**RESEARCH NOTE**

# Allelic variations of functional markers for polyphenol oxidase (*PPO*) genes in Indian bread wheat (*Triticum aestivum* L.) cultivars

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## Introduction

Polyphenol oxidase (PPO) activity causes undesirable browning and discolouration of products manufactured from bread wheat (*Triticum aestivum* L.) during processing or storage. PPO is a copper-containing metalloprotein which catalyses hydroxylation of *o*-monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones. Auto-oxidation and polymerization of quinones with amino acid group of cellular proteins results in dark and brown discolouration of products made from bread wheat grains (Anderson and Morris 2001). The darkening phenomena of such products may reduce the quality of products and thus affect consumer acceptance. Flour protein content has a negative association with flour PPO activity (Park *et al*. 1997) presumably because of reactivity of phenolic side groups (Demeke *et al*. 2001). In wheat, *PPO* genes belong to a multi-gene family and are classified into two clusters: kernel and nonkernel, based on gene expression sites (Anderson *et al*. 2006; Jukanti *et al*. 2006). Recently, Massa *et al*. (2007) reported 21 distinct *PPO* sequences in kernel-type genes in wheat and wild relatives. Many studies have implied that PPO activity is mainly conditioned by the genes located on homoeologous group 2 chromosomes in wheat and wild relatives (Demeke *et al*. 2001; Watanabe *et al*. 2004, 2006; Sun *et al*. 2005; He *et al*. 2007, 2009; Raman *et al*. 2007). The high activity *PPO* alleles on chromosomes 2AL and 2DL are most thoroughly studied and were first reported by Wrigley and McIntosh (1975). A relatively low PPO activity was also found to be associated with chromosomes 2B, 3D and 6B (Demeke *et al*. 2001; Fuerst *et al*. 2008). Functional Sequence tagged site (STS) markers for *PPO* genes on chromosomes 2A and 2D have been developed, based on DNA sequences in GenBank (Sun *et al*. 2005; He *et al*. 2007; Raman *et al*. 2007). Use of molecular markers associated with PPO activity has the potential to improve selection efficiency for low PPO activity. The objective of the present study was to validate some of the STS markers for PPO activity in Indian genotypes of wheat.

## Materials and methods

### *Plant materials*

Fifty seven Indian cultivars of wheat, released during 1969–2007 for different wheat cultivating agro-climatic zones of India viz., northwestern plain zone (NWPZ), northeastern plain zone (NEPZ), peninsular zone (PZ), central zone (CZ), north hill zone (NHZ) and southern hill zone (SHZ) were included in this study.

### *Phenol test*

Five seeds of each genotype were soaked in distilled water in a Petri plate for 19 h. The water was then drained and seeds were kept on a Whatman filter paper to remove the excess water. The seeds were transferred to centrifuge tubes containing 1 mL of 1% phenol. After 4 h, phenol solution was drained and seeds were dried on Whatman filter paper. The phenol colouration was classified in to four categories depending on the intensity of colour (see figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/).

### *DNA extraction and STS analysis*

Seeds were grown in Petri plates under dark conditions to ensure low carbohydrate content. Genomic DNA was extracted from one-week-old seedlings using CTAB method (Doyle and Doyle 1990). For STS analysis, a polymerase chain reaction (PCR) was performed in a 25-$\mu$L volume containing 100 ng of genomic DNA, 2.5 $\mu$L of 10× *Taq* PCR buffer, 1.5 mM of $MgCl_2$, 200 $\mu$M of each dNTP, 0.2 $\mu$M of each primer and 1.0 unit of *Taq* DNA polymerase in a PTC-200 thermal cycler (MJ Research, Massachussets, USA).

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The thermocycling program consisted of initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min with a final extension of 72°C for 10 min. PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide and visualized with UV light.

## Results and discussions

Many studies have independently shown that PPO activity is associated with homoeologous group 2 chromosomes (Wrigley and McIntosh 1975; Demeke *et al*. 2001; Sun *et al*. 2005; Anderson *et al*. 2006; Watanabe *et al*. 2006; He *et al*. 2007) and functional markers for *PPO* genes on chromosomes 2A and 2D have been developed based on DNA sequences in GenBank (Sun *et al*. 2005; He *et al*. 2007). In the present study, two codominant functional STS markers, *PPO18* (Sun *et al*. 2005) and *PPO33* (He *et al*. 2007) on chromosome 2A, and two complementary dominant STS markers, *PPO16* and *PPO29* (He *et al*. 2007) were tested for their occurrence in 57 Indian wheat cultivars from different agro-climatic zones of India.

