 2007; Demeke et al., 2001; He et al., 2007; Jimenez and Dubcovsky, 1999; Mares and Campbell, 2001; Raman et al., 2005, 2007; Simeone et al., 2002; Sun et al., 2005; Watanabe et al., 2004; Watanabe et al., 2006; Wrigley and McIntosh, 1975; Zeven, 1972; Zhang et al., 2005). The high PPO alleles on chromosomes 2AL and 2DL are the most thoroughly studied and were first reported by Wrigley and McIntosh (1975). Viewing this literature collectively, we hypothesize that a single genetic locus on the long arm of each homeologous pair of group 2 chromosomes is the primary basis for high kernel PPO activity.

Characterization of genomic PPO sequences in hexaploid wheat is complicated by three sets of homeologous chromosome pairs. Consequently we have chosen to compare a diversity of wheat progenitors, relatives, and substitution lines using PPO activity assays, Westerns, and Southern. Within each species, three accesses from diverse locations were selected for this study (Table 1). Diploid species containing progenitors of hexaploid wheat genomes A (Triticum monococcum and Triticum urartu), B (Aegilops speltoides), and D (Aegilops tauschii), were evaluated in addition to three tetraploid species containing A and B genome homologs (Triticum dicoccoides, Triticum dicoccum, and Triticum durum) (Table 1). The specific objectives of this study were to (1) correlate kernel staining, PPO enzyme activity, and protein antigenicity to a kernel-specific wheat PPO antibody in wheat progenitors and relatives, (2) characterize kernel- and non-kernel-type PPO genes in wheat progenitors and relatives using Southern blots, (3) evaluate association of kernel PPO activity with specific chromosomes in disomic substitution lines derived from three T. dicoccoides accesses in the background of T. durum ‘Langdon’, and (4) compare the first intron sequence from T. monococcum with homeologous sequences from hexaploid chromosome 2AL.

2. Experimental

2.1. Plant material

Wheat relatives used for this study were provided by Dr. Harold Bachelman at the USDA-ARS small grains collection in Aberdeen, ID (Table 1). Three T. dicoccoides accesses (‘Israel A’, PI 481521, and PI 478742) were chromosome donors for T. durum ‘Langdon’ disomic chromosome substitution lines developed by L.R. Joppa (Joppa and Cantrell, 1990; Xu et al., 2004). The substitution lines used in this study include two complete sets of chromosome substitution lines for PI 481521 and Israel A, and only the 2B substitution for PI 478742. ‘Langdon’, the T. dicoccoides chromosome donors, and the substitution lines were grown in two replicates (1–2 plants/replicate) in the greenhouse at the USDA-ARS, Northern Crop Science Laboratory (Fargo, ND). Hexaploid cultivars (‘Klasic’, ‘Chinese Spring’, ‘ID377s’, and ‘Penawawa’) were raised by the USDA-ARS Western Wheat Quality Laboratory in field plots near Pullman, WA.

2.2. Kernel staining, protein extraction, and soluble PPO assay

Lemma and palea were removed (when present) from kernels prior to kernel staining and protein extraction. Kernel staining has often been used as a visual indicator of PPO activity (e.g. Watanabe et al., 2004; Zeven, 1972). Whole kernels of wheat and its relatives were incubated in 10 mM L-DOPA in an end-over-end rotating mixer for 4 h, until differences in darkening were evident among wheat lines. Extraction of
PPO from whole kernels was similar to previously published procedures (Anderson et al., 2006; Fuerst et al., 2006a). Kernels were pulverized under liquid nitrogen and 100 mg of the powder was incubated in 1 ml of extraction buffer (50 mM MOPS pH 6.5, 0.1% SDS, 0.2% NP-40, 0.2 mM Pefabloc) in a microcentrifuge tube in an end-over-end shaker (8 rpm) at 4°C for 1 h. The suspension was homogenized for 60 sec in a polytron (model GLH, Omni International, Marietta, GA) and was again incubated 1 h in the shaker. The suspension was centrifuged at 4°C at 20,000 × g for 30 min and the supernatant was decanted and stored at −80°C. Protein was assayed using bicinchoninic acid (BCA protein assay kit, Pierce). Assays of PPO activity in extracts were conducted as previously described (Fuerst et al., 2006a), using 10 mM L-DOPA as substrate in 50 mM MOPS pH 6.5. Activity was initiated by addition of the enzyme extract and increase in absorbance was measured at 475 nm from 30 to 90 sec after initiation.

