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Research Article

Molecular marker assisted screening for kernel polyphenol oxidase activity in wheat *Triticum aestivum* L.

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Abstract
High level of kernel polyphenol oxidase (PPO) activity has been found to be the major factor behind time-dependent discolouration of wheat-based products, which ultimately leads to reduced consumers' preference. Till date, 6 genes belonging in 2 paralogous sets present in wheat (*Triticum aestivum* L.) chromosome 2 homeologues (2A, 2B and 2D) have been reported to govern kernel PPO activity. Among these 6 genes, perfect molecular markers have been developed for 2 genes (*PPOA1* and *PPOD1*) and the major role of *PPOA1* gene in governing kernel PPO activity in wheat has been reported. In the present study we have used the molecular markers for the *PPOA1* and *PPOD1* genes to characterize wheat genotypes for their kernel PPO activity. We have successfully converted the dominant marker assay for the *PPOD1* locus into a co-dominant assay using the already reported primers. Our molecular screening strategy could explain the kernel PPO activity of wheat genotypes in rapid, reliable and environment-independent manner. Furthermore, biochemical estimation of kernel PPO activity in wheat genotypes indicated the involvement of other genes in fine-tuning this important trait. Thus, the present study should facilitate the breeders in marker-assisted selection and breeding for developing wheat genotypes with low kernel PPO activity.

Keywords: Co-dominant screening; Marker-assisted breeding; Paralogous genes; Phenol test; Wheat kernel polyphenol oxidase activity

INTRODUCTION

Time-dependent darkening of wheat-based products, particularly noodles, is undesirable from the point of view of consumers' preference. The ubiquitous enzyme polyphenol oxidase (PPO, EC 1.14.18.1) has been reported to be the major factor behind this discolouration of different wheat-based products (Baik *et al.,* 1995; Mares and Campbell, 2001; Fuerst *et al.,* 2006). The nuclear-encoded, copper-containing, oxygen-dependent PPO enzyme has been reported to possess monophenolase and diphenolase activities (Gao *et al.,* 2009). Thus, in presence of oxygen, PPO is involved in hydroxylation of *o*-monophenols to *o*-diphenols and subsequently *o*--diphenols to *o*-quinones. The resulting quinones produce dark brown or black colouration after reacting with amines and thiol groups or through non-enzymatic self-polymerization (Mayer and Harel, 1979; Baik *et al.,* 1994; Anderson and Morris, 2001). In wheat kernel, PPO activity is mainly localized in the bran layer, which is generally removed during milling. But contaminating residual bran layer has been reported to be sufficient for causing product discolouration (Hatcher and Kruger, 1993; Rani *et al.,* 2001). In contrast, the nutritive value of wheat bran (Stevenson *et al.,* 2012) has been realized by the consumers to shift their choice from refined wheat flour to whole grain wheat flour, to a great extent. Furthermore, flour protein content has been reported to have negative correlation with PPO activity (Park *et al.,* 1997). In this way, breeding for development of wheat genotypes with low kernel PPO

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activity is rewarding, not only from the point of view of consumers' preference but also for the nutritional enrichment of wheat-based products.

The genetics behind kernel PPO activity in wheat has been studied in great details, where different *PPO* genes of wheat has been classified in 'kernel' and ' non-kernel' clusters (Jukanti *et al.,* 2004; Anderson *et al.,* 2006). The major genes governing kernel PPO activity have been reported to be present in wheat homeologous group 2 chromosomes (Jime´nez and Dubcovsky, 1999). The role of the *PPO* genes on chromosome 2AL and 2DL (i.e., *PPOA1* and *PPOD1*) in regulating kernel PPO activity has been established (Sun *et al.,* 2005; Chang *et al.,* 2007; He *et al.,* 2007; Wang *et al.,* 2009) and the *PPOA1* locus has been found to have major effect in determining kernel PPO activity (Raman *et al.,* 2007; Beecher and Skinner, 2011; Martin *et al.,* 2011; Nilthong *et al.,* 2013). In order to facilitate marker assisted selection (MAS), reproducible gene-based sequence tag site (STS) markers have been developed for the *PPOA1* and *PPOD1* loci. However, the possibility of erroneous prediction of alleles at *PPOD1* locus using the developed markers has been indicated (Nilthong *et al.,* 2013). In the recent past, 4 QTLs for PPO activity on chromosomes 2AL, 3BS, 4BL and 7DL has been documented to explain 64.1–89.0 % of the total phenotypic variance across environments (Zhai et al., 2016). Application of diversity arrays technology (DArT) in doubled haploid population has yielded markers (wPt-7024, wPt-0094 and wPt-2544) associated with major loci governing kernel PPO activity in bread wheat (Sadeque et al., 2018). Recently, genome-wide association study has identified several QTLs that regulate kernel PPO activity ; out of these QTLs, 12 located on homoeologous group 1 chromosomes, chromosomes 4B, 4D, and 7A and 7B have been postulated as new PPO loci (Zhai et al., 2020).