The codominant marker *PPO18* amplified two fragments, 685 and 876 bp, which corresponded to *Ppo-A1a* and *Ppo-A1b* alleles, respectively (figure 1). Majority of genotypes (77%) amplified 685-bp fragment (table 1). Similarly, *PPO33* marker amplified two fragments that were of 290 and 481-bp size. The 685 and 876-bp fragments of *PPO18* marker correspond to 290 and 481-bp fragments, respectively from *PPO33* marker.

Of the 57 cultivars, 41 amplified a 713-bp PCR fragment with *PPO16* marker, indicating the presence of *Ppo-D1a* allele, and 16 genotypes gave no amplification product, indicative of *Ppo-D1b* allele. *PPO29*, the complementary STS marker, amplified 490-bp fragments (*Ppo-D1b* allele) in 16 genotypes and remaining genotypes gave no PCR product (figure 2). The genotyping with complementary *PPO16* and *PPO29* markers can overcome the dominant nature of the markers and detect the heterozygous lines in marker-assisted breeding programmes.

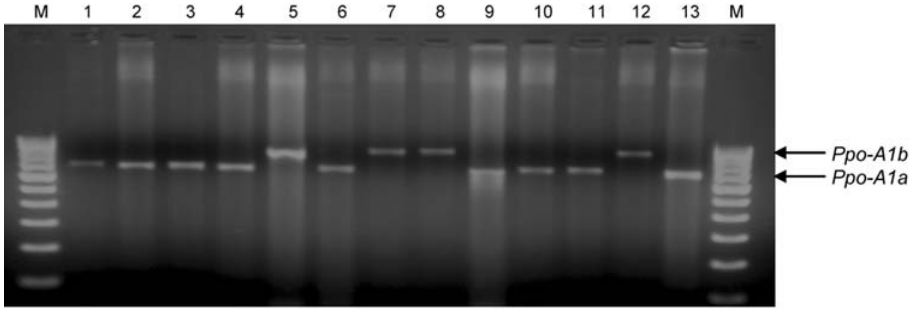


**Figure 1.** PCR detection of *Ppo-A1a* (713 bp) and *Ppo-A1b* (490 bp) alleles with *PPO18* marker. M, DNA marker; 1, HI 977; 2, HD 2329; 3, GW 366; 4, DBW 14; 5, GW 173; 6, DBW 17; 7, DL 788-2; 8, HD 2687; 9, GW 322; 10, HD 2189; 11, HD 2733; 12, K 8027; 13, K 0307.

**Table 1.** Allelic variations at *Ppo* loci on chromosomes 2A and 2D, and phenol colour reactions in Indian wheat genotypes.

| | | | | PCR fragment size (bp) *PPO* of marker | | | |
|---|---|---|---|---|---|---|---|
| Cultivars | Zone | Phenol colour reaction | Year of release | *PPO18* | *PPO33* | *PPO29* | *PPO16* |
| Kharchia 65 | All zones | +++ | 1970 | 685 | 290 | – | 713 |
| KRL 19 | All zones | ++ | 2000 | 876 | 481 | – | 713 |
| Lok-1 | CZ | +++ | 1982 | 685 | 290 | – | 713 |
| GW 322 | CZ | +++ | 2002 | 685 | 290 | – | 713 |
| GW 366 | CZ | +++ | 2006 | 685 | 290 | 490 | – |
| MP 4010 | CZ | +++ | 2003 | 876 | 481 | 490 | – |
| DL 788-2 | CZ | +++ | 1997 | 876 | 481 | 490 | – |
| GW 173 | CZ | ++ | 1994 | 876 | 481 | – | 713 |
| Raj 4037 | CZ | + | 2003 | 876 | 481 | – | 713 |
| HD 2864 | CZ | + | 2004 | 876 | 481 | – | 713 |
| HI 1500 | CZ | ++ | 2003 | 685 | 290 | – | 713 |
| GW 190 | CZ | +++ | 1994 | 685 | 290 | – | 713 |

