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A novel STS marker for polyphenol oxidase activity in bread wheat

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Abstract

The enzyme activity of polyphenol oxidase (PPO) in grain has been related to undersirable brown discoloration of bread wheat (*Triticum aestivum* L.) based end-products, particularly for Asian noodles. Breeding wheat cultivars with low PPO activity is the best approach to reduce the undesirable darkening. Molecular markers could greatly improve selection efficiency in breeding programs. Based on the sequences of PPO genes (GenBank Accession Numbers AY596268, AY596269 and AY596270) conditioning PPO activity during kernel development, 28 pairs of primers were designed using the software 'DNAMAN'. One of the markers from AY596268, designated as *PPO18*, can amplify a 685-bp and an 876-bp fragment in the cultivars with high and low PPO activity, respectively. The difference of 191-bp size was detected in the intron region of the PPO gene. The STS marker *PPO18* was mapped to chromosome 2AL using a DH population derived from a cross Zhongyou 9507 × CA9632, a set of nulli-tetrasomic lines and ditelosomic line 2AS of Chinese Spring. QTL analysis indicated that the PPO gene co-segregated with the STS marker *PPO18* and is closely linked to *Xgwm312* and *Xgwm294* on chromosome 2AL, explaining 28 – 43% of phenotypic variance for PPO activity across three environments. A total of 233 Chinese wheat cultivars and advanced lines were used to validate the correlation between the polymorphic fragments of *PPO18* and grain PPO activity. The results showed that *PPO18* is a co-dominant, efficient and reliable molecular marker for PPO activity and can be used in wheat breeding programs targeted for noodle quality improvement.

Introduction

Polyphenol oxidase (PPO) is a widely distributed enzyme in plant species (Flurkey 1989). It catalyzes hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones that react with amines and thiol groups or polymerize non-enzymatically into dark or brown products (Demeke et al. 2001; Okot-Kotber et al. 2002; Jukanti et al. 2004). High PPO activity in

grain and/or flour is responsible for the undesirable time-dependent darkening of bread wheat (*Triticum aestivum* L.) based end-products such as noodles (Kruger et al. 1992, 1994a, 1994b; Baik et al. 1995; Crosbie et al. 1996; Miskelly 1996; Hatcher et al. 1999; Morris et al. 2000; Anderson and Morris 2001, 2003). Noodles are the major wheat products consumed in Asian countries, and over 40% of Chinese wheat is used to make various types of noodles (Liu et al. 2003; He et al. 2004). White-salted udon noodles, yellow alkaline (ramen), and Chinese white noodles are the most popular types (Nagao 1996; Liu et al. 2003; He et al. 2004). Different noodle types vary in firmness and texture requirement, but all types require good brightness, white is the preferred color for udon and Chinese white noodles, and yellowness is preferred for yellow alkaline noodles. The time-dependent darkening is undesirable for all Asian noodle types. Fresh Chinese and Japanese noodles are especially sensitive to darkening during storage (Nagao 1996; Ge et al. 2003). PPO activity varies among wheat genotypes and is also affected by environment (Baik et al. 1994; Park et al. 1997; Ge et al. 2003). Cultivars with low PPO activity are desirable for the consumers and food manufacturers. It is, therefore, necessary for breeding programs targeted for noodle products to develop cultivars with very low PPO activity.

PPO genes have been cloned and sequenced in several plant species (Bucheli et al. 1996; Thipyapong et al. 1997). Recently, efforts have been made to clone PPO genes from bread wheat to understand the molecular mechanism underlying darkening of wheat based end-products and novel sequence information has been published (Demeke and Morris 2002; Anderson 2004; Jukanti et al. 2004). Demeke and Morris (2002) performed PCR on wheat genomic DNA with oligonucleotide primers designed from conserved copper binding regions of other plant PPO genes and obtained putative DNA sequence of wheat PPO gene (GenBank Accession Number AF507945). Anderson (2004) obtained a PPO gene sequence from a wheat cDNA library (GenBank Accession Number AY515506). In addition, sequence information of some other PPO genes was obtained from full-length sequencing of EST clones (Jukanti et al. 2004, GenBank Accession Numbers AY596266, AY596267, AY596268, AY596269, and AY596270).