### 2.3. Western blots

Protein extracts were the same as those used in PPO assays, above. Proteins were separated by 7.5% SDS-PAGE and transblotted to PVDF (Bio-Rad) as previously described (Anderson and Davis, 2004) but using different pre-stained protein standards (SeeBlue Plus2, Invitrogen, San Diego, CA). Transblots were incubated in TBST blocking buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% bovine serum albumin for 1 h prior to incubation in a 1:7,500 dilution of wheat PPO antibody for at least 1 h. Rabbit polyclonal wheat PPO antibody raised against recombinant wheat kernel PPO from accession BT009357 (Anderson et al., 2006) was a gift from J.V. Anderson. Blots were washed 4 × 10 min with TBST prior to incubation with a 1:10,000 dilution of goat anti-rabbit conjugated alkaline phosphatase (Bio-Rad) secondary antibody for 1 h. Blots were washed an additional 4 × 15 min in TBST, followed by 2 × 5 min in water, and developed by addition of NBT & BCIP (Sigma-Aldrich). The procedure was repeated four times with similar results. Optical density was determined with a Syngene (Frederick, MD) ‘GeneGenius’ Bio Imaging System and ‘GeneSnap’ imaging software.

### 2.4. Southern analysis

Southern blot analysis was performed by standard methods described previously (Beecher et al., 2002). Briefly, genomic DNA was prepared from young greenhouse-grown leaf tissue and digested with HindIII overnight (Riede and Anderson, 1996). Digested DNA was fractionated on a 0.7% agarose gel

