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ORIGINAL PAPER

Allelic variation of polyphenol oxidase (PPO) genes located on chromosomes 2A and 2D and development of functional markers for the PPO genes in common wheat

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Abstract Polyphenol oxidase (PPO) activity is highly related to the undesirable browning of wheat-based end products, especially Asian noodles. Characterization of PPO genes and the development of their functional markers are of great importance for marker-assisted selection in wheat breeding. In the present study, complete genomic DNA sequences of two PPO genes, one each located on chromosomes 2A and 2D and their allelic variants were characterized by means of in silico cloning and experimental validation. Sequences were aligned at both DNA and protein levels. Two haplotypes on chromosome 2D showed 95.2% sequence identity at the DNA level, indicating much more sequence diversity than those on chromosome 2A with 99.6% sequence identity. Both of the PPO genes on chromosomes 2A and 2D contain an open reading frame (ORF) of 1,731 bp, encoding a PPO precursor peptide of 577 amino acids with a predicted molecular mass of ~64 kD. Two complementary dominant STS markers, *PPO16* and *PPO29*, were developed based on the PPO gene haplotypes located on chromosome 2D; they amplify a 713-bp fragment in cultivars with low PPO activity and a 490-bp fragment in those with high PPO activity, respectively. The two markers were mapped on chromosome 2DL using a doubled haploid population derived from the cross Zhongyou 9507/CA9632, and a set of nullisomic–tetrasomic lines and ditelosomic line 2DS of Chinese Spring. QTL analysis indicated that the PPO gene co-segregated with the two STS markers and was closely linked to SSR marker *Xwmc41* on chromosome 2DL, explaining from 9.6 to 24.4% of the phenotypic variance for PPO activity across

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three environments. In order to simultaneously detect PPO loci on chromosomes 2A and 2D, a multiplexed marker combination *PPO33/PPO16* was developed and yielded distinguishable DNA patterns in a number of cultivars. The STS marker *PPO33* for the PPO gene on chromosome 2A was developed from the same gene sequences as *PPO18* that we reported previously, and can amplify a 481-bp and a 290-bp fragment from cultivars with low and high PPO activity, respectively. A total of 217 Chinese wheat cultivars and advanced lines were used to validate the association between the polymorphic fragments and grain PPO activity. The results showed that the marker combination *PPO33/PPO16* is efficient and reliable for evaluating PPO activity and can be used in wheat breeding programs aimed for noodle and other end product quality improvement.

Introduction

Polyphenol oxidase (PPO), a ubiquitous enzyme in plants, is associated with browning and discoloration of pasta (Simeone et al. 2002), pan bread (McCallum and Walker 1990), steamed bread (Dexter et al. 1984) and particularly Asian noodles (Kruger et al. 1992; Baik et al. 1995; Mares and Campbell 2001; Fuerst et al. 2006). PPOs catalyze the oxidation of phenols to produce dark colored products undesirable for most classes of noodles in oriental Asia (Feillet et al. 2000). Therefore, developing wheat cultivars with low PPO activity is one of the most important objectives in wheat breeding programs.

To date, full-length sequences of PPO genes have been cloned in several plant species including broad bean (Cary et al. 1992), tomato (Shahar et al. 1992), potato (Hunt et al. 1993), grape (Dry and Robinson 1994), apple (Boss et al. 1995), sugar cane (Bucheli et al. 1996), apricot (Chevalier et al. 1999), poplar (Constabel et al. 2000) and banana (Gooding et al. 2001). All of these PPO genes have two conserved copper-binding domains, CuA and CuB, forming the central domain of the catalytic site. Based on the conserved copper-binding sequences of other plant PPO genes, Demeke and Morris (2002) cloned the first partial sequence of a wheat PPO gene (GenBank accession number AF507945). Subsequently, Jukanti et al. (2004) obtained five additional partial sequences of wheat PPO genes by means of in silico cloning and three of them (AY596268, AY596269 and AY596270) were believed to be expressed in kernels. Peptide sequences of the first purified wheat kernel PPO (Anderson and Morris 2003) were homologous to those encoded by AY596268 (Jukanti et al. 2004), supporting the hypothesis that the gene for AY596268 is expressed in kernels. Jukanti et al. (2006) subsequently purified another kernel protein, also homologous to that encoded by AY596268, confirming the significance of this gene. Recently, Anderson et al. (2006) and Jukanti et al. (2006) reported that kernel PPO transcript levels in developing kernels of several cultivars were positively correlated with PPO activity of mature kernels. The wheat PPO cDNA, AY515506 (Anderson et al. 2006), was most likely derived from the same gene as AY596270, based upon 99% sequence identity (Jukanti et al. 2004). The full-length wheat kernel cDNA clone, BT009357, sharing 96% similarity with AY596269, was used to produce recombinant protein and antibody (Anderson et al. 2006). Western and Northern blots indicated that kernel PPO protein levels were greatest during kernel development, and declined rapidly during maturation, using BT009357 and its corresponding antibody (Anderson et al. 2006) and AY596268 and its corresponding antibody (Jukanti et al. 2006).

Many studies to date implied that PPO activity is mainly conditioned by the genes located on homoeologous group 2 chromosomes in wheat. Udall (1997) mapped a QTL for PPO activity on chromosome 2A in a recombinant inbred line population derived from the cross NY6432-18/Clark's Cream (NY18/CC), that accounted for over 40% of the phenotypic variance. Jimenez and Dubcovsky (1999) reported that genes located in the wheat homoeologous group 2 played an important role in PPO activity, and chromosome 2D was specifically implicated as a high PPO locus in cv. Chinese Spring (Anderson and Morris 2001; Anderson et al. 2006) and in a doubled haploid (DH) population derived from Sunco/Tasman (Mares and Campbell 2001). Demeke et al. (2001) also detected a QTL significantly associated with wheat PPO activity on chromosome 2D in the M6/Opata85 mapping population. Raman et al. (2005) found a major QTL for PPO activity on chromosome 2AL in a DH population derived from Chara/WW2449, explaining 82–84% of the genetic variation. In addition, a QTL on chromosome 2B was reported by Demeke et al. (2001) in the population derived from NY18/CC, accounting for 12–13% of the phenotypic variance. The molecular markers linked with the QTLs mentioned above were either RFLPs or SSRs, which were not specific for the PPO genes in question. Consequently, it is desirable to develop functional STS markers for PPO genes with high levels of precision in distinguishing different alleles associated with high vs. low PPO activity.

Previously, we detected two QTLs on chromosomes 2AL and 2DL in a DH population derived from Zhongyou 9507/CA9632 (Zhang et al. 2005), and developed a functional STS marker *PPO18* for the PPO gene on chromosome 2A based on the DNA sequence of AY596268 (Sun et al. 2005). However, no functional markers for the PPO gene on chromosome 2D have been developed so far. The objectives of this study were to characterize the PPO genes on chromosomes 2A and 2D, and to develop functional STS markers for the PPO gene on chromosome 2D.

Materials and methods

Plant materials

In total, 217 Chinese wheat cultivars and advanced lines were used for the validation of new STS markers. A DH population with 71 lines derived from the cross Zhongyou 9507/CA9632 was employed to map both the PPO genes and the newly developed STS markers. Zhongyou 9507 is a wheat cultivar with high PPO activity, while CA9632 has low PPO activity. This DH population was used for QTL mapping of PPO genes with 143 SSRs, four STS and 26 AFLP markers in our previous study (Zhang et al. 2005). A set of Chinese Spring nullisomic–tetrasomic lines (except nullisomic 2A and nullisomic 4B lines) and ditelosomic line 2DS were kindly provided by Prof. R.A. McIntosh, University of Sydney, and were employed to verify the chromosome location of STS markers. The nullisomic 2A–tetrasomic 2B line was selected from the self-pollinating progenies of the monosomic 2A–tetrasomic 2B (M2A–T2B) line, kindly provided by Prof. Peidu Chen of Nanjing Agricultural University. In addition, a durum wheat cultivar Langdon and an *Aegilops tauschii* line Ae38 available in the National Genbank of China were used to confirm the location of STS markers as well.