In the present study, we analyzed the allelic variation at *PPOA1* and *PPOD1* loci in 14 wheat genotypes and attempted to correlate their allelic status with kernel PPO activity. Using PPO33 marker for the *PPOA1* locus, we document the identification of *PPOA1b* low PPO allele in selected wheat genotypes. At the same time, we propose the strategy of using 3 already reported primers (for PPO16 and PPO29 markers) in a single polymerase chain reaction (PCR) for co-dominant screening of the *PPOD1* locus to identify the *PPOD1a* low PPO allele.

MATERIALS AND METHODS

Seed materials

Freshly harvested seeds (kernels) of 14 wheat (*Triticum aestivum* L.) genotypes were obtained from Wheat Section, Bihar Agricultural University, Sabour, Bhagalpur.

Bioinformatics analysis

Sequence information of the wheat polyphenol oxidase (*PPO*) *A1a*, *A1b*, *D1a* and *D1b* alleles was retrieved from National Centre for Biotechnology Information (NCBI) database (GenBank accession No. EF070147, EF070148, EF070149 and EF070150, respectively). Sequence alignment was performed using ClustalW (https://embnet.vital-it.ch/software/ClustalW.html) and pictorial representation of aligned sequences was prepared using ESPript3 (http://espript.ibcp.fr/ESPript/ESPript/).

Genotyping at *PPOA1* and *PPOD1* loci

Genomic DNA from *in vitro* germinated wheat seedlings was isolated using a rapid method (Kumar *et al.,* 2017). The primers for PPO33 (Forward: 5´-CCAGATACACAACTGCTGGC-3´, Reverse: 5´-TGATCTTGAGGTTCTCGTCG-3´), PPO16 (Forward: 5´-TGCTGACCGACCTTGACTCC-3´, Reverse: 5´-CTCGTCACCGTCACCCGTAT-3´) and PPO29 (Forward: 5´-TGAAGCTGCCGGTCATCTAC-3´, Reverse: 5´-AAGTTGCCCATGTCCTCGCC-3´) markers were used in this study, as reported earlier (He *et al.,* 2007). PCR-based genotyping at *PPOA1* locus was done with the PPO33 marker, whereas genotyping at *PPOD1* locus involved the use of PPO16 marker (for detection of the *PPOD1a* allele) and PPO29 marker (for detection of the *PPOD1b* allele). For simultaneous detection of *PPOD1a* and *PPOD1b* alleles in co-dominant manner, the combination of PPO16 Forward, PPO29 Forward and PPO16 Reverse primers was used. Each reaction mix (total 12 µl) contained 2 µl of isolated genomic DNA, 1X PCR buffer [10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1 % (v/v) Triton X-100], 0.1 mM of dNTP mix, 0.4 µM of each primers, 5 % (v/v) dimethyl sulfoxide (DMSO) and 1 U of *Taq* DNA polymerase (Xcelris). The polymerase chain reaction was carried out in an automated thermal cycler (Veriti, Applied Biosystems) with initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at an appropriate temperature for 40 s and extension at 72 °C for 1 min and ended with a final extension at 72 °C for 10 min followed by hold at 4 °C for 2 min. For the PPO33 marker system, annealing temperature was kept at 61 °C, whereas for the PPO16, PPO29 and PPO16 Forward + PPO29 Forward + PPO16 Reverse marker system, the annealing temperature was kept at 64 °C. Following PCR, the amplicons were separated through 1 or 1.2 % (w/v) agarose gel electrophoresis in the presence of ethidium bromide and imaged in gel documentation system (Genei, Bangalore).