<table>
<thead>
<tr>
<th>Lane number</th>
<th>Species</th>
<th>Genome</th>
<th>Accession identifier</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>T. monococcum</em></td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PI 167615</td>
<td>Balikesir, Turkey</td>
</tr>
<tr>
<td>2</td>
<td><em>T. monococcum</em></td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PI 345181</td>
<td>Santiago, Chile</td>
</tr>
<tr>
<td>3</td>
<td><em>T. monococcum</em></td>
<td>Crl 2433</td>
<td></td>
<td>Thuringia, Germany</td>
</tr>
<tr>
<td>4</td>
<td><em>T. urartu</em></td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PI 428187</td>
<td>Mardin, Turkey</td>
</tr>
<tr>
<td>5</td>
<td><em>T. urartu</em></td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PI 428272</td>
<td>El Beqaa, Lebanon</td>
</tr>
<tr>
<td>6</td>
<td><em>T. urartu</em></td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PI 487268</td>
<td>Al Hasakah, Syria</td>
</tr>
<tr>
<td>7</td>
<td><em>Ae. speltoides</em></td>
<td>S</td>
<td>PI 219867</td>
<td>Arbil, Iraq</td>
</tr>
<tr>
<td>8</td>
<td><em>Ae. speltoides</em></td>
<td>D</td>
<td>PI 393493</td>
<td>Israel</td>
</tr>
<tr>
<td>9</td>
<td><em>Ae. speltoides</em></td>
<td>D</td>
<td>PI 487232</td>
<td>Idlib, Syria</td>
</tr>
<tr>
<td>10</td>
<td><em>Ae. tauschii</em></td>
<td>D</td>
<td>Clae 8</td>
<td>Mazandaran, Iran</td>
</tr>
<tr>
<td>11</td>
<td><em>Ae. tauschii</em></td>
<td>D</td>
<td>PI 220641</td>
<td>Kondoz, Afghanistan</td>
</tr>
<tr>
<td>12</td>
<td><em>Ae. tauschii</em></td>
<td>D</td>
<td>PI 554314</td>
<td>Van, Turkey</td>
</tr>
<tr>
<td>13</td>
<td><em>T. dicoccoides</em></td>
<td>AB</td>
<td>PI 190919</td>
<td>Zaragoza, Afghanistan</td>
</tr>
<tr>
<td>14</td>
<td><em>T. dicoccoides</em></td>
<td>AB</td>
<td>PI 300989</td>
<td>Israel</td>
</tr>
<tr>
<td>15</td>
<td><em>T. dicoccoides</em></td>
<td>AB</td>
<td>PI 478745</td>
<td>Israel</td>
</tr>
<tr>
<td>16</td>
<td><em>T. dicoccum</em></td>
<td>AB</td>
<td>Crl 9311</td>
<td>Georgia</td>
</tr>
<tr>
<td>17</td>
<td><em>T. dicoccum</em></td>
<td>AB</td>
<td>Crl 14454</td>
<td>Sidamo, Ethiopia</td>
</tr>
<tr>
<td>18</td>
<td><em>T. dicoccum</em></td>
<td>AB</td>
<td>PI 94654</td>
<td>Bulgaria</td>
</tr>
<tr>
<td>19</td>
<td><em>T. durum</em></td>
<td>AB</td>
<td>Crl 1494</td>
<td>North Dakota, United States</td>
</tr>
<tr>
<td>20</td>
<td><em>T. durum</em></td>
<td>AB</td>
<td>Crl 15064</td>
<td>Baghan, Afghanistan</td>
</tr>
<tr>
<td>21</td>
<td><em>T. durum</em></td>
<td>AB</td>
<td>PI 94729</td>
<td>Saratov, Russian Federation</td>
</tr>
<tr>
<td>22</td>
<td><em>T. aestivum</em></td>
<td>ABD</td>
<td>‘Klasic’, PI 486139</td>
<td>United States</td>
</tr>
<tr>
<td>23</td>
<td><em>T. aestivum</em></td>
<td>ABD</td>
<td>‘Chinese Spring’ Crl 14108</td>
<td>United States</td>
</tr>
<tr>
<td>24</td>
<td><em>T. aestivum</em></td>
<td>ABD</td>
<td>‘ID377’, PI 591045</td>
<td>United States</td>
</tr>
<tr>
<td>25</td>
<td><em>T. aestivum</em></td>
<td>ABD</td>
<td>‘Penawawa’, PI 495916</td>
<td>United States</td>
</tr>
<tr>
<td>26</td>
<td><em>T. durum</em></td>
<td>AB</td>
<td>‘Langdon’, Crl 13165</td>
<td>United States</td>
</tr>
</tbody>
</table>

<sup>a</sup> See abbreviations.

<sup>b</sup> Accession numbers from USDA National Small Grains Collection, Aberdeen, Idaho, USA.
for 30 h at 15 V, and blotted to a positively charged nylon membrane (Osmonics, Inc.). The amount of DNA loaded was proportional to the number of genomes present to insure appropriate representation of a single locus among the various species: 7 μg/lane for diploids, 14 μg/lane for tetraploids, and 21 μg/lane for hexaploids. Blots were hybridized to [32P]-labeled probes prepared by a random primer method (Invitrogen). The kernel-type probe was cDNA clone BT009357, while the non-kernel-type probe was clone AF507945 (gifts from J.V. Anderson and C.F. Morris, respectively). Following hybridization, the membranes were washed twice at low stringency (2× SSPE, 0.1% SDS) and then two times at high-stringency (0.2× SSPE, 0.1% SDS). All washes were 15 min at 65°C. Washed membranes were exposed to Kodak Biomax MS film at −80°C using an intensifying screen.