Field trials and grain PPO activity assay

During the 2001–2002 and 2002–2003 cropping seasons, 217 Chinese wheat cultivars and advanced lines were sown in a randomized complete block design with three replicates in the Anyang experimental station of the Chinese Academy of Agricultural Sciences, located in Henan Province. Each plot consisted of two 2-m rows spaced 25 cm apart, with 100 plants in each row. Field trials for the DH population were conducted with two replicates each in Beijing, Jinan and Anyang in the 2001–2002 cropping season and were described in detail in our previous study (Zhang et al. 2005). Test plots were managed according to local practices. All field trials were kept free of weeds and diseases, with two applications of broad-range herbicides and fungicides, respectively. The procedure for estimating PPO activity in wheat grains followed the method described by Anderson and Morris (2001) with the following minor modifications: 4.5 ml of 10 mM L-DOPA (3,4-dihydroxyphenylalanine) in 50 mM MOPS [3-(*N*-morpholino) propane sulfonic acid] buffer, pH 6.5, with 15 seeds constantly rotated in a 50-ml centrifuge tube for 0.5 h at room temperature to allow the reaction to take place. Absorbance (A_{475}) was measured on 1.0 ml of incubated solution at 475 nm using a TU-1800PC UV-VIS spectrophotometer (Beijing Purkinje General Instrument Co., Ltd) with a UV-WIN (a WINDOWS platform) software package against a solvent blank. The L-DOPA solution was made fresh daily. One unit of PPO activity was defined as a change of 1 absorbance (A_{475}) unit min^{-1} g^{-1} 10^{-3} in a 1-cm path at 475 nm.

Strategies for cloning and sequence analysis of PPO genes

In silico cloning was employed for obtaining the lacking 5′ coding sequences (CDS) of PPO genes. Both AY596268 and AY515506 were used for BLAST search against the GenBank wheat EST database. All ESTs sharing high similarity with the upstream sequences of reference genes were subjected to the construction of tentative full-length PPO gene sequence. Primers were designed based on the constructed sequences using the software Premier Primer 5 (http://www.premierbiosoft.com) and synthesized by Beijing Augct Biological Technology Co., Ltd. (http://www.augct.com).

Genomic DNA was extracted from a single seed using a method modified from Lagudah et al. (1991). PCR reactions were performed in an MJ Research PTC-200 thermal cycler in a total volume of 20 µl including 20 mM of Tris–HCl (pH 8.4), 20 mM of KCl, 150 µM of each of dNTP, 1.5 mM of $MgCl_2$, 8 pmol of each primer, 1 unit of *Taq* DNA polymerase (TIANGEN Biotech (Beijing) Co., Ltd, www.tiangen.com) and 50 ng of genomic DNA. Reaction conditions were 95°C for 5 min, followed by 40 cycles of touch-down PCR in 95°C for 30 s, 66–54°C for 30 s with 0.3°C decreasing per cycle and 72°C for 1 min, with a final extension of 72°C for 5 min. The PCR products were separated by electrophoresis in a 1.5% agarose gel. The bands were stained with ethidium bromide and visualized using UV light. The targeted fragments were recovered and cloned into pMD18-T vector and sequenced by Shanghai Sangon Biological Engineering & Technology and Service Co., Ltd. (http://www.sangon.com).

To obtain full-length coding sequences of the two PPO genes and their allelic variants, ten pairs of primer were generated based on the nucleotide sequences of AY596268 and AY515506. Each primer combination (except *PPO30*, whose PCR products were sequenced for determining its amplified locus in comparison to AY596268 and AY515506) was confirmed to be genome-specific using Chinese Spring nullisomic–tetrasomic lines of N2A–T2B (from M2A–T2B), N2B–T2A and N2D–T2B. The PCR reaction and DNA sequencing was repeated 2–4 times for each primer set. The complete genomic DNA sequence of the PPO genes and their deduced amino-acids were aligned using the software DNAMAN (http://www.lynnon.com). Intron positions were determined by the alignment of amplified genomic DNA sequences and their corresponding

cDNA sequences (AY596268 or AY515506) using DNAMAN as well.

STS analysis

Fourteen wheat cultivars were used for screening the newly developed STS markers, all of which possessed the same haplotype of the PPO gene on chromosome 2A, as indicated by the 876-bp PCR fragment amplified by *PPO18* (Sun et al. 2005), seven of these cultivars had high PPO activity while the other seven showed low PPO activity, which were assumed to be associated with the PPO gene on chromosome 2D. Based on the sequences of the PPO genes on chromosome 2D characterized in this study, a total of 13 primer sets were designed to identify the nucleotide polymorphisms for the PPO gene in the 14 cultivars. The PCR reaction was conducted as previously described, except that 100 μM of each of dNTP and 5 pmol of each primer was used.

Multiplex PCR

For the purpose of detecting PPO loci on chromosomes 2A and 2D simultaneously in a single PCR reaction, a new STS marker, designated *PPO33*, was developed from DNA sequence of the same gene as *PPO18* developed previously in our lab (Sun et al. 2005). Fifteen cultivars with different PPO activity were used for a preliminary test of the correlation between band patterns and PPO activity, which was then validated with 217 Chinese wheat cultivars and advanced lines. The PCR conditions were the same as those for STS analysis mentioned above except that 2 pmol of each primer of *PPO33* and 3 pmol of each primer of *PPO16* were used.

Statistical analysis

For the 217 cultivars and advanced lines, the PPO activity of each genotype was measured in each of two cropping seasons and averaged to verify the association between PPO activity and STS markers. Analysis of variance (ANOVA) was performed with the SAS System for Windows version 9.0 (SAS Institute Inc., Cary, NC, USA). The differences of PPO activity in the genotypes with different PCR band profiles were tested using Fischer's protected LSD. Mapmaker/Exp 3.0 (Lander et al. 1987) was employed for linkage analysis and the genetic distance (centimorgan, cM) was generated with Kosambi function (Kosambi 1944). QTL analysis was conducted using the software QTL Cartographer V2.0 (Wang et al. 2004), and a LOD score of 3.0 was set as the threshold for declaring significant linkage and QTL detection.

Results

Characterization of complete DNA sequence of PPO genes

BLAST search indicated that a wheat EST, CD908212, showed 95% sequence identity with the upstream sequence of AY515506 in a 298-bp overlap**.** The tentative sequence of the PPO gene was then constructed based on the EST sequences of CD908212 and AY515506. Two primer sets, *PPO30* and *PPO43*, designed from the tentative sequence yielded 615 and 578-bp PCR fragments, respectively.

The upstream sequence of the 615-bp PCR fragment amplified by *PPO30* demonstrated 100% identity with CD908212 in a 447-bp overlap, while the downstream sequence of the PCR fragment showed 100% identity with AY596268 in a 107-bp overlap, implying that CD908212 is the upstream sequence of AY596268. The complete DNA sequence of the PPO gene was thus constructed with CD908212 and AY596268, and was designated as *Ppo-A1*, since AY596268 was mapped on chromosome 2AL previously (Sun et al. 2005). The gene *Ppo-A1* has an in-frame ATG codon with a context in agreement with Kozak's rule near its 5′-end (Kozak 1996), indicating that the trinucleotide ATG is the initiation codon of *Ppo-A1* (Fig. 1). The DNA sequence of *Ppo-A1* contains a 1,731-bp ORF, a 44-bp 5′untranslated region (UTR), and a 280-bp 3′UTR, comprising 2,055 base pairs in total. *Ppo-A1* has two allelic variants in common wheat (Sun et al. 2005), and designated as *Ppo-A1a* and *Ppo-A1b* (Fig. 1, GenBank accessions EF070147 and EF070148, respectively). Complete genomic DNA sequences (from start codon to stop codon) of *Ppo-A1a* and *Ppo-A1b* were 1,961 and 2,152 bp, respectively, sharing 99.6% sequence identity (Table 1). A difference of 191 bp was present in the first intron between *Ppo-A1a* and *Ppo-A1b* (Table 2 and Fig. 1). Seven SNPs were found between the two haplotypes, with one in the first intron and the other six in exons. The deduced preprotein sequences of *Ppo-A1a* and *Ppo-A1b* were consisted of 577 amino-acid residues, with a predicted molecular weight of ~64 kD. The precursor polypeptides were composed of mature proteins (referred to the processed proteins lacked of transit peptide) of 496 amino-acid residues with a molecular mass of 55.4 kD and a transit peptide of 81 amino acids with a molecular weight of 8.6 kD (Table 2). The deduced polypeptides of *Ppo-A1a* and *Ppo-A1b* shared 99.1% sequence identity, with five different amino-acid residues (Fig. 2).