Analysis of kernel polyphenol oxidase activity

Polyphenol oxidase activity of wheat kernels was analyzed through the whole kernel assay method, as de-

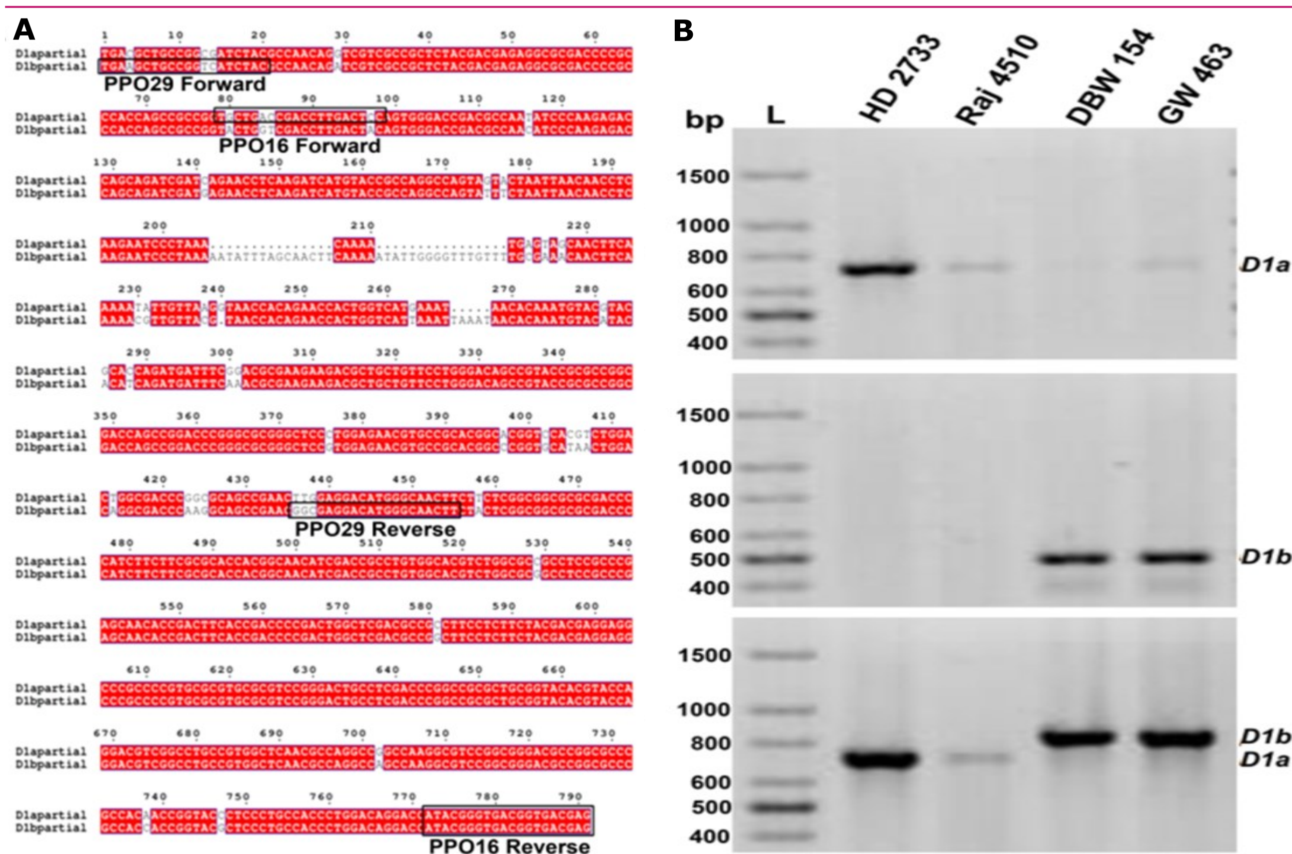

Fig. 1. *Development of a strategy for co-dominant screening of alleles at PPOD1 locus. (A) Alignment of partial sequences of PPOD1a and PPOD1b alleles. Region corresponding to PPO16 Forward, PPO16 Reverse, PPO29 Forward and PPO29 Reverse primers are shown inside boxes. (B) Inverse image of ethidium bromide stained 1 % agarose gel showing dominant detection of PPOD1a allele (top gel), dominant detection of PPOD1b allele (middle gel) and co-dominant detection of PPOD1a/PPOD1b allele (bottom gel) in 4 wheat genotypes. L = 100 bp DNA ladder.*

scribed earlier (Singh *et al.,* 2009) with minor modifications. Briefly, 4 randomly selected kernels of each wheat genotype were soaked for 16 h in double-distilled water. Afterwards, the kernels were placed on filter paper soaked in 1 % (w/v) phenol solution and placed in a Petri-dish. After 4 h, the darkening of the kernels was observed and visually scored in a scale of 1 (low) to 4 (high).