2.5. Whole-kernel PPO assays of T. durum ‘Langdon’ chromosome substitution lines

Kernel PPO was determined according to Approved Method 22-85 (AACC, 2000) with five kernels per 1.5 ml 10 mM L-DOPA substrate. Each ‘Langdon’ chromosome substitution line and their chromosome donors were assayed, including two greenhouse replicates of each line and four samples (five kernels each) for each replicate. PPO activity was measured after 1 h as absorbance at 475 nm. Data were analyzed as a randomized block design (greenhouse replicates as blocks) using SAS Version 9.0 (SAS Institute, Cary, NC). Blocks were analyzed for analysis of variance and mean separation using the general linear models procedure in SAS with α set equal to 0.05. Mean separation was evaluated using Duncan’s multiple range test.

3. Results

3.1. Kernel staining, PPO assays, and Western blots

Soluble PPO was extracted from 26 wheat cultivars and relatives and characterized by kernel staining (Fig. 1A), assays of PPO extracts with L-DOPA substrate (Fig. 1B), and Western blots (Fig. 1C). All three of the T. monococcum accessions (lanes 1–3) evaluated had dark kernel staining, high PPO activity, and strong antigenicity, indicating that there is at least one locus of high kernel PPO activity on the A genome. Accessions of other diploid species, T. urartu, Ae. speltoides, and Ae. tauschii (lanes 4–12), had low to moderate PPO activity except for Ae. speltoides PI 393493 (lane 8) which had very high PPO activity and strong antigenicity. Tetraploid accessions of T. dicoccoides, T. dicoccum, and T. durum (lanes 13–21) had low to moderate PPO activity compared to T. monococcum, except for T. durum PI 15064 (lane 20) which had very high PPO. T. durum ‘Langdon’ (lane 26) had the lowest PPO activity of any accession tested. Although ‘Langdon’ was omitted in our Western blot (Fig. 1C), earlier studies using the same methods showed very low antigenic staining intensity in ‘Langdon’ compared to the hexaploids evaluated here (Anderson et al., 2006). PPO activity was highly variable among Triticum aestivum cultivars: ‘Chinese Spring’ (lane 23) had the lowest PPO, ‘ID377s’ (lane 24) was intermediate, and ‘Klasic’ (lane 22) and ‘Penawawa’ (lane 25) were highest, similar to previous reports (Anderson et al., 2006; Fuerst et al., 2006a,b). In the Westerns, only one major antigenic band, possibly a doublet, was observed at ~72 kD in all genotypes evaluated (Fig. 1C). Despite the genetic diversity included here, both antigenic staining and PPO activity were usually correlated with kernel staining intensity, especially...
when comparing within a species (Fig. 1A). Extracted PPO activity was well-correlated ($r^2 = 0.61$) with antigenic staining intensity (Fig. 2).

### 3.2. Southern blots

Genomic DNA from 25 wheat cultivars and relatives was digested with HindIII and probed with kernel-type accession BT009357 (Fig. 3). DNA loading was proportional to the number of genomes present. Consequently, the exposure of hexaploid wheat (lanes 22–25) was the darkest. Since only one restriction enzyme was used in this procedure, conclusions regarding the number of genes present must be qualified. Each diploid wheat relative had two or more kernel-type PPO hybridizing bands. *T. monococcum* appeared to have more kernel-type PPO bands than *T. urartu*, *Ae. speltoides*, and *Ae. tauschii*. Tetraploid *T. dicoccoides*, *T. dicoccum*, and *T. durum*, generally had more kernel-type bands than the diploid species, whereas the number of bands in hexaploid *T. aestivum* was generally comparable to the tetraploid species.

An additional Southern blot of eight wheat relatives and cultivars confirmed the presence of two or more kernel-type PPO hybridizing sequences in diploid wheat species (Fig. 4A, lanes 1–4), except that the *Ae. tauschii* accession (lane 4) appeared to have a single band. They also indicate that *T. durum* ‘Langdon’ (lane 6) may have two or more kernel-type PPO genes, despite its very low PPO activity (Fig. 1B). The same blot was probed with non-kernel-type accession AF507945 (Fig. 4B). In general, the tetraploid (lanes 5 and 6) and hexaploid (lanes 7 and 8) accessions all had at least three non-kernel-type PPO bands, which exceeded the number of non-kernel-type bands in the diploid accessions (lanes 1–4).