Jukanti et al. (2004) suggested the presence of at least six PPO genes in bread wheat falling into two clusters with three similar sequences each. Based on the tissues used for cDNA library preparation, three genes AY596268, AY596269 and AY596270 are expressed during kernel development and may, therefore, influence PPO activity in flour; the remaining three genes belonging to the second cluster were isolated from non-kernel cDNA libraries and may not be expressed at high levels during grain development.

PPO activity is a physiological–biochemical trait that cannot be selected based on morphological characteristics. Identification and use of molecular markers associated with PPO activity has the potential to accelerate the efficiency of selection for low PPO activity. Udall (1997) identified a QTL for PPO activity in a recombinant inbred line population derived from a cross between NY18 and CC, and found an RFLP marker *Xcdo373* on wheat chromosome 2A that was closely linked to the QTL, accounting for over 40% of the variation of PPO activity. Jimenez and Dubcovsky (1999) reported that genes located in the wheat chromosome homologous group 2 played an important role in PPO activity. Mares and Campbell (2001) detected a QTL for grain PPO activity on chromosome 2D in a doubled haploid population derived from Sunco/Tasman. Demeke et al. (2001) used three inbred line populations to study the distribution, chromosome location, and number of loci involved in wheat PPO, and found polygenic inheritance in two populations (M6/Opata85, NY18/CC) and monogenic inheritance in the third population (ND2603/Butte86). They identified a QTL significantly associated with wheat PPO activity on chromosome 2D in the M6/Opata85 mapping population. Raman et al. (2004) also found a major QTL for PPO activity on the chromosome 2AL in a DH population derived from Chara/WW2449 and suggested that the SSR markers *Xgwm294* and *WMC170* may be used for marker-assisted selection. Zhang et al. (2005) detected a QTL on chromosome 2AL that was closely linked to *Xgwm312* and *Xgwm294*, and explained 38% of the phenotypic variance of grain PPO activity. Nevertheless, all the molecular markers identified previously are either RFLPs or SSRs, which are based on techniques with high costs. It is, therefore, desirable to develop a less expensive, and reliable molecular marker for a

large scale characterization of PPOs in wheat breeding programs.

Development and identification of molecular markers associated with PPO activity in wheat kernels would allow selection of wheat lines in early generations and greatly improve the selection efficiency. The objectives of the present study were to develop an efficient, reliable STS marker for PPO activity, and to identify the chromosome location of the corresponding PPO gene.

Materials and methods

Plant materials

In total, 106 Chinese wheat cultivars and 127 advanced lines from four major wheat regions were used for screening new STS markers and for validating their function. A doubled haploid (DH) population with 71 lines derived from the cross Zhongyou 9507 × CA9632 was used to map the PPO gene as well as the newly developed STS marker. Zhongyou 9507 is a wheat cultivar with high PPO activity, while CA9632 is one with low PPO activity. This DH population was used for QTL mapping of PPOs with 143 SSRs, 4 STS and 26 AFLP markers in our previous study (Zhang et al. 2005). A set of Chinese Spring nullisomic–tetrasomic lines (except for nullisomic 2A and nullisomic 4B lines) and Chinese Spring ditelosomic line 2AS, kindly provided by Prof. McIntosh from the University of Sydney, were employed to verify the location of the STS marker *PPO18*. The nullsomic2A–tetrasomic2B seeds used for DNA extraction were chosen from the self-pollinating progenies of monosomic2A–tetrasomic2B (M2A–T2B) line, kindly provided by Prof. Peidu Chen of Nanjing Agricultural University.

Field trials and grain PPO activity assay

During the 2001 – 2002 and 2002 – 2003 crop seasons, 106 Chinese wheat cultivars and 127 advanced lines were sown in a randomized complete block design with three replicates in the Anyang experimental station of Chinese Academy of Agricultural Sciences, located in Henan Province. Each plot consisted of two 2-m rows spaced 25 cm apart, with 100 plants of each row. Field trials for the DH population were conducted with two replicates in Beijing, Jinan and Anyang, respectively, in the 2001 – 2002 crop season, which 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 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 g^{-1} 10^{-3} in a 1-cm path at 475 nm.