RESULTS

Co-dominant screening of the *PPOD1* locus

Two complementary dominant marker systems (PPO16 and PPO29) have been documented for screening the presence of *PPOD1a* and *PPOD1b* alleles in bread wheat (He *et al.*, 2007). In the present study, the partial reference sequences of the *PPOD1a* and *PPOD1b* alleles were aligned in such a way that the sequence alignment could reveal the regions corresponding to the PPO16 and PPO29 marker primers (Fig. 1.A). Interestingly, it was found that the PPO16 Reverse primer could bind perfectly in both *PPOD1a* and *PPOD1b* alleles. As PPO16 Forward and PPO29 Forward primers were designed to specifically bind *PPOD1a* and *PPOD1b* alleles, respectively, the use of (PPO16 Forward + PPO29 Forward + PPO16 Reverse) primers in a single reaction indicated the possibility of co-dominant screening at *PPOD1* locus. Using this primer combination, a 713 bp band specific to *PPOD1a* allele and an 826 bp band specific to *PPOD1b* allele were expected.

For evaluation of this system, firstly wheat genotypes containing different allelic versions at *PPOD1* locus were screened through the PPO16 and PPO29 marker, separately. The wheat genotypes HD 2733 and Raj 4510 produced the *PPOD1a* allele-specific 713 bp band with the PPO16 marker, whereas the genotypes DBW 154 and GW 463 produced the *PPOD1b* allele-specific 490 bp band with the PPO29 marker (Fig. 1.B, top and middle gel). Interestingly, the genotype GW 463 was found to generate a faint band of 713 bp with the PPO16 marker, which could mislead towards the prediction of the presence of *PPOD1a* allele in this genotype. Only after obtaining the result with the complementary dominant marker PPO29, the proper allelic version at *PPOD1* locus present in this genotype could be confirmed as *PPOD1b*. However, the use of PPO16 Forward + PPO29 Forward + PPO16 Reverse

primer combination in a single reaction could easily distinguish between the allelic versions of *PPOD1* locus present in the aforementioned 4 wheat genotypes. In the wheat genotypes HD 2733 and Raj 4510, containing the *PPOD1a* allele, a sharp 713 bp expected band was observed, whereas in the wheat genotypes DBW 154 and GW 463, containing the *PPOD1b* allele, a sharp 826 bp band was obtained (Fig. 1.B., bottom gel). Thus, the use of PPO16 Forward + PPO29 Forward + PPO16 Reverse primer combination in a single reaction was found to be suitable for co-dominant screening of the *PPOD1* locus in wheat.

Testing of heterozygosity at the *PPOD1* locus

The primer combination of PPO16 Forward + PPO29 Forward + PPO16 Reverse was found to be helpful for co-dominant screening for the presence of *PPOD1a*/*PPOD1b* allele in wheat through a single reaction. The co-dominant nature of this primer combination prompted us to check the utility of this system in detecting heterozygosity at *PPOD1* locus in wheat. For this purpose, equal amount of genomic DNA from the genotypes HD 2733 (containing the *PPOD1a* allele), DBW 154 (containing the *PPOD1b* allele) and GW 463 (containing the *PPOD1b* allele) were mixed in different combinations to artificially create heterozygosity at the *PPOD1* locus. Single PCR with the PPO16 Forward + PPO29 Forward + PPO16 Reverse primer combination using the HD 2733 + DBW 154 mixed genomic DNA (representing the *PPOD1a*/*PPOD1b* heterozygous genotype) was found to reveal the presence of both the *PPOD1a* allele-specific 713 bp and *PPOD1b* allele-specific 826 bp bands (Fig. 2).

In a similar manner, use of this primer combination was found to generate *PPOD1a* allele-specific 713 bp and *PPOD1b* allele-specific 826 bp bands, when the HD 2733 + GW 463 mixed genomic DNA (representing the *PPOD1a*/*PPOD1b* heterozygous genotype) was used as template for PCR. On the other hand, PCR using DBW 154 + GW 463 mixed genomic DNA (representing the *PPOD1b*/*PPOD1b* homozygous genotype) was observed to generate only the *PPOD1b* allele-specific 826 bp band (Fig. 2). Hence, the aforementioned primer combination was found suitable for screening for heterozygosity at the *PPOD1* locus, which may arise in the segregating lines of a crossing programme involving parental wheat genotypes carrying different allelic versions at the *PPOD1* locus.