#### 3.3. Whole-kernel PPO assays of *T. durum* ‘Langdon’ chromosome substitution lines

We compared whole-kernel PPO activities in *T. durum* ‘Langdon’ disomic chromosome substitutions from three *T. dicoccoides* accessions (Fig. 5). ‘Langdon’ had very low PPO activity compared to the *T. dicoccoides* chromosome donors, and most chromosome substitution lines were similar to ‘Langdon’. ‘Israel A’ had the lowest PPO activity among the three *T. dicoccoides* chromosome donors. PPO activity was associated with ‘Israel A’ chromosome 2A and a small increase in activity was associated with chromosome 2B. High PPO activity was associated with PI 481521 chromosome 2A and a small but non-significant increase in PPO activity was associated with chromosome 7B. Not all chromosome substitutions, including the 2A, are available for PI 478742 (Xu et al., 2004). Therefore we limited the evaluation to chromosome 2B, which was associated with a small increase in PPO activity (Fig. 5).

---

**Fig. 2.** Correlation between extracted kernel PPO activity and antigenic staining intensity in the Western blot.

**Fig. 3.** Southern blot comparing the same wheat relatives as in Fig. 1. Genomic DNA was digested with HindIII and probed with $[^{32}P]$-labeled kernel-type accession BT009357. Lanes are as indicated in Table 1. Molecular size standard positions in kilobase pairs are shown at left.
3.4. Alignment of intron DNA sequences

A previous study mapped the AY596268 sequence to the long arm of chromosome 2A, and two variants of the first intron were observed in the alleles PPO-A1a (EF070147) and PPO-A1b (EF070148) (He et al., 2007; Sun et al., 2005). The shorter intron of PPO-A1a was associated with higher PPO activity while the longer intron of PPO-A1b was associated with lower PPO activity. We compared partial gene sequences including the intron 1 region from PPO-A1a and PPO-A1b (He et al., 2007) with a PCR clone isolated from T. monococcum ‘DV92’ (this study) (Fig. 6). The T. monococcum intron had 94% nucleotide identity with the high PPO allele PPO-A1a from T. aestivum (Fig. 6). Excluding the 191 bp insertion in the PPO-A1b intron, PPO-A1a and PPO-A1b introns had 99% nucleotide identity.

4. Discussion

PPO is a major cause of undesirable darkening in certain wheat products such as raw Asian noodles (Fuerst et al., 2006b; Kruger et al., 1994; Mares and Panozzo, 1999; Morris et al., 2000, 2002). Reducing or eliminating PPO-based darkening is a plausible goal for improving this aspect of wheat quality. The development of a rapid screen for kernel PPO activity has facilitated the reduction of this activity as new wheat lines are developed (AACC, 2000; Anderson and Morris, 2001), while marker-assisted selection may allow additional improvements in the future (Chang et al., 2007; He et al., 2007; Raman et al., 2007; Simeone et al., 2002; Sun et al., 2005).

A diversity of wheat-related germplasm was evaluated in this study (Table 1; Figs. 1–6). All wheat relatives had
measurable PPO activity ranging from the highest in *T. aestivum* ‘Penawawa’, to the lowest in *T. durum* ‘Langdon’ (Fig. 1B). Among the diploid wheat types, all *T. monococcum* accessions (lanes 1–3) had high PPO activity, whereas only two other non-hexaploid accessions, *Ae. speltoides* PI 393493 (lane 8) and *T. durum* CItr 15064 (lane 20), showed similar activity. Based on these observations, we hypothesized that *T. monococcum* contains the ancestral form of the high PPO trait in hexaploid wheat that is associated with chromosome 2A (discussed below). None of the three accessions of *Ae. tauschii* (lanes 10–12), the D genome donor of hexaploid wheat, had such a high level of PPO activity. Although many *T. durum* cultivars including ‘Langdon’ have very low PPO activity (Fuerst et al., 2006a,b), a few high PPO durum wheat cultivars have been reported elsewhere (Bernier and Howes, 1994; Simeone et al., 2002), similar to what we observed for CItr 15064 (lane 20).