STS analysis

Genomic DNA was extracted from young leaves using the method described by Doyle and Doyle (1987). Eleven, seven and 10 pairs of primers were designed using the software DNAMAN based on the sequence information of PPO ESTs AY596268, AY596269 and AY596270, respectively (Jukanti et al. 2004), and used for screening the cultivars with low and high PPO activity. It was expected that the amplified fragments with 400 – 600 bp of each could cover all of the EST sequences. One pair of the STS primers from the sequence of AY596268 showing good polymorphisms between the cultivars with high and low PPO activity (Figure 1) was designated as *PPO18* with forward primer 5′-AACTGCTGGCTCTTC-TTCCCA-3′ and reverse primer 5′-AAGAAGT-TGCCCATGTCCGC-3′. PCR primers were synthesized by Beijing Augct Biological Technology Co. Ltd (http://www.augct.com). PCR reaction was performed in an MJ Research PTC-200

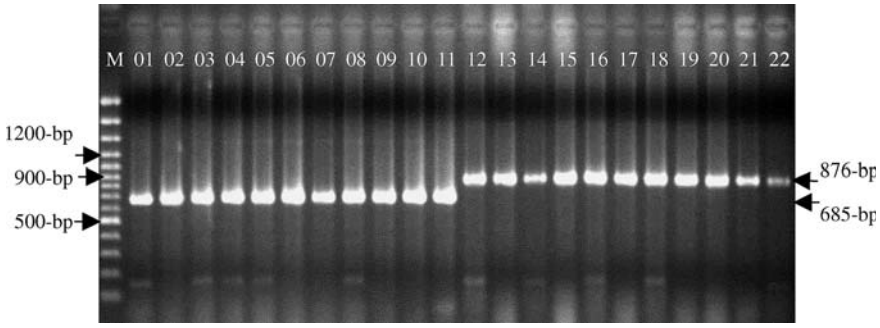

Figure 1. Polymorphic test of PCR fragments amplified with *PPO18* in 11 cultivars with high and 11 cultivars with low PPO activity. M – 100-bp DNA ladder; 1 – Nongda116 (*41.7); 2 – Yunmai 39 (42.1); 3 – Jimai 24 (44.6); 4 – Zhoumai 13 (45.6); 5 – Shan 623 (50.5); 6 – Huaimai 17 (40.5); 7 – PH85-1-1 (44.8); 8 – CA9550 (49.4); 9 – Fengyou 7 (56.6); 10 – Yumai 25 (41.7); 11 – Xuzhou 25 (52.0); 12 – Dongfanghong 3 (7.3); 13 – Lu 22 (10.4); 14 – Nongda 3291 (13.7); 15 – Zhongmai 9 (14.2); 16 – Ji Z76 (15.5); 17 – Yunfengzao 898 (9.3); 18 – Xu 858 (14.3); 19 – Yuandong 107 (16.6); 20 – Zhongyu 6 (17.1); 21 – Yumai 2 (17.3); 22 – Linhan 917 (16.2); * indicates PPO activity: A_{475}/min g^{-1} 10^{-3}.

thermal cycler in a total volume of 20 μl including 20 mM of Tris–HCl (pH 8.4), 20 mM of KCl, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 10 pmol of each primer, 1.5 units of *Taq* polymerase and 80 ng of template DNA. Temperature conditions for PCR amplification were 94 °C for 5 min, followed by 36 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, with a final extension of 72 °C for 8 min. Amplified PCR fragments were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized using UV light.

Sequencing of PCR products

The PCR products amplified from 11 cultivars with high PPO activity and 11 with low PPO activity (Figure 1) were sequenced by Bioasia Biological Technology Co., Ltd (http://www.bioasia.cn), and each PCR fragment was sequenced from two directions. Sequence alignment was conducted using the software DiAlign available in Genomatix (http://www.genomatix.de).

Statistical analysis

Linkage analysis was performed using Map Manager QTX (Manly et al. 2001). The Kosambi function was used for calculating the map distance. QTL analysis was conducted using the software QTL Cartographer V2.0 (Wang et al. 2004), and a LOD score of 3.0 was used as the threshold for declaration of linkage and QTL detection. For the 233 cultivars and advanced lines, the PPO activity of each genotype was measured in each of two crop seasons and averaged to validate the association between PPO activity and STS marker *PPO18*.

Results

Polymorphic test of STS markers

Among the 28 pairs of primers tested, clear polymorphisms were detected between the cultivars with high and low PPO activity for the PCR fragments amplified with *PPO18* (Figure 1). A 685- and 876-bp PCR fragment was amplified in 11 cultivars with high PPO activity and 11 with low PPO activity, respectively.