The 578-bp PCR fragment amplified by *PPO43* showed 100% identity with AY515506 in a 455-bp overlap, and extended the upstream sequence of AY515506 by 123 bp at the 5′-end. *PPO43* developed from the tentative sequence of CD908212 and AY515506 was assigned to chromosome 2D with a set of Chinese Spring nulli–tetrasomic lines, and

Fig. 1 Alignment of the alleles *Ppo-A1a* and *Ppo-A1b* located on wheat chromosome 2A. The SNPs are *shadowed*. The introns *underlined*. The start and terminate codons are *boxed*

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Ppo-A1a   GCAGCATGGAGAGCAGTCGCATGCCACTGAGTGCCACCCCTCGCATGCCATGCAGCCTCCAAACCCTTGC    70
Ppo-A1b   GCAGCATGGAGAGCAGTCGCATGCCACTGAGTGCCACCCCTCGCATGCCATGCAGCCTCCAAACCCTTGC    70

Ppo-A1a   GCGCCGCAACCTTCTCCGTGGCCTTCACCTCCGGAAGGACGCGAGGCAGCCACGGCGTCTCTCAGTCTCA   140
Ppo-A1b   GCGCCGCAACCTTCTCCGTGGCCTTCACCTCCGGAAGGACGCGAGGCAGCCACGGCGTCTCTCAGTCTCA   140

Ppo-A1a   TGCGAGGCGACCGGCGGCTGCCGCGTCGACCGCCGTGAGGTGCTCCTCGGTCTCGGCGGCGCCGCGGCTG   210
Ppo-A1b   TGCGAGGCGACCGGCGGCTGCCGCGTCGACCGCCGTGAGGTGCTCCTCGGTCTCGGCGGCGCCGCGGCTG   210

Ppo-A1a   CCGGTCTGGCCACGGACAAAGGTCGAGGCGCGATCGCCGCGCCCATCCAGGCCCCGGACCTCCGCAACTG   280
Ppo-A1b   CCGGTCTGGCCACGGACAAAGGTCGAGGCGCGATCGCCGCGCCCATCCAGGCCCCGGACCTCCGCAACTG   280

Ppo-A1a   CCAAACGCCCGAGCTCCCGAACACGCCGCCGGACACCAACTGCTGCCCGACGCCCGGCACCGGCATCACC   350
Ppo-A1b   CCAAACGCCCGAGCTCCCGAACACGCCGCCGGACACCAACTGCTGCCCGACGCCCGGCACCGGCATCACC   350

Ppo-A1a   GACTTCGTGCTGCCGCCCGTCTCCTCGCCGCTCCGCGTGCGTCCGGCAGCGCACCTGGTGGACGCGGGGT   420
Ppo-A1b   GACTTCGTGCTGCCGCCCGTCTCCTCGCCGCTCCGCGTGCGTCCGGCAGCGCACCTGGTGGACGCGGGGT   420

Ppo-A1a   ACCTGGCCAAGTACGAGAGGGCCGTGGCGCTCATGAAGCAGCTGCCCGCCGACGACCCGCGCAGCTTCGA   490
Ppo-A1b   ACCTGGCCAAGTACGAGAGGGCCGTGGCGCTCATGAAGCAGCTGCCCGCCGACGACCCGCGCAGCTTCGA   490

Ppo-A1a   GCAGCAGTGGCACGTGCACTGCGCCTACTGCGACGCCGCCTACGACCAGGTCGGGTTCCCGGACCTGGAG   560
Ppo-A1b   GCAGCaGTGGCACGTGCACTGCGCCTACTGCGACGCCGCCTACGACCAGGTCGGGTTCCCGGACCTGGAG   560

Ppo-A1a   CTCCAGATACACAACTGCTGGCTCTTCTTCCCATGGCACAG...................   611
Ppo-A1b   CTCCAGATACACAACTGCTGGCTCTTCTTCCCATGGCACAGGTTCGTATGGAGCAATGCTACACCTAGG   630

Ppo-A1a   ......................................................................   611
Ppo-A1b   TAAAGTTACGTACAGATTTTACGTAATAGGCAACGTGTAGGACTGCGATTGGATGGATGGATGTGGCAGG   700

Ppo-A1a   ......................................................................   611
Ppo-A1b   GGCCCACACGGGTGAAAATCAGGGGGGGCGATAGATTTGTTAGGTAGGTTACGTAACTCTTAGCAGGTTG   770

Ppo-A1a   ................................GTTCGTATGGTCAATGGGTTATGGATGGGACGACCTGCACCTTTCTGA   649
Ppo-A1b   GTTTCGTAGGTGTAGTATTATTGTTCGTATGGTCAATGGGTTATGGATGGGACGACCTGCACCTTTCTGT   840

Ppo-A1a   GCTGAACGTCAAGGAGCCGTCACTTGTCCCTGCGTGGGTTTGCTGAACGTGCAGGTTCTACCTCTACTTC   719
Ppo-A1b   GCTGAACGTCAAGGAGCCGTCACTTGTCCCTGCGTGGGTTTGCTGAACGTGCAGGTTCTACCTCTACTTC   910

Ppo-A1a   CACGAGAGGATCCTCGGCAAGCTCATCGGCGACGACACCTTCGCGCTGCCCTTCTGGAACTGGGACGCGC   789
Ppo-A1b   CAGGAGAGGATCCTCGGCAAGCTCATCGGCGACGACACCTTCGCGCTGCCCTTCTGGAACTGGGACGCGC   980

Ppo-A1a   CGGCCGGCATGACGCTGCCGGCCATCTACGCCGACAGGTCGTCGCCGCTCTACGACGAGAGGCGCGACCC   859
Ppo-A1b   CGGCCGGCATGACGCTGCCGGCCATCTACGCCGACAGGTCGTCGCCGCTCTACGACGAGAGGCGCGACCC  1050

Ppo-A1a   CGCGCACCAGCCGCCGGTGCTGGTCGACCTTGACTCCAGTGGGTCCGACACCAATATCCCAAGAGACCAG   929
Ppo-A1b   CGCGCACCAGCCGCCGGTGCTGGTCGACCTTGACTCCAGTGGGTCCGACACCAATATCCCAAGAGACCAG  1120

Ppo-A1a   CAGATCGACGAGAACCTCAAGATCATGTACCGCCAGGCCAGTAGTACCAACTAACAACCTCAAGAATCCC   999
Ppo-A1b   CAGATCGACGAGAACCTCAAGATCATGTACCGCCAGGCCAGTAGTACCAACTAACAACCTCAAGAATCCC  1190

Ppo-A1a   TGAAAAAATTAGCAACTTCAAAAACATTGTTAACGTAACCACAGAGTTAACCACTGGTCATTAAAATAAC  1069
Ppo-A1b   TGAAAAAATTAGCAACTTCAAAAACATTGTTAACGTAACCACAGAGTTAACCACTGGTCATTAAAATAAC  1260

Ppo-A1a   ACAAATGTACGTACGCACCAGATGATTTCGAACGCGAAGAAGACGCTGCTGTTCCTGGGACAGCCGTACC  1139
Ppo-A1b   ACAAATGTACGTACGCACCAGATGATTTCGAACGCGAAGAAGACGCTGCTGTTCCTGGGACAGCCGTACC  1330

Ppo-A1a   GCGCCGGCGACCAGCCGGACCCGGGCGCCGGCTCCCTGGAGAACGTGCCGCACGGCACGGTCCACGTCTG  1209
Ppo-A1b   GCGCCGGCGACCAGCCGGACCCGGGCGCCAGCTCCCTGGAGAACGTGCCGCACGGCACGGTCCACGTCTG  1400

Ppo-A1a   GACTGGCGACCCAAGGCAGCCCAACTTGGCGGACATGGGCAACTTCTTCTCGGCGGCGCGCGACCCCATC  1279
Ppo-A1b   GACTGGCGACCCAAGGCAGCCCAACTTGGCGGACATGGGCAACTTCTTCTCGGCGGCGCGCGACCCCATC  1470

Ppo-A1a   TTCTTCGCGCACCACGGCAACATCGACCGCCTGTGGCACGTCTGGCGCGGCCTCCGCCCGAGCAACACTG  1349
Ppo-A1b   TTCTTCGCGCACCACGGCAACATGGACCGCCTGTGGCACGTCTGGCGCGGCCTCCGCCCGAGCAACACTG  1540