Screening at *PPOA1* and *PPOD1* loci of different wheat genotypes

Allelic variation at the *PPOA1* and *PPOD1* loci of 14 wheat genotypes was screened in the present study. For the *PPOA1* locus, the co-dominant marker PPO33 (He *et al.*, 2007) was used, whereas, for the *PPOD1* locus, the PPO16 Forward + PPO29 Forward + PPO16 Reverse primer combination was used. The *PPOA1b* allele contained 191 bp insertion in the 1st intron of the gene, which has been explored by the PPO33 marker to differentiate between the *PPOA1a* and *PPOA1b* alleles. The PPO33 marker, designed from the conserved sequence flanking this insertion has been reported to generate *PPOA1a* allele-specific small and *PPOA1b* allele-specific large amplicons (He *et al.*, 2007). When the 14 wheat genotypes of the present study were subjected to PCR using the PPO33 marker, only 4 geno-

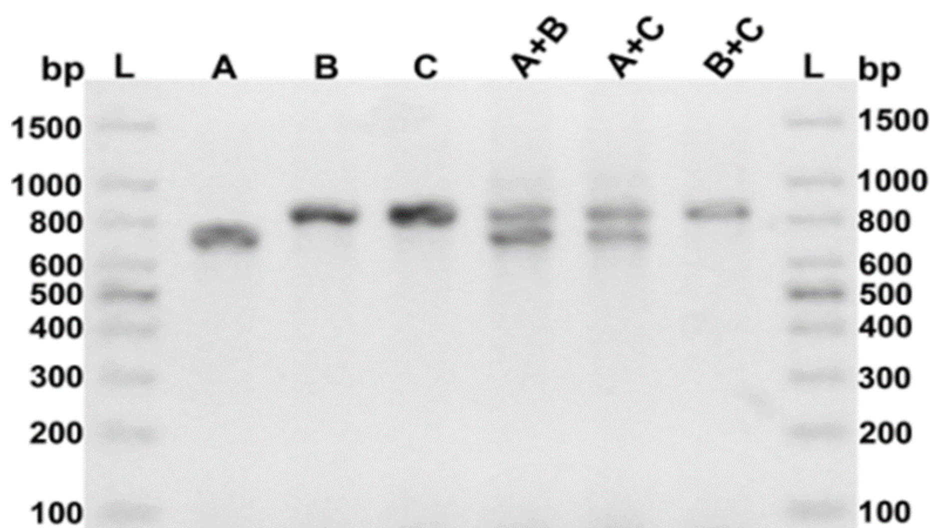

Fig. 2. *Inverse image of ethidium bromide stained 1 % agarose gel showing co-dominant detection of PPOD1a and PPOD1b alleles in both homozygous and heterozygous conditions using the (PPO16 Forward + PPO29 Forward + PPO16 Reverse) primer combination. A = HD 2733; B = DBW 154; C = GW 463; A+B = mixed genomic DNA of HD 2733 and DBW 154; A+C = mixed genomic DNA of HD 2733 and GW 463; B+C = mixed genomic DNA of DBW 154 and GW 463; L = 100 bp DNA ladder.*