DNA sequence of PCR product amplified with PPO18

Based on DNA sequence of AY596268 and annealing sites of forward and reverse primers of *PPO18*, a 458-bp fragment would have been amplified (Figure 2). However, the amplified fragments in the cultivars with either high or low PPO activity are much bigger than the expected size. DNA sequence alignment indicated that two insertion sequences are present in the PCR fragments amplified from the genomic DNA of the tested

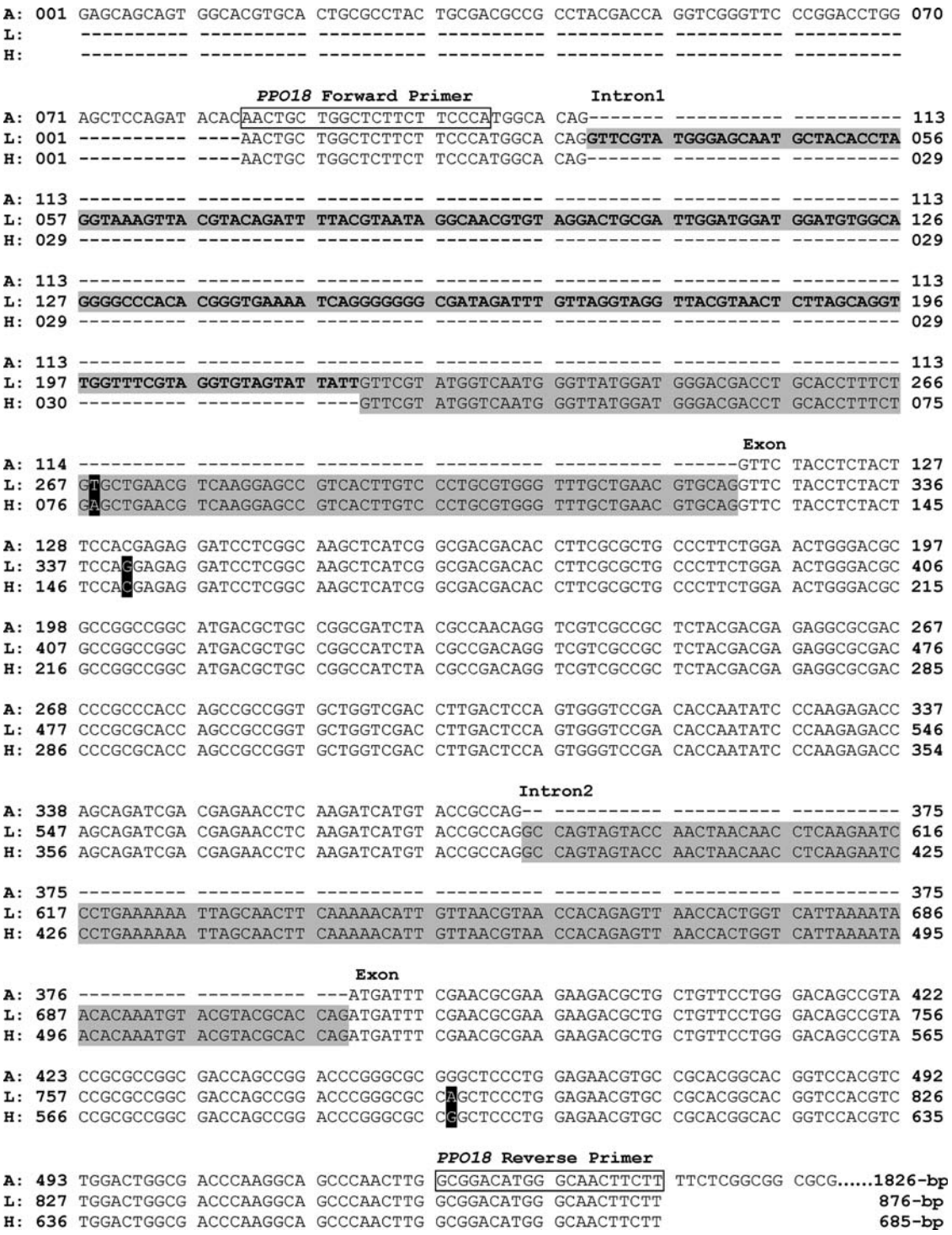

Figure 2. DNA sequence alignment of wheat PPO gene AY596268 (A) and the PCR fragments amplified with *PPO18* in the cultivars with low PPO activity (L) and the cultivars with high PPO activity (H). The bold sequence indicates a 191-bp difference between the fragments L and H. The gray shaded sequences indicate the DNA sequence of introns. The black shading bases are SNPs between the sequences of PCR fragments of L and H. The two boxes indicate the oligo-nucleotide sequences of forward primer and complementary sequence of reverse primer, respectively.