Western blots (Fig. 1C) were conducted with a polyclonal antibody to a recombinant PPO from the kernel-type EST sequence BT009357 (Anderson et al., 2006). All accessions showed one major antigenic band at ~72 kD, possibly a doublet, which stained at varying intensities. Anderson et al. (2006) showed that hexaploid cultivars had two antigenic bands, one at ~58 kD and one at ~60–62 kD, using the same antibody and Western blot procedures used here. Anderson et al. (2006) showed that the staining intensity varied similarly among several of the same cultivars and that the two bands were usually of similar intensity, comparable to the possible doublet that we see (Fig. 1C). Although our methods were virtually identical, two differences in procedures probably contributed to the differences in band separation and apparent molecular weight: (1) different molecular weight standards were used and (2) the extraction procedure by Anderson et al. (2006) was only 10 min vs. a 2 h extraction and homogenization procedure here. Our procedure may have extracted substances that interfered with electrophoresis.

The high correlation ($r^2 = 0.61$) between extracted PPO and antigenic staining intensity (Fig. 2) was surprising, considering the diverse genetics and kernel morphology (Fig. 1A) evaluated. This strong relationship suggests a possible direct and fairly simple relationship between the quantity of kernel-type PPO protein and the activity. This is somewhat unexpected due to varying PPO isoforms and activation levels (Anderson et al., 2006). Such variances may be responsible for the “outlier” in Fig. 2, *T. monococcum* CItr 2433, which had the darkest antigenic staining intensity but intermediate PPO activity (Fig. 1, lane 3). The strong correlation between PPO activity and antigenic staining intensity suggests the possibility that an accurate ELISA test for PPO activity, possibly using kernel leachates as in Fuerst et al. (2006a), could be developed for research or germplasm screening purposes.

Jukanti et al. (2004) classified six wheat PPO sequences as either kernel- or non-kernel-type genes. Massa et al. (2007) explored a diversity of germplasm from wheat relatives and further classified the kernel-type PPO genes into at least four paralogous groups; they also demonstrated that diploid wheat progenitors had multiple kernel-type PPOs. Our observations of multiple PPO genes in diploid and tetraploid wheat progenitors (Figs. 3 and 4) are consistent with those of Massa et al. (2007). Our Southern blots suggest that each diploid wheat relative has two or more kernel-type PPO genes (Fig. 3, lanes 1–12; Fig. 4, lanes 1–4). There was an apparent
correlation between the number of potential kernel-type PPO genes and level of PPO activity. For example, *T. monococcum* accessions all had high PPO activity (Fig. 1B) and appeared to have more kernel-type genes than the other diploid species (Figs. 3 and 4A). Likewise, ‘Langdon’ appeared to have two genes (Fig. 4A, lane 6) and had lower PPO activity (Fig. 2B) while other *T. durum* accessions had higher PPO activity (Fig. 2B) and three or more potential PPO genes (Fig. 3). Also, ‘Chinese Spring’ had fewer genes than ‘Klasic’ (Fig. 3, lanes 23 and 22, respectively; Fig. 4A, lanes 7 and 8, respectively) and likewise had lower PPO activity (Fig. 1B, lanes 23 and 22, respectively). The correlation between apparent gene number and PPO activity implies the possibility that multiple loci may contribute to the total PPO activity observed in kernels of wheat and its relatives. This is contrary to our hypothesis that a single homeologous locus on each genome controls PPO activity (discussed further, below).