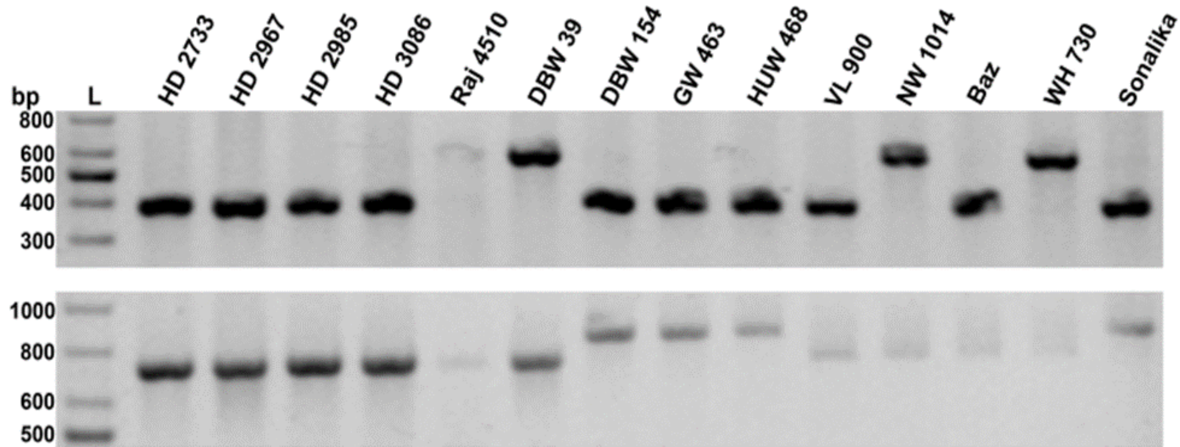

Fig. 3. *Inverse image of ethidium bromide stained 1.2 % (top) and 1 % (bottom) agarose gels showing detection of allelic versions at the PPOA1 and PPOD1 loci present in 14 wheat genotypes used in the present study. L = 100 bp DNA ladder.*

types (i.e., Raj 4510, DBW 39, NW 1014 and WH 730) were observed to generate the *PPOA1b* allele-specific ~583 bp band (Fig. 3, top). The other 10 genotypes were found to generate *PPOA1a* allele-specific ~392 bp band.

The strategy of using the PPO16 Forward + PPO29 Forward + PPO16 Reverse primer combination explored to analyse the allelic variation present at the *PPOD1* locus of the 14 wheat genotypes revealed that the *PPOD1b* allele-specific ~826 bp band was present in only 4 genotypes i.e., DBW 154, GW 463, HUW 468 and Sonalika. The other 10 genotypes were found to generate *PPOD1a* allele-specific ~713 bp band.

Trait-marker relation for polyphenol oxidase *(PPO)* activity in wheat genotypes

The *PPOA1b* and *PPOD1a* alleles at *PPOA1* and *PPOD1* loci have been documented to be associated with low polyphenol oxidase activity in wheat kernels (Sun *et al.,* 2005; He *et al.,* 2007). In the present study, genotyping of the 14 wheat lines revealed the utility of molecular markers in assessing kernel PPO activity, through phenol test followed by visible scoring (Fig. 4). As summarized in Table 1, presence of the low *PPO* allelic combination (*PPOA1b* + *PPOD1a*) was found in 4 genotypes (i.e., Raj 4510, DBW 39, NW 1014 and WH 730). All of these genotypes, except the genotype WH 730 were observed to possess low kernel polyphenol oxidase activity (Fig. 4). The high *PPO* allelic combination (*PPOA1a* + *PPOD1b*) was present in 3 genotypes (i.e., DBW 154, GW 463 and HUW 468), all with high kernel PPO activity (Fig. 4). Presence of the high *PPO* allele (*PPOA1a*) at the *PPOA1* locus was found to be associated with high kernel activity in all of the tested wheat genotypes, except one (i.e., VL 900). Thus, the screening of wheat genotypes at *PPOA1* and *PPOD1* loci using the previously reported PPO33 marker and the presently developed primer combination strategy, respectively, was observed to be highly informative about the kernel PPO activity of the genotypes.

DISCUSSION

In the present study, we studied the kernel PPO activity in wheat genotypes using phenol test and molecular markers. Kernel PPO activity has been found to be an important undesirable feature in wheat-based products, particularly noodles (reviewed in Morris, 2018). Kernel PPO activity in Indian wheat genotypes have been estimated, where genotypes belonging to North Eastern Plain Zone (NEPZ) and North Western Plain Zone (NWPZ) have been identified to have lower kernel PPO activity, making them suitable for use in producing better wheat-based products (Salaria et al., 2018). In our study, for the screening of the allelic variation at *PPOD1* locus, 2 complementary dominant markers (PPO16 and PPO29) have been reported (He *et al.,* 2007). Notably, the possibility of erroneous allelic prediction at *PPOD1* locus using dominant markers has already been reported (Nilthong *et al.,* 2013). Furthermore, the capability of a co-dominant marker system to distinguish between homozygous and heterozygous segregants offers an added advantage in marker assisted breeding (MAB) programmes. Hence, in this study, we tried to optimize co-dominant screening of the *PPOD1* locus in wheat. On the basis of sequence alignment, we developed a co-dominant marker system, where PPO16 Forward, PPO29 Forward and PPO16 Reverse primers are used together. Using this strategy, we were able to amplify the *PPOD1a* allele-specific ~713 bp band and the *PPOD1b* allele-specific ~826 bp band in different wheat genotypes in a co-dominant manner (Fig. 2, 3). Furthermore, the suitabil-