**Table 1** (*contd*)

| Cultivars | Zone | Phenol colour reaction | Year of release | PCR fragment size (bp) *PPO* of marker | | | |
|---|---|---|---|---|---|---|---|
| | | | | *PPO18* | *PPO33* | *PPO29* | *PPO16* |
| DBW 16 | NEPZ | +++ | 2006 | 685 | 290 | – | 713 |
| K 9107 | NEPZ | +++ | 1996 | 685 | 290 | – | 713 |
| HD 2733 | NEPZ | +++ | 2001 | 685 | 290 | – | 713 |
| DBW 14 | NEPZ | +++ | 2002 | 685 | 290 | – | 713 |
| NW 2036 | NEPZ | +++ | 2002 | 685 | 290 | – | 713 |
| K 8027 | NEPZ | + | 1989 | 876 | 481 | – | 713 |
| HD 2888 | NEPZ | ++ | 2005 | 685 | 290 | – | 713 |
| HUW 234 | NEPZ | ++ | 1986 | 876 | 481 | – | 713 |
| NW 1014 | NEPZ | ++ | 1998 | 876 | 481 | – | 713 |
| K 0307 | NEPZ | +++ | 2006 | 685 | 290 | – | 713 |
| HS 240 | NHZ | +++ | 1989 | 685 | 290 | – | 713 |
| VL 738 | NHZ | ++++ | 1997 | 685 | 290 | 490 | – |
| VL 804 | NHZ | +++ | 2002 | 876 | 481 | 490 | – |
| VL 616 | NHZ | +++ | 1986 | 685 | 290 | – | 713 |
| HS 277 | NHZ | ++++ | 1992 | 685 | 290 | 490 | – |
| VL 829 | NHZ | +++ | 2003 | 685 | 290 | – | 713 |
| HS 295 | NHZ | +++ | 1992 | 685 | 290 | – | 713 |
| HPW 42 | NHZ | +++ | 1992 | 685 | 290 | – | 713 |
| HS 375 | NHZ | ++++ | 2002 | 685 | 290 | 490 | – |
| Sonalika | NWPZ | ++++ | 1969 | 685 | 290 | 490 | – |
| WH 542 | NWPZ | +++ | 1992 | 685 | 290 | 490 | – |
| HD 2687 | NWPZ | ++ | 1999 | 876 | 481 | – | 713 |
| PBW 343 | NWPZ | ++++ | 1996 | 685 | 290 | – | 713 |
| PBW 373 | NWPZ | +++ | 1996 | 685 | 290 | – | 713 |
| Raj 3765 | NWPZ | ++ | 1996 | 685 | 290 | – | 713 |
| UP 2425 | NWPZ | +++ | 1999 | 685 | 290 | – | 713 |
| PBW 175 | NWPZ | ++ | 1989 | 876 | 481 | – | 713 |
| PBW 396 | NWPZ | ++++ | 2000 | 685 | 290 | 490 | – |
| HD 2329 | NWPZ | ++++ | 1985 | 685 | 290 | 490 | – |
| DBW 17 | NWPZ | +++ | 2006 | 685 | 290 | – | 713 |
| PBW 502 | NWPZ | +++ | 2004 | 685 | 290 | – | 713 |
| UP 2565 | NWPZ | ++++ | 2003 | 685 | 290 | 490 | – |
| PBW 527 | NWPZ | +++ | 2007 | 685 | 290 | – | 713 |
| HD 2285 | NWPZ | +++ | 1984 | 685 | 290 | – | 713 |
| NI 5439 | PZ | ++ | 1973 | 685 | 290 | – | 713 |
| Raj 4083 | PZ | +++ | 2006 | 685 | 290 | – | 713 |
| HD 2189 | PZ | +++ | 1980 | 685 | 290 | – | 713 |
| NIAW 917 | PZ | +++ | 2005 | 685 | 290 | 490 | – |
| NIAW 34 | PZ | ++ | 1997 | 876 | 481 | – | 713 |
| HD 2781 | PZ | ++ | 2002 | 685 | 290 | – | 713 |
| HI 977 | PZ | ++++ | 1988 | 685 | 290 | 490 | – |
| PBW 533 | PZ | ++ | 2005 | 685 | 290 | – | 713 |
| MACS 2496 | PZ | ++++ | 1991 | 685 | 290 | – | 713 |
| HW 2044 | SHZ | ++ | 1999 | 685 | 290 | 490 | – |
| HPW 155 | | ++++ | | 685 | 290 | 490 | – |

–, Absence of PCR fragment.