Southern blot analyses of kernel-type PPOs in these wheat relatives have not been reported elsewhere, to the best of our knowledge. Based on the observation of two or more kernel-type genes in diploid relatives of the A, B, and D genomes, the number of potential genes in hexaploid wheat was less than additive (Figs. 3 and 4A). In part, this may be explained by the possibility of overlapping bands in hexaploid wheat. Nonetheless, hexaploid wheat clearly has several kernel-type PPO genes as seen here and as reported by Massa et al. (2007).

There was no apparent relationship between the potential number of genes and PPO activity when probing with the non-kernel-type accession AF507945. For example, ‘Chinese Spring’ and ‘Klasic’ had identical Southern blots (Fig. 4B, lanes 7 and 8, respectively) but very different kernel PPO activity (Fig. 1B, lanes 23 and 22, respectively). Likewise, the extremely low PPO cultivar ‘Langdon’ had at least as many potential non-kernel PPO genes as the higher PPO accession *T. dicoccoides* (Fig. 4B, lanes 6 and 5, respectively). As expected, there was no detectable mRNA complementary to AF507945 in developing wheat kernels (Anderson et al., 2006).

High PPO activity in wheat kernels has been widely associated with chromosomes 2AL and 2DL (Anderson et al., 2006; Chang et al., 2007; Demeke et al., 2001; He et al., 2007; Jimenez and Dubcovsky, 1999; Mares and Campbell, 2001; Raman et al., 2005, 2007; Simeone et al., 2002; Sun et al., 2005; Watanabe et al., 2004; Watanabe et al., 2006; Wrigley and McIntosh, 1975; Zhang et al., 2005). PPO activity has occasionally been associated with chromosome 2BL (Demeke et al., 2001; Watanabe et al., 2004). The high PPO trait was present on only one of these chromosomes in most cultivars and, in these cases, was inherited as a dominant single gene trait. However, the high PPO trait was present on both the 2AL and 2DL chromosomes of ‘Timstein’ and ‘Zhongyou 9507’ (Wrigley and McIntosh, 1975; Zhang et al., 2005). Viewing this literature collectively, we hypothesize that a single genetic locus on the long arm of each homoeologous group 2 chromosome pair is the primary basis for high kernel PPO activity. The high PPO trait may also be associated with a homeologous locus among other Triticeae members. For example, the high PPO trait was inherited as a dominant single gene trait associated with syntenous chromosome 2 of barley (Takeda and Chang, 1996), chromosome 2R of rye (Wrigley and McIntosh, 1975), and chromosome 4 of rice (Raman et al., 2005).

‘Langdon’ had very low PPO activity and most disomic chromosome substitution lines were similar to ‘Langdon’, indicating that relatively few chromosomes contributed to kernel PPO activity (Fig. 5). Substantial PPO activity was associated with chromosome 2A in both *T. dicoccoides* accessions for which this substitution was available (Fig. 5). Not all chromosome substitutions, including the 2A, are available for PI 478742 (Xu et al., 2004). Therefore we only evaluated chromosome 2B of PI 478742. A small increase in PPO activity, relative to the ‘Langdon’ background, was associated with chromosome 2B of PI 478742 and PI 481521. Activity associated with chromosome 2B was much lower than that associated with chromosome 2A (Fig. 5). We are aware of only two other reports in which PPO activity was associated with chromosome 2B (or 2BL) (Demeke et al., 2001; Watanabe et al., 2004). The presence of a high PPO trait in *Ae. speltoides* PI 393493 (Fig. 1B, lane 8), which contains the putative donor of the B genome of wheat, suggests the potential for a high PPO trait to be associated with the B genome in other tetraploid and hexaploid accessions.