Ppo-A1a   ACTTCACCGACCCCGACTGGCTCGACGCCGCCTTCCTCTTCTACGACGAGGAGGCCCGCCCCGTGCGCGT  1419
Ppo-A1b   ACTTCACTGACCCCGACTGGCTCGACGCCGCCTTCCTCTTCTACGACGAGGAGGCCCGCCCCGTGCGCGT  1610

Ppo-A1a   GCGCGTCCGGGACTGCCTCGACCCGGCCGCGCTGCGGTACATGTACCAGGACGTCGGCCTGCCGTGGCTC  1489
Ppo-A1b   GCGCGTCCGGGACTGCCTCGACCCGGCCGCGCTGCGGTACACGTACCAGGACGTCGGCCTGCCGTGGCTC  1680

Ppo-A1a   AACGCCAGGCCGGCCAAGGCGTCCGGCGGGACGCCGGCGCCCGCCACCACCGGTACCCTCCCTGCCACCC  1559
Ppo-A1b   AACGCCAGGCCGGCCAAGGCGTCCAGCGGGACGCCGGCGCCCGCCACCACCGGTACCCTCCCTGCCACCC  1750

Ppo-A1a   TGGACAGCACCATACGGGTGACCGTGACGAGGCCCAGGGTGTCGAGGAGCCGCCGGGAAAAGGACGAGGA  1629
Ppo-A1b   TGGACAGCACCATACGGGTGACCGTGACGAGGCCCAGGGTGTCGAGGAGCCGCCGGGAAAAGGACGAGGA  1820

Ppo-A1a   GGAGGAGGTGCTGGTCGTGGAGGGGATCGAGATCGCCGACCATTTCAACAAGTTCATCAAGTTCGACGTG  1699
Ppo-A1b   GGAGGAGGTGCTGGTCGTGGAGGGGATCGAGATCGCCGACCATTTCAACAAGTTCATCAAGTTCGACGTG  1890

Ppo-A1a   CTGGTGAACGAGCCCGAGGGAGGGGTGGACGGCACGCCGGCGACGGCGACGGGGTACTGCGCCGGGAGCT  1769
Ppo-A1b   CTGGTGAACGAGCCCGAGGGAGGGGTGGACGGCACGCCGGCGACGGCGACGGGGTACTGCGCCGGGAGCT  1960

Ppo-A1a   TCGCGCACACGCCGCACATGGTCCGGCCCGAGGAGACGAGGAAGGGGTCGGTCAAGACGGTGGCGAGGTT  1839
Ppo-A1b   TCGCGCACACGCCGCACATGGTCCGGCCCGAGGAGACGAGGAAGGGGTCGGTCAAGACGGTGGCGAGGTT  2030

Ppo-A1a   CGGCGTGTGCGACCTGATGGACGACATCGGAGCGGACGGCGACCAGACGGTGGTCGTGTCGCTCGTACCC  1909
Ppo-A1b   CGGCGTGTGCGACCTGATGGACGACATCGGAGCGGACGGCGACCAGACGGTGGTCGTGTCGCTCGTACCC  2100

Ppo-A1a   AGGTGCGGCGGTGAGCTGGTCACCGTAGGCGGCGTCAGCATCAGCTACCTCAAGTGAAGTTACGTAATGT  1979
Ppo-A1b   AGGTGCGGCGGTGAGCTGGTCACCGTAGGCGGCGTCAGCATCAGCTACCTCAAGTGAAGTTACGTAATGT  2170

Ppo-A1a   GGTCCGCTCTCGCGTCGGTGGCTCGCCGTGGTATAGAATCTATGTAATGGTGATGGTGATGAATTATAAA  2049
Ppo-A1b   GGTCCGCTCTCGCGTCGGTGGCTCGCCGTGGTATAGAATCTATGTAATGGTGATGGTGATGAATTATAAA  2240
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thus we designated the PPO gene constructed with *PPO43*-generated PCR fragment and AY515506 as *Ppo-D1*. The constructed DNA sequence of *Ppo-D1* consisted of an ORF of 1,731-bp, a 5-bp 5′UTR, and a 443-bp 3′UTR. The amplified genomic DNA sequence of *Ppo-D1* revealed the presence of two haplotypes, *Ppo-D1a* and *Ppo-D1b* (GenBank accessions EF070149 and EF070150, respectively), with 1,961 and 1,999 bp, respectively (Fig. 3). Both

Table 1 Sequence comparison of four haplotypes of wheat PPO genes on chromosomes 2A and 2D (below diagonal) and their deduced amino-acid sequences (above diagonal) (%, irrespective of the sequence length)

Allele	*Ppo-A1a*	*Ppo-A1b*	*Ppo-D1a*	*Ppo-D1b*
Ppo-A1a	100	99.1	93.1	94.1
Ppo-A1b	99.6	100	92.5	93.6
Ppo-D1a	95.3	95.1	100	93.6
Ppo-D1b	95.3	95.2	95.2	100

The four haplotypes *Ppo-A1a*, *Ppo-A1b*, *Ppo-D1a* and *Ppo-D1b* have been registered in GenBank (i.e., Accessions EF070147–EF070150)

Table 2 Characteristics of four haplotypes of wheat PPO genes on chromosomes 2A and 2D and their deduced amino-acid sequences

Allele	ORF (bp)	Intron (bp)		SNP	InDel	GC content[a]	Deduced amino acids	Mr (kD)[b]
		First	Second					
Ppo-A1a	1,731	102	125	7	1	68.6%	577	8.6, 55.3
Ppo-A1b	1,731	293	125			68.3%	577	8.6, 55.4
Ppo-D1a	1,731	103	124	95	5	68.1%	577	8.7, 55.5
Ppo-D1b	1,731	106	159			68.2%	577	8.7, 55.4

[a] Calculated from the corresponding cDNA sequences

[b] Predicted molecular weight of transit peptide and mature proteins, respectively

of them contained three exons and two introns. *Ppo-D1a* and *Ppo-D1b* shared 95.2% sequence identity (Table 1), with 95 SNPs between the two haplotypes, of which 81 SNPs were present in exons and the remaining 14 SNPs in introns (Table 2 and Fig. 3). Four short insertion sequences were found in the introns of *Ppo-D1b*, with one in the first intron and the other three in the second intron, and a single nucleotide deletion was found in the first intron of *Ppo-D1b* (Fig. 3). The two haplotypes of *Ppo-D1* encoded polypeptides of 577 amino acids, with similar molecular masses and cleavage sites to those of *Ppo-A1a* and *Ppo-A1b* (Table 2 and Fig. 2). The two polypeptides encoded by *Ppo-D1a* and *Ppo-D1b* exhibited 93.6% sequence identity (Table 1).

Although the two PPO genes on chromosomes 2A and 2D differed in length and sequences, they all contained a 1,731-bp ORF coding for 577 amino acids (Table 2 and Fig. 2), demonstrating their close phylogenetic relationship. Their deduced protein sequences contained two copper-binding domains typical of PPOs and conserved His residues supposed to be involved in copper binding (Fig. 2). The GC content of *Ppo-A1* and *Ppo-D1* ranged from 68.1 to 68.6%, showing GC-rich characteristics.