The phenol colour reaction of kernel was allocated to four classes: very dark, (++++); dark, (+++); slightly dark, (++); faint, (+) (see figure 1 in electronic supplementary material). The reactions of very dark and dark were classified as strong reactions, where as slightly dark and faint were classified as weak reactions. A similar criterion of classification for phenol test was followed by Watanabe *et al*. (2004). Kernels of 17 genotypes showed weak phenol colour reaction and 40 genotypes gave a strong colour reaction with phenol. All genotypes (10) from NHZ wheat growing zone showed strong phenol colour reaction (table 1), whereas four genotypes from each of CZ, NEPZ, PZ wheat growing zone

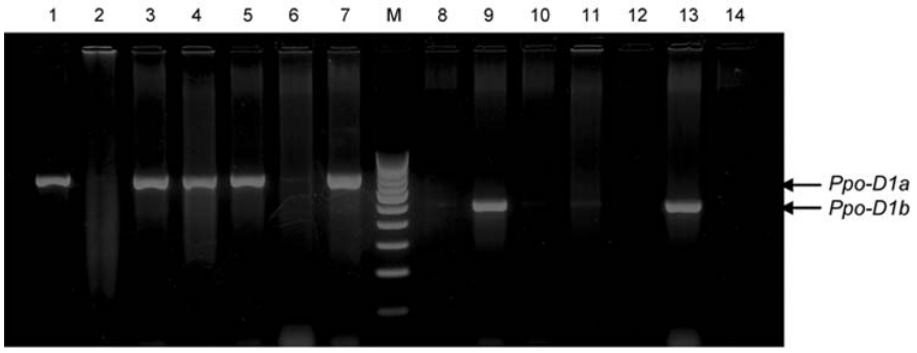


**Figure 2.** PCR detection of *Ppo-D1a* (713 bp) and *Ppo-D1b* (490 bp) alleles with *PPO16* and *PPO29* markers, respectively. M, DNA marker; 1&8, PBW 502; 2&9, HI 977; 3&10, HD 2687; 4&11, DBW 17; 5&12, Raj 4037; 6&13, HD 2329; 7&14, PBW347.

showed weak phenol colour reaction. Only three out of 15 cultivars showed weak colour reaction in NWPZ, the major wheat growing zone in India.

Among the wheat genotypes whose seeds gave low (+ or ++) phenol colour reaction rating, the results showed that 59% have alleles for low PPO activity at both *Ppo-A1* and *Ppo-D1* loci and 94% have alleles for low PPO activity at *Ppo-D* locus. Ten genotypes amplified both PCR fragments of 876 and 713 bp with *PPO18* and *PPO16* markers, respectively, indicating the presence of *Ppo-A1b* and *Ppo-D1a* alleles, and showed a weak colour reaction with phenol (table 1). The codominant marker *PPO18* amplified a 685-bp fragment in the genotypes with high PPO activity (*Ppo-A1a* allele) and a 876-bp fragment in those with low PPO activity (*Ppo-A1b* allele) (Sun *et al*. 2005; He *et al*. 2007). *PPO16* marker amplified 713-bp fragment in genotypes with low *PPO* activity (*Ppo-D1a* allele) and no PCR product in those with high *PPO* activity (*Ppo-D1b* allele), whereas *PPO29* marker amplified complementary 490-bp fragment in genotypes with high PPO activity and no PCR product in genotypes with low *PPO* activity (He *et al*. 2007).

Six cultivars viz., Raj 3765, HD 2888, NI 5439, HD 2781, PBW 533 and HI 1500 also showed weak phenol colour reaction, but possessed high PPO activity (*Ppo-A1a*) and low PPO activity (*Ppo-D1a*) alleles on chromosome 2A and 2D, respectively. Two genotypes viz., MP 4010 and DL 788-2, with low PPO activity (*Ppo-A1b*) and high PPO activity (*Ppo-D1b*) alleles showed strong phenol reaction. The *PPO18* markers on chromosome 2A explained 28%-43% of phenotypic variation for PPO activity (Sun *et al*. 2005), whereas *PPO16* and *PPO29* markers on chromosome 2D explained 9.6%–24.4% phenotypic variance (He *et al*. 2007). The remaining genotypes showed strong phenol colour reaction. The genotypes with both *Ppo-A1a* and *Ppo-D1b* alleles showed very dark colour reaction with phenol except HW 2044 (table 1). It will be interesting to find the single nucleotide polymorphism (SNP) in the *PPO* genes of these genotypes viz. Raj 3765, HD 2888, NI 5439, HD 2781, PBW 533, HI 1500, MP 4010, DL 788-2 and HW 2044, which may affect the activity of *PPO* genes. The results suggest that selection for low *PPO* activity alleles at both *Ppo-A1* and *Ppo-D1* loci with markers may help in marker assisted selection for low PPO activity in wheat improvement programmes. The markers for PPO activity are derived from polymorphic site within coding sequences of genes and are effective in determining the target phenotype in different genetic backgrounds; thus, may be effectively integrated in the marker assisted breeding to overcome the negative effects of PPO activity on quality of wheat products.

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