cultivars in comparison to AY596268. The first insertion sequence occurred at the position of the 113th base of AY596268 in a size of 293 bp amplified from the cultivars with low PPO activity and 102 bp from the cultivars with high PPO activity, respectively, which have the typical characteristics of exon–intron boundary 'GT-AG'. This indicated a 191-bp difference for DNA sequences of PPO genes between the cultivars with low PPO activity and those with high activity. The second insertion sequence occurred at the position of the 375th base of AY596268 with a size of 125 bp in the cultivars with either high or low PPO activity, which has a characteristics starting at 5′-GC and ending at 3′-AG. In addition, three single nucleotide polymorphisms (SNP) were detected in the DNA sequences of PCR fragments between the 11 cultivars with low and 11 with high PPO activity.

Chromosome location of PPO18 *and the corresponding PPO gene*

Linkage analysis indicated that *PPO18* is closely linked to SSR markers *Xgwm312* and *Xgwm294* on chromosome 2AL (Röder et al. 1998), with a genetic distance of 1.4 cM and 5.8 cM, respectively (Figure 3). QTL analysis revealed a major QTL for PPO activity that co-segregated with *PPO18* and accounted for 28–43% of the phenotypic variance across three environments. The QTL was derived from the female parent Zhongyou 9507 with high PPO activity. These results indicated that the PPO gene (AY596268) is located on chromosome 2AL. The test of nulli–tetrasomic lines and ditelosomic line 2AS amplified with *PPO18* confirmed that the STS marker is located on chromosome 2AL (Figure 4).

Validation of the STS marker PPO18

The separation of PCR fragments amplified with *PPO18* in 23 widely grown Chinese commercial wheat cultivars, part of the 233 cultivars and lines analyzed in this study, is shown in Figure 5. Of 233 genotypes tested, 89 genotypes with PPO activity less than 25 A_{475}/min g^{-1} 10^{-3} are always associated with the 876-bp fragment, whereas, 54 genotypes with PPO activity more than 40 A_{475}/min g^{-1} 10^{-3} are merely related to the 685-bp fragment (Figure 6). Among the cultivars and lines with PPO activity between 25 and 40 A_{475}/min g^{-1} 10^{-3}, the 876-bp fragment was detected in 32 genotypes, while the 685-bp fragment was found in 58 genotypes. Nevertheless, a clear tendency can still be seen for these genotypes with middle PPO activity (25–40 A_{475}/min g^{-1} 10^{-3}) that a 685-bp fragment was amplified from most of the cultivars with higher PPO activity (35–40 A_{475}/min g^{-1} 10^{-3}).

Discussion

Molecular mechanism for the different expression of PPO genes in cultivars with low and high PPO activity

The sequence of the PPO gene AY596268 was obtained from wheat EST CA716843 (Jukanti et al. 2004), which is supposed to be mature mRNA without any introns. The sequence alignments revealed two insertion sequences from the genomic DNA amplified with *PPO18* (Figure 2). These insertion sequences are, obviously, introns of the PPO gene. The sequence of intron 1 has a typical 5′-GT and 3′-AG boundary, which is in agreement with previous reports (Dibb and Newman 1989; Yan et al. 2000; Xing and Shuai 2002; Alexei et al. 2003), whereas, intron 2 has a GC-AG characteristics that was not reported previously in plant genes (Figure 2). However, the GC-AG alternative intron isoforms were detected in human and animal genes previously (Thanaraj and Clark 2001; Xing and Shuai 2002). Dibb and Newman (1989) found that introns tended to arise at sites with a consensus sequence of (C/A)AG|R in the study of actin and tubulin genes, which is consistent with the present study.