Identification and validation of STS markers for the gene *Ppo-D1*

Among 13 primer sets tested, *PPO16* (Table 3) designed from *Ppo-D1a* exhibited a polymorphic band profile with a 713-bp fragment amplified in the cultivars with low PPO activity, but without any PCR product in those with rela-

```
Ppo-A1a   MESSRMPLSATPRMPCSLQTLARRNLLRGLHLRKDARQPRRLSVSCEATGGCRVDRREVLLGLGGAAAAG   70
Ppo-A1b   ----------------------------------------------------------------------   70
Ppo-D1a   -----VLP--NH--------FP---V--A--R---------I-I-------G------------------   70
Ppo-D1b   -----------S--S-------------A--R-----------I-------R------------------   70

Ppo-A1a   LATDKGRGAIAAPIQAPDLRNCQTPELPNTPPDTNCCPTPGTGITDFVLPPVSSPLRVRPAAHLVDAGYL  140
Ppo-A1b   ----------------------------------------------------------------------  140
Ppo-D1a   ----Q--------------------D---------------------E---A---------------E--  140
Ppo-D1b   ----Q--------------S-----A---------------------E---A---------------E--  140

Ppo-A1a   AKYERAVALMKQLPADDPRSFEQQWHVHCAYCDAAYDQVGFPDLELQIHNCWLFFPWHRFYLYFHERILG  210
Ppo-A1b   ----------------------------------------------------------------Q-----  210
Ppo-D1a   -----------------------------------F---------I-V-------------V--------  210
Ppo-D1b   ----------------------------------------------------------------------  210

Ppo-A1a   KLIGDDTFALPFWNWDAPAGMTLPAIYADRSSPLYDERRDPAHQPPVLVDLDSSGSDTNIPRDQQIDENL  280
Ppo-A1b   ----------------------------------------------------------------------  280
Ppo-D1a   ----------------------------N-------------------T------T-A---------Q--  280
Ppo-D1b   ---------------------K--V---N-----------------------Y--T-A------------  280

Ppo-A1a   KIMYRQMISNAKKTLLFLGQPYRAGDQPDPGAGSLENVPHGTVHVWTGDPRQPNLADMGNFFSAARDPIF  350
Ppo-A1b   --------------------------------S-------------------------------------  350
Ppo-D1a   ---------D----------------------------------------A----E--------------  350
Ppo-D1b   ----------------------------------V------P--N---------GE-----Y--------  350

Ppo-A1a   FAHHGNIDRLWHVWRGLRPSNTDFTDPDWLDAAFLFYDEEARPVRVRVRDCLDPAALRYMYQDVGLPWLN  420
Ppo-A1b   ------M----------------------------------------------------T----------  420
Ppo-D1a   ---------------R-------------------------------------------T----------  420
Ppo-D1b   --------------------------------G--------------------------T----------  420

Ppo-A1a   ARPAKASGGTPAPATTGTLPATLDSTIRVTVTRPRVSRSRREKDEEEEVLVVEGIEIADHFNKFIKFDVL  490
Ppo-A1b   -------S--------------------------------------------------------------  490
Ppo-D1a   ------------------------R------------------E--------------------V-----  490
Ppo-D1b   ------------------------R---------------------------------------V-----  490

Ppo-A1a   VNEPEGGVDGTPATATGYCAGSFAHTPHMVRPEETRKGSVKTVARFGVCDLMDDIGADGDQTVVVSLVPR  560
Ppo-A1b   ----------------------------------------------------------------------  560
Ppo-D1a   --------GS------------------------M---P-------------------D-----------  560
Ppo-D1b   --------G-------------------------M---P-------------------------------  560

Ppo-A1a   CGGELVTVGGVSISYLK                                                      577
Ppo-A1b   -----------------                                                      577
Ppo-D1a   -----------------                                                      577
Ppo-D1b   ---D---I-------V-                                                      577
```

Fig. 2 Alignment of deduced amino-acid sequences of the four haplotypes of PPO genes on chromosomes 2A and 2D. Conserved copper-binding domains (adapted from Demeke and Morris 2002) are *shadowed*. *Arrow* indicates the predicted cleavage site of the transit peptide (determined from alignment with other plant PPOs)