The genomic sequence for kernel-type PPOs was mapped to chromosome 2AL and contained two introns (Sun et al., 2005). The first intron was 191 bp longer in cultivars with low kernel PPO activity than in cultivars with high PPO activity. Sun et al. (2005) hypothesized that chromosome 2AL-based PPO activity levels are regulated by this difference in intron length. The chromosome 2AL alleles were designated as *PPO*-*A1a* (high PPO activity) and *PPO*-*A1b* (low PPO activity) by He et al. (2007) and as *TaPPO-A1b* and *TaPPO-A1a*, respectively, by Chang et al. (2007). At least five types of the second intron were present in kernel-type PPO genes, in a diversity of wheat relatives (Massa et al., 2007), but relationships between the second intron and PPO activity have not been determined. The importance of the 2AL gene has been confirmed at the protein level. Peptide sequences of wheat kernel PPO purified from high PPO cultivars (Anderson and Morris, 2003b; Jukanti et al., 2006) were homologous to AY596268 (Jukanti et al., 2004), substantiating the significance of this gene.

Based on the high PPO activity observed in *T. monococcum* (Fig. 1B), we hypothesized that the *T. monococcum* contains the ancestral form of the high PPO trait associated with the 2AL *PPO-A1a* allele of hexaploid wheat. Our results indicated that *T. monococcum* ‘DV92’ contains an intron sequence having 94% nucleotide identity with that of *PPO-A1a* (Fig. 6). This shorter intron form associated with high PPO activity (Chang et al., 2007; He et al., 2007; Sun et al., 2005) is therefore likely to be the ancestral form of the PPO gene. *PPO-A1a* and *PPO-A1b* share 99% identity when the 191 bp intron insertion of *PPO-A1b* is excluded from the comparison. The intron insertion present in the *PPO-A1b* allele is therefore a relatively recent genetic alteration. In related work, the alleles for both high and low PPO activity associated with
chromosome 2D were detected in the D genome ancestor, *Ae. tauschii* (Chang et al., 2007).

Many observations are consistent with the hypothesis that a single genetic locus on homeologous group 2 chromosomes plays a major role in determining wheat kernel PPO activity. In particular, chromosome 2AL contributes to very high PPO activity (Chang et al., 2007; Raman et al., 2005, 2007; Simeone et al., 2002; Sun et al., 2005; Watanabe et al., 2006; Wrigley and McIntosh, 1975; Zeven, 1972; Zhang et al., 2005) and this is supported by our observations in ‘Langdon’ chromosome substitution lines (Fig. 5). *T. monococcum* has very high PPO activity (Fig. 1B) and appears to be the ancestral origin of the high PPO allele, *PPO-A1a*, of hexaploid wheat (Fig. 6). A locus on chromosome 2DL also contributes substantially to kernel PPO activity but not as much as the 2AL locus (Chang et al., 2007; He et al., 2007; Zhang et al., 2005). The 2DL allele for low PPO activity still contributes significant PPO activity, since several wheat accessions with an apparent null 2DL allele had much lower PPO activity than those with the low PPO activity allele (Chang et al., 2007). Finally, a locus on chromosome 2BL appears to contribute to kernel PPO activity (Watanabe et al., 2004) but this may be a minor factor in kernel PPO activity, as seen in *T. dicoccum* (Fig. 5).

Contrary to the simple hypothesis that kernel PPO activity is controlled by a single genetic locus on homeologous group 2 chromosomes, we observed that accessions with higher PPO activity (Fig. 1B) tended to have more kernel-type PPO genes (Figs. 3 and Fig. 4A). This implies the possibility that additional genetic loci contribute to kernel PPO activity. Perhaps such genetic loci make minor contributions to PPO activity, such as we observed for chromosome 7B of PI 481521 (Fig. 5). Alternatively, since this observation is merely a correlation, it may be a coincidence that more kernel-type genes were observed among the higher PPO wheat cultivars and relatives that we selected. The published literature as well as our own results strongly suggest that relatively few of the many kernel-type PPO genes contribute to high kernel PPO activity.

Acknowledgements

Special thanks to James V. Anderson for providing the wheat PPO clone (BT009357), to Leonard Joppa for developing the substitution lines, and to Justin D. Faris for providing the original seed of ‘Langdon’ durum disomic substitution lines. This research was supported in part by U.S. Department of Agriculture NRI Grant Award 2002-35503-12670.

References