DNA transcription can be influenced by introns (Fedorova and Fedorov 2003). At the position of intron 1, the PPO gene in the cultivars with low PPO activity has 191 bases more than that in the cultivars with high PPO activity where PPO gene is expressed. The additional 191-bp insertion sequence might influence the splicing of premature mRNA, which could cause the PPO gene unable to express (Suzu et al. 1998). In addition, a point mutation from C to G and G to A substitution occurred at the 341th and 788th base, respectively, of the DNA sequence amplified from the cultivars with low PPO activity, in comparison to the sequence of AY596268 and the one amplified from

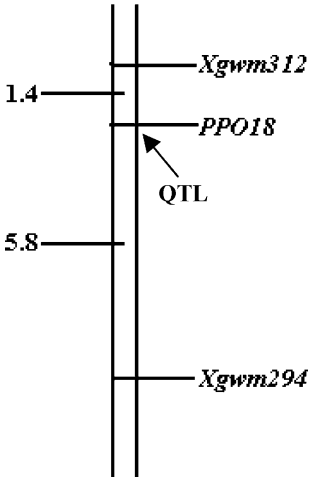

Figure 3. Linkage map constructed with *PPO18* and two SSR markers on chromosome 2AL using Map Manger QTX. The arrow indicates the position of PPO gene (QTL).

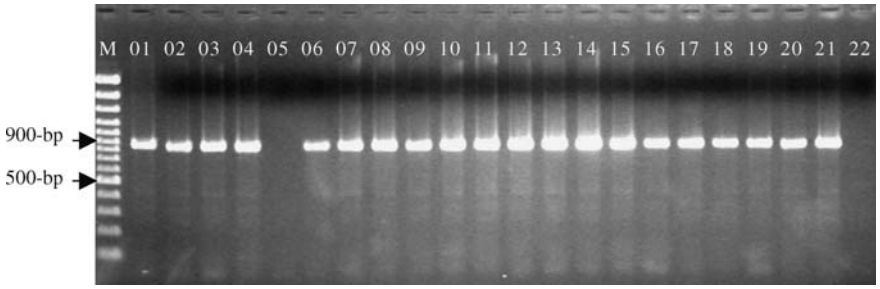

Figure 4. PCR amplification of Chinese Spring nulli–tetrasomic lines and ditelosomic line 2AS using the STS primer *PPO18*. M – 100-bp DNA ladder; Lanes 1 ~ 22 – Chinese Spring, nullisomic1A–tetrasomic1D (N1A–T1D), N1B–T1D, N1D–T1B, N2A–T2B (derived from M2A–T2B line), N2B–T2A, N2D–T2B, N3A–T3D, N3B–T3D, N3D–T3A, N4A–T4B, N4D–T4B, N5A–T5B, N5B–T5A, N5D–T5B, N6A–T6B, N6B–T6D, N6D–T6B, N7A–T7D, N7B–T7D, N7D–T7A, and ditelosomic line 2AS.

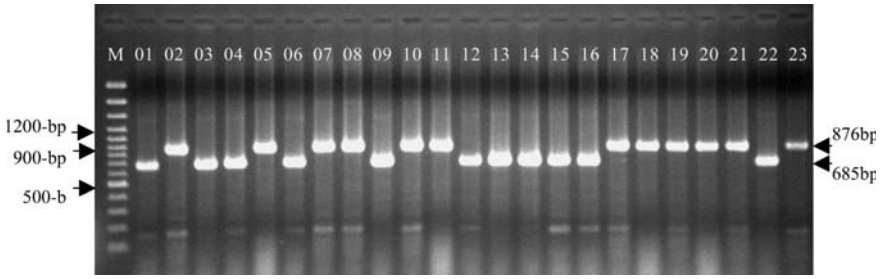

Figure 5. Electrophoresis of PCR fragments amplified with *PPO18* in 23 widely grown Chinese wheat cultivars. M – 100-bp DNA ladder; Lanes: 1 – Yumai 21 (PPO activity: 33.8 A_{475}/min g^{-1} 10^{-3}); 2 – Jimai 38 (14.5); 3 – Zhengmai 9023 (45.2); 4 – Yumai 70 (46.3); 5 – Yumai 47 (12.1); 6 – Zhongzuo8131 (37.6); 7 – Jimai19 (17.1); 8 – Lumai 23 (10.2); 9 – Nongda 152 (51.9); 10 – Mianyang 26 (18.3); 11 – Xinong 2611 (22.9); 12 – Zhongyou 9507 (48.8); 13 – Yumai 49 (40.1); 14 – Lumai 21 (43.0); 15 – Shan 354 (55.1); 16 – Yangmai 158 (45.0); 17 – CA9632 (20.3); 18 – Gaocheng 8901 (20.2); 19 – PH82-2 (22.8); 20 – Yumai 54 (12.4); 21 – Yangmai 9 (19.1); 22 – Linfen 133 (40.1); 23 – Wan 38 (21.7).