Fig. 3 Alignment of the alleles *Ppo-D1a* and *Ppo-D1b* located on wheat chromosome 2D. The SNPs are *shadowed*. The introns *underlined*. The start and terminate codons are *boxed*

```
Ppo-D1a   GCAGCATGGAGAGCAGTCGCGTGCTACCGAGTGCCAACCATCGCATGCCATGCAGCCTCCAAACCTTTCC     70
Ppo-D1b   GCAGCATGGAGAGCAGTCGCATGCCACTGAGTGCCACCTCTCGCATGTCATGCAGCCTCCAAACCCTTGC     70

Ppo-D1a   GCGACGCAACGTTCTCCGTGCCCTTCACCGCCGCAAGGACGCAAGGCAGCCACGTCGTATCTCAATCTCA    140
Ppo-D1b   GCGCCGCAACCTTCTCCGTGCCCTTCACCGCCGGAAGGACGCGAGGCAGCCACGGCGTCTCTCAATCTCA    140

Ppo-D1a   TGCGAGGCGACCGGCGGCGGTCGCGTCGACCGCCGTGAGGTGCTCCTCGGCCTCGGCGGCGCCGCAGCTG    210
Ppo-D1b   TGTGAGGCGACCGGCGGCCGCCGCGTCGACCGCCGTGAGGTGCTCCTCGGCCTCGGCGGCGCCGCAGCTG    210

Ppo-D1a   CGGGGCTGGCCACGGACCAAGGTCGAGGCGCGATCGCCGCGCCCATCCAGGCCCCGGACCTCCGCAACTG    280
Ppo-D1b   CCGGGCTGGCCACGGACCAAGGTCGAGGCGCGATCGCCGCGCCCATCCAGGCCCCGGACCTCAGCAACTG    280

Ppo-D1a   CCAAACGCCCGACCTCCCGAACACGCCGCCGGACACCAACTGCTGCCCGACGCCCGGCACCGGCATCACC    350
Ppo-D1b   CCAAACGCCCGCCCTCCCGAACACGCCGCCGGATACCAACTGCTGCCCGACGCCCGGCACCGGCATCACC    350

Ppo-D1a   GACTTTGAGCTGCCGCCCGCTTCCTCGCCGCTCCGCGTGCGTCCGGCAGCGCACCTGGTGGACGCGGAGT    420
Ppo-D1b   GACTTCGAGCTGCCGCCCGCCTCCTCGCCGCTCCGCGTGCGTCCGGCCGCGCACCTGGTGGACGCGGAGT    420

Ppo-D1a   ACCTGGCCAAGTACGAGAGGGCCGTGGCGCTCATGAAGCAGCTGCCTGCCGATGACCCGCGCAGCTTCGA    490
Ppo-D1b   ACCTGGCCAAGTACGAGAGGGCCGTGGCGCTCATGAAGCAGCTGCCCGCCGATGACCCCCGCAGCTTCGA    490

Ppo-D1a   GCAGCAGTGGCACGTGCACTGCGCCTACTGCGACGCCGCCTTCGACCAGGTCGGGTTCCCGGACCTTGAG    560
Ppo-D1b   GCAGCAGTGGCACGTGCACTGTGCCTACTGCGACGCCGCCTACGACCAGGTCGGGTTCCCGGACCTGGAG    560

Ppo-D1a   ATCCAGGTGCACAACTGCTGGCTCTTCTTCCCATGGCACAGGTTCGTAT....GGTCAATGGGTTAATGG    626
Ppo-D1b   CTCCAGATACACAACTGCTGGCTCTTCTTCCCATGGCACAGGTTCGTATATATGGTCAATGGGTTA.TGG    629

Ppo-D1a   GTGCGACGACTTGCACCTTTCTGTGCTGAACCTCAAGGAGCCGTCACTTGTCCCTGCGTGCGTTTGCTGA    696
Ppo-D1b   GTGAGACGACCTGCACCTTTCTGTGCTGAACCTCAAGGAGCCGTCACTTGTCCCTGCGTGCGTTTGCTGA    699

Ppo-D1a   ACGTGCAGGTTCTACGTCTACTTCCACGAGAGGATCCTCGGCAAGCTCATCGGCGACGACACCTTCGCGC    766
Ppo-D1b   ACGTGCAGGTTCTACCTCTACTTCCACGAGAGGATCCTCGGCAAGCTCATCGGCGACGACACCTTCGCGC    769

Ppo-D1a   TGCCCTTCTGGAACTGGGACGCGCCGGCCGGCATGACGCTGCCGGCGATCTACGCCAACAGGTCGTCGCC    836
Ppo-D1b   TGCCGTTCTGGAACTGGGACGCGCCGGCCGGCATGAAGCTGCCGGTCATCTACGCCAACAGATCGTCGCC    839

Ppo-D1a   GCTCTACGACGAGAGGCGCGACCCCGCCCACCAGCCGCCGGTGCTGACCGACCTTGACTCCAGTGGGACC    906
Ppo-D1b   GCTCTACGACGAGAGGCGCGACCCCGCCCACCAGCCGCCGGTACTGGTCGACCTTGACTACAGTGGGACC    909

Ppo-D1a   GACGCCAATATCCCAAGAGACCAGCAGATCGATCAGAACCTCAAGATCATGTACCGCCAGGCCAGTAGTA    976
Ppo-D1b   GACGCCAACATCCCAAGAGACCAGCAGATCGATGAGAACCTCAAGATCATGTACCGCCAGGCCAGTATTT    979

Ppo-D1a   CTAATTAACAACCTCAAGAATCCCTAAA...............CAAAA...............TGAGTA   1015
Ppo-D1b   CTAATTAACAACCTCAAGAATCCCTAAAAATATTTAGCAACTTCAAAAATATTGGGGTTTGTTTTGCGAA   1049

Ppo-D1a   GCAACTTCAAAAATATTGTTAAGGTAACCACAGAACCACTGGTCATGAAAT.....AACACAAATGTACG   1080
Ppo-D1b   ACAACTTCAAAAACGTTGTTACG.TAACCACAGAACCACTGGTCATTAAATTAAATAACACAAATGTACA   1118

Ppo-D1a   TACGCACCAGATGATTTCGGACGCGAAGAAGACGCTGCTGTTCCTGGGACAGCCGTACCGCGCCGGCGAC   1150
Ppo-D1b   TACACATCAGATGATTTCAAACGCGAAGAAGACGCTGCTGTTCCTGGGACAGCCGTACCGCGCCGGCGAC   1188

Ppo-D1a   CAGCCGGACCCGGGCGCGGGCTCCCTGGAGAACGTGCCGCACGGCACGGTCCACGTCTGGACTGGCGACC   1220
Ppo-D1b   CAGCCGGACCCGGGCGCGGGCTCCGTGGAGAACGTGCCGCACGGCCCGGTGCATAACTGGACAGGCGACC   1258

Ppo-D1a   CGGCGCAGCCGAACTTGGAGGACATGGGCAACTTCTTCTCGGCGGCGCGCGACCCCATCTTCTTCGCGCA   1290
Ppo-D1b   CAAGGCAGCCGAACGGCGAGGACATGGGCAACTTCTACTCGGCGGCGCGCGACCCCATCTTCTTCGCGCA   1328

Ppo-D1a   CCACGGCAACATCGACCGCCTGTGGCACGTCTGGCGCCGCCTCCGCCCGAGCAACACCGACTTCACCGAC   1360
Ppo-D1b   CCACGGCAACATCGACCGCCTGTGGCACGTCTGGCGCGGCCTCCGCCCGAGCAACACCGACTTCACCGAC   1398

Ppo-D1a   CCCGACTGGCTCGACGCCGCCTTCCTCTTCTACGACGAGGAGGCCCGCCCCGTGCGCGTGCGCGTCCGGG   1430
Ppo-D1b   CCCGACTGGCTCGACGCCGGCTTCCTCTTCTACGACGAGGAGGCCCGCCCCGTGCGCGTGCGCGTCCGGG   1468

Ppo-D1a   ACTGCCTCGACCCGGCCGCGCTGCGGTACACGTACCAGGACGTCGGCCTGCCGTGGCTCAACGCCAGGCC   1500
Ppo-D1b   ACTGCCTCGACCCGGCCGCGCTGCGGTACACGTACCAGGACGTCGGCCTGCCGTGGCTCAACGCCAGGCC   1538

Ppo-D1a   GGCCAAGGCGTCCGGCGGACGCCGGCGCCCGCCACAACCGGTACCCTCCCTGCCACCCTGGACAGGACC   1570
Ppo-D1b   AGCCAAGGCGTCCGGCGGACGCCGGCGCCCGCCACCACCGGTACGCTCCCTGCCACCCTGGACAGGACC   1608

Ppo-D1a   ATACGGGTGACGGTGACGAGGCCCAGAGTGTCCAGGAGCCGCCGGGAGAAGGAGGAGGAGGAGGAGGTGC   1640
Ppo-D1b   ATACGGGTGACGGTGACGAGGCCCAGAGTGTCGAGGAGCCGCCGGGAGAAGGACGAGGAGGAGGAGGTGC   1678

Ppo-D1a   TGGTCGTGGAGGGGATCGAGATCGCCGACCATTTCAACAAGTTCGTCAAGTTCGACGTGTTGGTGAACGA   1710
Ppo-D1b   TGGTCGTGGAGGGGATCGAGATCGCCGACCATTTCAACAAGTTCGTCAAGTTCGACGTGCTGGTGAACGA   1748

Ppo-D1a   GCCCGAGGGCGGAGTGGGCAGCACGCCGGCGACGGCGACGGGGTACTGCGCTGGGAGCTTCGCGCATACG   1780
Ppo-D1b   GCCCGAGGGCGGAGTGGGCGGCACGCCGGCGACGGCGACGGGGTACTGTGCCGGGAGCTTCGCGCACACG   1818

Ppo-D1a   CCGCACATGGTCCGGCCCGAGGAGATGAGGAAGGGACCGGTCAAGACGGTGGCGAGGTTCGGCGTGTGCG   1850
Ppo-D1b   CCGCACATGGTCCGGCCCGAGGAGATGAGGAAGGGGCCGGTCAAGACGGTGGCGAGGTTCGGCGTGTGCG   1888

Ppo-D1a   ACCTGATGGACGACATCGGGGCGGACGACGACCAGACGGTGGTGGTGTCGCTCGTACCCAGGTGCGGCGG   1920
Ppo-D1b   ACCTGATGGACGACATCGGGGCGGACGGCGACCAGACGGTGGTCGTGTCGCTCGTACCCAGGTGCGGCGG   1958

Ppo-D1a   TGAGCTGGTCACCGTTGGCGGCGTCAGCATCAGCTACCTCAAGTGAAGTTACCTAATGTGGTCCGCTCTC   1990
Ppo-D1b   TGATCTGGTCACCATCGGCGGCGTCAGCATCAGCTACGTCAAGTGAAGTTACATAATGTGGTCCGCTCTC   2028

Ppo-D1a   GCGTCGGTGGCTCGCCGTG                                                       2009
Ppo-D1b   GCGTCGGTGGCTCGCCGTG                                                       2047
```

tively high PPO activity (Fig. 4). In contrast, *PPO29* (Table 3) developed from *Ppo-D1b* yielded a 490-bp fragment in the cultivars with relatively high PPO activity and no PCR product in those with low PPO activity, demonstrating that *PPO29* is the complementary marker of *PPO16* (Figs. 4, 5). In a test of 217 Chinese wheat cultivars and advanced lines using *PPO16*, the 713-bp fragment was detected in 129 genotypes, but not in the remaining 88 genotypes. The former cultivars were proven to have significantly lower mean PPO activity than the latter by statistical analysis (Table 4). A complementary result was found in a test of 217 accessions with *PPO29*.

Table 3 STS markers for wheat PPO genes developed in the current and previous studies, and primer sets used for cloning the upstream sequences of PPO genes on chromosomes 2A and 2D

Marker	Primers (5′–3′)	Target gene	Size of PCR fragments (bp)
PPO16	Forward: TGCTGACCGACCTTGACTCC	*Ppo-D1a*	713
	Reverse: CTCGTCACCGTCACCCGTAT		
PPO29	Forward: TGAAGCTGCCGGTCATCTAC	*Ppo-D1b*	490
	Reverse: AAGTTGCCCATGTCCTCGCC		
PPO18	Forward: AACTGCTGGCTCTTCTTCCCA	*Ppo-A1a* and *Ppo-A1b*	685 and 876
	Reverse: AAGAAGTTGCCCATGTCCGC		
PPO33	Forward: CCAGATACACAACTGCTGGC	*Ppo-A1a* and *Ppo-A1b*	290 and 481
	Reverse: TGATCTTGAGGTTCTCGTCG		
PPO30	Forward: CAGCCAGAAAGCAAACACCG	*Ppo-A1*	615
	Reverse: CATGGGAAGAAGAGCCAGCA		
PPO43	Forward: GCAGCATGGAGAGCAGTCGC	*Ppo-D1*	578
	Reverse: GCAGTTGTGCACCTGGATCTCA		

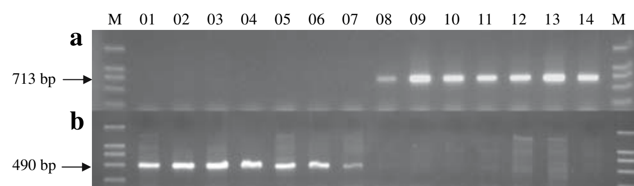

Fig. 4 Polymorphic test of PCR fragments amplified by *PPO16* (**a**) and its complementary marker *PPO29* (**b**) in seven cultivars with high PPO activity and seven with low PPO activity. M DNA ladder DL2000; 01 CA9722 (33.02); 02 Jing 411 (33.25); 03 Ji 3475 (44.10); 04 98 Zhong 18 (34.27); 05 Shan 9314 (40.00); 06 Xinong 336 (40.01); 07 Xinong 8925-13 (33.28); 08 Dongfeng 9801 (12.53); 09 Lumai 22 (10.29); 10 Lumai 23 (10.25); 11 Yangmai 5 (11.45); 12 Chuan 89-114 (9.32); 13 Mianyang 940112 (9.35); 14 Dongfanghong 3 (7.32). The *number in parentheses* indicates PPO activity: A_{475} min^{-1} g^{-1} 10^{-3}

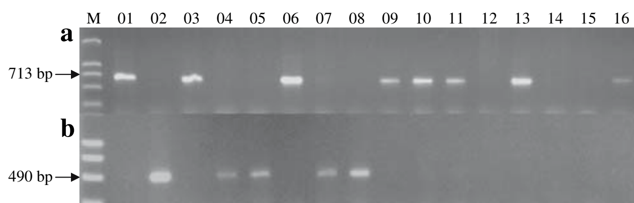

Fig. 5 Electrophoresis of PCR fragments amplified with *PPO16* (**a**) and its complementary marker *PPO29* (**b**) in ten Chinese wheat cultivars, Chinese Spring nullisomic–tetrasomic lines N2A–T2B (derived from M2A–T2B line) and N2D–T2B, Chinese Spring ditelosomic lines 2AS and 2DS, durum and *Ae. tauschii* line. M DNA ladder DL2000; 01 Beijing 837 (20.46); 02 CA8686 (28.00); 03 CA9532 (13.67); 04 CA9550 (49.42); 05 CA9553 (26.71); 06 CA9641 (13.70); 07 CA9719 (41.79); 08 CA9722 (33.02); 09 Dongfeng 611 (17.14); 10 Dongfeng 9801 (12.53); 11 N2A–T2B; 12 N2D–T2B; 13 DT2AS; 14 DT2DS; 15 Langdon; 16 *Ae. tauschii* line Ae38. The *number in parentheses* indicates PPO activity: A_{475} min^{-1} g^{-1} 10^{-3}

Chromosomal localization of STS markers

Linkage analysis indicated that *PPO16* and *PPO29* are closely linked to an SSR marker, *Xwmc41*, on chromosome 2DL, with a genetic distance of 2 cM (Fig. 6). QTL analysis detected a major QTL for PPO activity on chromosome 2D, that co-segregated with *PPO16* and *PPO29*, explaining from 9.6 to 24.4% of the phenotypic variance in the DH population across three environments. The location of *PPO16* on chromosome 2DL was confirmed by amplifying the genomic DNA from a set of Chinese Spring nullisomic–tetrasomic lines and ditelosomic line 2DS (Fig. 7), in which no PCR product was detected in N2D–T2B (lane 07) and DT2DS (lane 23). Furthermore, *PPO16* amplified a 713-bp fragment in *A. tauschii* line Ae38 (DD, lane 16), but not in durum wheat Langdon (AABB, lane 15), providing further evidence for this conclusion (Fig. 5).

Multiplex PCR with *PPO33/PPO16*

Since the STS marker *PPO18* for the PPO gene on chromosome 2A (Sun et al. 2005) was not sufficiently compatible with either *PPO16* or *PPO29* in a single PCR reaction, a new primer set, designated *PPO33* (Table 3), was developed based on the sequence of *Ppo-A1*. *PPO33* amplifies a 481-bp and a 290-bp fragment from cultivars with low and high PPO activity, respectively. The *PPO33/PPO16* combination yielded four different band patterns in various genotypes, recorded as a/−, a/+, b/−, b/+ (Fig. 8), where "a" and "b" indicate the 290 and 481-bp fragments amplified by *PPO33*, respectively, and "+" and "−" denote the presence and absence, respectively, of the 713-bp fragment amplified by *PPO16*. The results indicated significant differences in PPO activity among the four genotypes of a/−, a/+, b/− and b/+ (Table 4). The accessions with band pattern a/− showed very high PPO activity, while those with band pattern b/+ displayed very low PPO activity. The accessions with band patterns a/+ and b/− have more diverse phenotypic values; however, the former still have significantly higher mean PPO activity than the latter.

Table 4 Statistical analysis of the association between PCR band patterns and PPO activity in 217 wheat accessions tested with different markers

Marker or marker combination	Band type[a]	Accession numbers tested	Mean PPO activity[b,c]	Standard deviation	Range
PPO18 and *PPO33*	a	106	40.5a	7.0	26.1–57.8
	b	111	20.6b	7.6	7.3–44.1
PPO16	+	129	27.5a	11.0	7.3–57.8
	–	88	34.5b	12.4	9.5–57.3
PPO33/PPO16	a/–	37	44.4a	7.4	32.6–57.3
	a/+	67	38.4b	5.9	26.1–57.8
	b/–	51	27.3c	6.8	9.5–44.1
	b/+	62	15.7d	3.7	7.3–26.6

[a] ”*a*” and “*b*” indicates the shorter and longer fragments, respectively, amplified by *PPO18* or *PPO33*; “+” and “–“ denotes the presence and absence of the 713-bp fragment amplified by *PPO16*, respectively

[b] Different letters followed the mean PPO activity indicate highly significant differences among different genotypes ($P < 0.01$; Fisher’s protected LSD)

[c] The data of PPO activity was from our previous study (Sun et al. 2005)

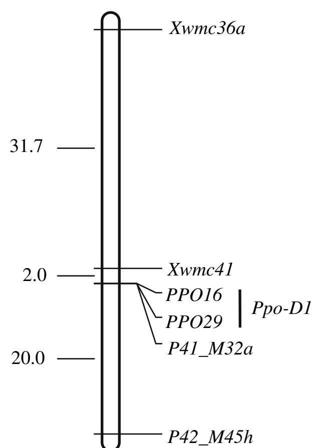

Fig. 6 Linkage map constructed with *PPO16*, *PPO29*, two SSR markers and two AFLP markers on wheat chromosome 2DL in the DH population derived from Zhongyou 9507/CA9632

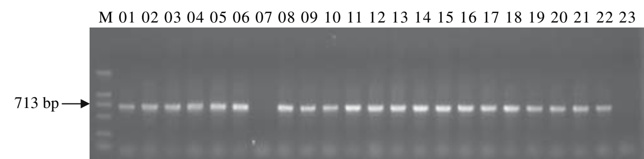

Fig. 7 PCR amplification of Chinese Spring and its nullisomic–tetrasomic lines and ditelosomic line 2DS with the STS marker *PPO16*. M DNA ladder DL2000; 01 Chinese Spring; 02 Chinese Spring nullisomic 1A–tetrasomic 1D (N1A–T1D); 03 N1B–T1D; 04 N1D–T1B; 05 N2A–T2B (derived from M2A–T2B line); 06 N2B–T2A; 07 N2D–T2B; 08 N3A–T3D; 09 N3B–T3D; 10 N3D–T3A; 11 N4A–T4B; 12 DT4BS; 13 N4D–T4B; 14 N5A–T5B; 15 N5B–T5A; 16 N5D–T5B; 17 N6A–T6B; 18 N6B–T6D; 19 N6D–T6B; 20 N7A–T7D; 21 N7B–T7D; 22 N7D–T7A; 23 DT2DS

Discussion

Cloning of PPO genes

Since wheat PPO genes characteristically have GC-rich sequences, it is difficult to isolate full-length sequence using the conventional RACE technique (Demeke and Morris 2002). In recent years, more and more wheat ESTs have been deposited in GenBank, which makes it possible to clone the full-length sequences of wheat PPO genes in silico in combination with PCR amplification. In the present study, CD908212 was found in a BLAST search using AY515506 as the reference sequence, and was then combined with AY515506 to generate the tentative full-length sequence of the PPO gene on chromosome 2D. However, the PCR fragment of *PPO30* designed on the basis of the constructed sequence showed that CD908212 was actually the upstream sequence of AY596268, a PPO gene on chromosome 2A (Sun et al. 2005). This inconsistency was due to the high sequence homology of PPO genes on chromosomes 2A and 2D (Table 1) (Massa et al. 2007). Cross-amplification also happened when *PPO43* was used to amplify the tentative sequence constructed with CD908212 and AY515506, which yielded the upstream sequence of AY515506 on chromosome 2D. Therefore, experimental validation is very important for cloning genes in silico, and PCR products must be carefully compared with constructed sequences to determine the actual gene sequences.