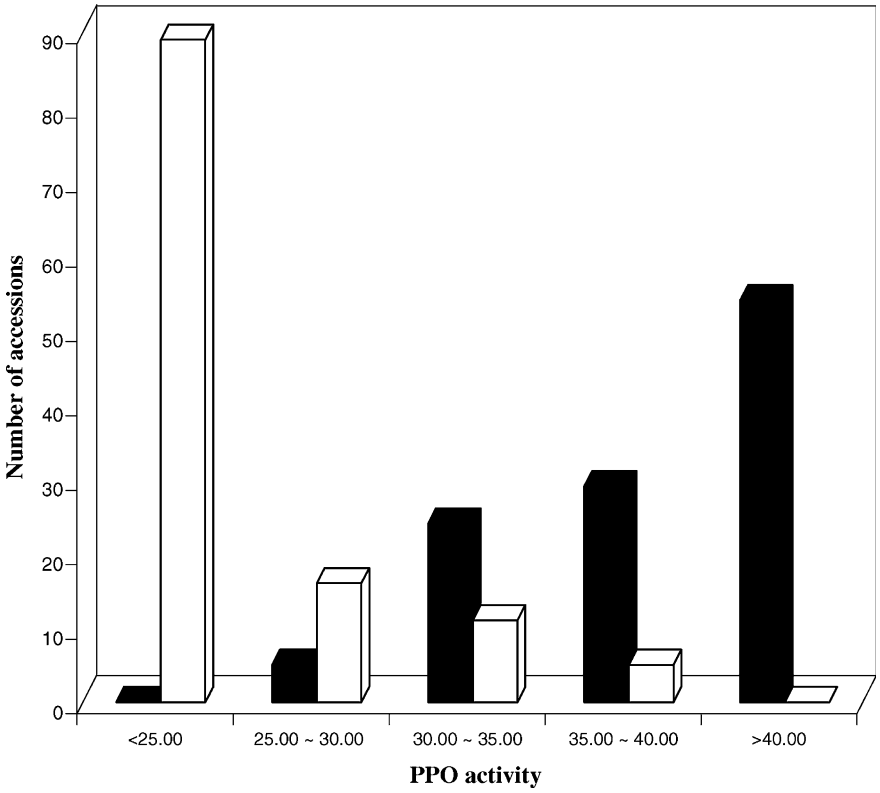

Figure 6. Association between PPO activity (A_{475}/min g^{-1} 10^{-3}) and the size of PCR fragments amplified with *PPO18* in 233 Chinese wheat cultivars and advanced lines. White columns indicate the number of accessions with 876-bp PCR fragment; black columns indicate the number of accessions with 685-bp PCR fragment.

the cultivars with high PPO activity (Figure 2). The single nucleotide substitutions might lead to the changes of histidine to glutamine (CAC to CAG) and glycine to serine (GGC to AGC) at two positions, respectively, which could be another reason for the PPO gene failing to express in the cultivars with low PPO activity.

The DNA sequence variation is generally very low in bread wheat (Bryan et al. 1999). We tested 28 primers and found only one showing polymorphisms between the cultivars with low and high PPO activity. Ma et al. (2004) suggested designing allele-specific primers in the intron regions with large variation in bread wheat, which was confirmed by the present study.

Verification of the location of PPO18

Gene mapping with molecular markers was proved to be effective in a large number of studies during the past decade (Asins 2002; Huang and Röder 2004). However, a biased estimation of gene effect and gene location might be made, especially for quantitative traits, if the mapping population is relatively small (Zeng 1994). In the present study, STS marker *PPO18* and its corresponding PPO gene were mapped to chromosome 2AL with SSR markers *Xgwm312* and *Xgwm294* (Röder et al. 1998). To verify the location of *PPO18*, we used a set of nulli–tetrasomic lines and ditelosomic line 2AS. The results confirmed that *PPO18* was located on chromosome 2AL, which was consistent with the linkage analysis with molecular data. The PPO gene on chromosome 2AL is a major gene that can be mapped with molecular data much more easily than polygenic minor genes, although the mapping population was relatively small. Raman et al. (2004) also located a major QTL for PPO activity on chromosome 2AL with SSR marker *Xgwm294* and deletion stocks. They found that *Xgwm294* associated with PPO

activity maps in a deletion bin delimited by C-2A0 to 2AL-0.85.

Acknowledgements

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