The transcriptional cDNA sequence of *Ppo-A1* is 2,055 bp, which is in accordance with the size of cloned PPO genes in other plant species reported previously (Shahar et al. 1992; Hunt et al. 1993; Dry and Robinson 1994; Boss et al. 1995; Chevalier et al. 1999; Constabel et al. 2000). Demeke and Morris (2002) detected a PPO transcript of ~2 kb in immature wheat kernels by northern-blot analysis. Recently, Jukanti et al. (2006) reported a transcript of approximately 2 kb in immature wheat kernels

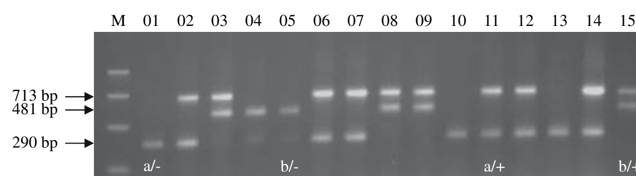

Fig. 8 Multiplexed PCR amplification with marker combination *PPO33*/*PPO16* in 15 Chinese wheat cultivars with diverse PPO activity. M DNA ladder DL2000; 01 Zhengmai 9023 (45.40); 02 Fengyou 6 (34.92); 03 85 Zhong 33 (18.64); 04 99G66 (29.94); 05 Jinmai 67 (21.95); 06 Jingnong 97-86 (40.89); 07 Jingnong 8318 (43.99); 08 Nongda 3291 (13.70); 09 Nongda 3214 (16.67); 10 Nongda 116 (41.70); 11 Yuandong 9428 (39.27); 12 Yuandong 971 (38.46); 13 Yumai 70 (46.29); 14 Zhongyou 9507 (48.03); 15 CA9641 (13.70). The *number in parentheses* indicates PPO activity: A_{475} min^{-1} g^{-1} 10^{-3}. The four possible genetic combinations are illustrated as "*a*" and "*b*" (that indicate the 290-bp and 481-bp fragments amplified by *PPO33*, respectively), and "+" and "−" (that indicate the presence and absence of 713-bp fragment amplified by *PPO16*, respectively)

using PPO gene AY596268 as a probe, which corresponds to *Ppo-A1* characterized here and in our previous study (Sun et al. 2005).

Plant PPO genes encode ~70 kD unprocessed proteins containing a ~10 kD transit peptide that targets the enzyme to the thylakoid lumen; mature proteins are typically ~55–65 kD (van Gelder et al. 1997). The deduced amino-acid sequences encoded by the four haplotypes *Ppo-A1a*, *Ppo-A1b*, *Ppo-D1a* and *Ppo-D1b* have mature proteins of ~55.4 kD and transit peptides of ~8.6 kD, which are consistent with those calculated from the cDNA sequences in other plants (Shahar et al. 1992; Dry and Robinson 1994; Boss et al. 1995; Thygesen et al. 1995; van Gelder et al. 1997; Chevalier et al. 1999; Constabel et al. 2000). A PPO protein homologous to that encoded by AY596268 was purified both by Anderson and Morris (2003) and Jukanti et al. (2006), with apparent molecular masses reported as 67 and 58 kD. The discrepancy may relate to the different SDS-PAGE methods and markers used in the two investigations.

The introns of *Ppo-A1* and *Ppo-D1* genes

Many plant PPO genes reported do not have any introns (Newmann et al. 1993; Dry and Robinson 1994; Thygesen et al. 1995). Nevertheless, Gooding et al. (2001) reported introns in banana PPO, while Sun et al. (2005) and Massa et al. (2007) found PPO genes with introns in wheat. In this study, both *Ppo-A1* and *Ppo-D1* have two introns. Interestingly, the species with intron-free PPO genes are all dicotyledonous while banana and wheat are monocotyledonous. The second introns of *Ppo-A1* or *Ppo-D1* are all bordered with GC-AG, instead of the typical consensus structure of GT-AG (Figs. 1, 3) (Massa et al. 2007). GC-AG intron structure was reported to be in 1% of *Arabidopsis thaliana* genes (Brown et al. 1996), and also found in several other plants including soybean (Katinakis and Verma 1985), cucumber (Schwartz et al. 1991), black cherry (Hu and Poulton 1997) and maize (Lal et al. 1999). Coincidently, the intron of banana PPO gene is also bordered with GC-AG structure (Gooding et al. 2001).

Introns can influence gene transcription by alternative splicing (Fedorova and Fedorov 2003). The first intron of *Ppo-A1b* has a 191-bp insertion sequence compared with that of *Ppo-A1a*. This insertion may influence the splicing of premature mRNA and interfere with PPO gene expression (Sun et al. 2005), resulting in a lower PPO activity in the genotypes with *Ppo-A1b* than those with *Ppo-A1a* (Table 4). Similarly, Massa et al. (2007) found four different length polymorphisms for intron 2; in this case the shorter (125 vs. 362 bp) of the two types present in *Triticum asetivum* was associated with lower PPO activity.

Sequence divergence of PPO gene haplotypes

The haplotypes *Ppo-A1a* and *Ppo-A1b* on chromosome 2A showed 99.6% homology, whereas *Ppo-D1a* and *Ppo-D1b* on chromosome 2D exhibited much more divergence both in DNA and in protein sequence (Table 1). The PCR profiles of *PPO16* developed from *Ppo-D1a* and *PPO29* from *Ppo-D1b*, however, confirmed that *Ppo-D1a* and *Ppo-D1b* are two haplotypes at the same locus by linkage analysis. Considering hexaploid wheat was formed only approximately 8000 years ago (Huang et al. 2002), this large number of SNPs and InDels at *Ppo-D1* locus were unlikely to have resulted from mutation events. The possible reason may be attributed to different D-genome donors of *Ae. tauschii* (DD). Hexaploid wheat originated from the hybridization of cultivated allotetraploid emmer wheat (*T. turgidum* ssp. *dicoccum*, AABB) with diploid *A. tauschii* (DD) approximately 8000 years ago (Huang et al. 2002). During the past decades, increasing evidence suggested that this event occurred at least twice and more than one *Aegilops* species participated in the formation of hexaploid wheat (Dvorak et al. 1998; Talbert et al. 1998; Lelley et al. 2000; Caldwell et al. 2004; Giles and Brown 2006). Chang et al. (2007) used a pair of primers designed from AY515506 to investigate the genetic diversity at the PPO gene locus in *A. tauschii* and found four polymorphic alleles. In our study, five *A. tauschii* lines were tested using *PPO16*, and two of them yielded a 713-bp fragment while the others did

not (data not shown), indicating clearly the divergence at this PPO locus on the D-genome of *A. tauschii*.

Having originated from different ancestors, *Ppo-D1a* and *Ppo-D1b* might have different expression profiles and post-translational modifications, and the diverged amino-acid sequences may lead to different three-dimensional conformations of mature proteins, resulting in the difference of PPO activities between two haplotypes of *Ppo-D1*. More recently, results of Massa et al. (2007) indicate the existence of at least five kernel-associated PPO genes in hexaploid wheat, three in durum cv. Langdon, and three in *T. monococcum* cv. DV92. The potential influence of these additional PPO genes on kernel PPO activity has not been determined.

Implementation of functional STS markers for PPO activity

Application of functional markers is especially important for an accurate discrimination of different alleles in marker-assisted selection (Andersen and Lübberstedt 2003). In the present study, we developed two complementary dominant functional markers, *PPO16* and *PPO29*, based on the DNA sequences of a PPO gene on chromosome 2D, which can effectively discriminate two haplotypes of *Ppo-D1* gene. In order to detect *Ppo-A1* and *Ppo-D1* loci simultaneously, a new STS primer *PPO33* was designed, which can amplify PCR fragments shorter than *PPO18* (Sun et al. 2005) and thus may be multiplexed with *PPO16* in a single PCR reaction. The marker combination *PPO33/PPO16* can be efficiently used to identify genotypes with low PPO activity in wheat breeding.

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