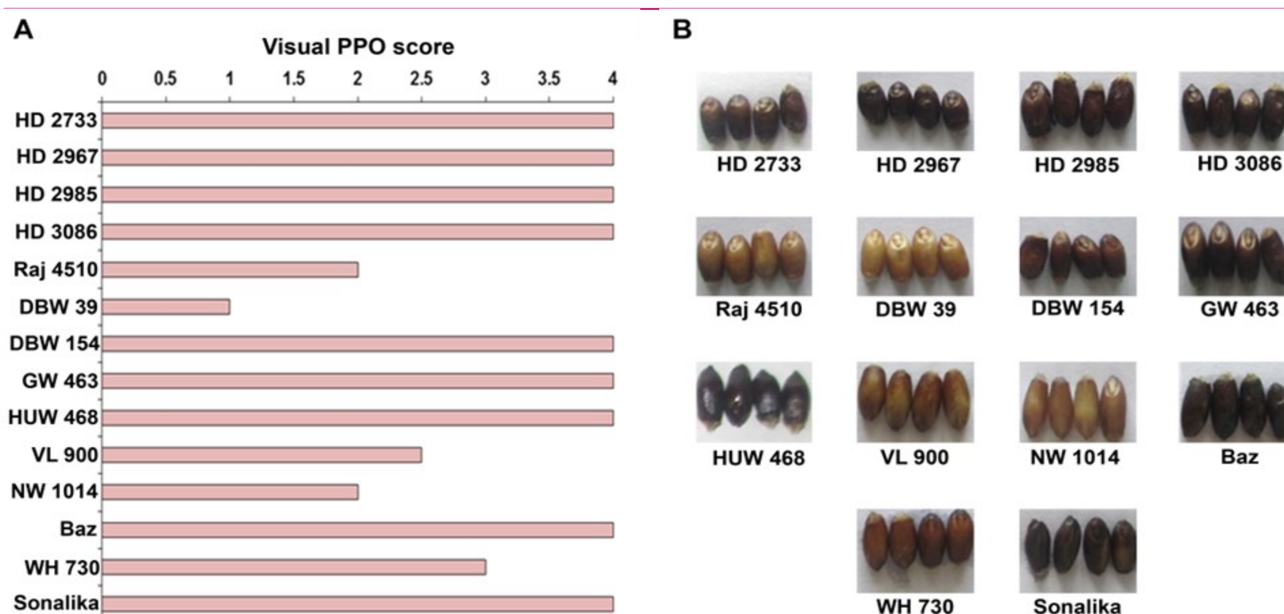

Fig. 4. *Kernel polyphenol oxidase activity of 14 wheat genotypes used in the present study.* ***(A)*** *Bar diagram, showing visual score (1 to 4 scale) of kernels from different wheat genotypes after phenol test.* ***(B)*** *Images of enzymatic browning of kernels from different wheat genotypes after phenol test.*

Table 1. Allelic status at *PPOA1* and *PPOD1* loci along with kernel PPO activity in 14 wheat genotypes.

Sl. No.	Genotype	*PPOA1* allele	*PPOD1* allele	Visual PPO score
1	HD 2733	*A1a*	***D1a***	4
2	HD 2967	*A1a*	***D1a***	4
3	HD 2985	*A1a*	***D1a***	4
4	HD 3086	*A1a*	***D1a***	4
5	Raj 4510	***A1b***	***D1a***	2
6	DBW 39	***A1b***	***D1a***	1
7	DBW 154	*A1a*	*D1b*	4
8	GW 463	*A1a*	*D1b*	4
9	HUW 468	*A1a*	*D1b*	4
10	VL 900	*A1a*	***D1a***	2.5
11	NW 1014	***A1b***	***D1a***	2
12	Baz	*A1a*	***D1a***	4
13	WH 730	***A1b***	***D1a***	3
14	Sonalika	*A1a*	*D1b*	4

*Alleles reported to be associated with low kernel PPO activity are marker by bold underline

ity of this system in differentiating homozygosity and heterozygosity at the *PPOD1* locus was also tested and found appropriate (Fig. 2). Hence, we advocate the utility of this system as an important component in marker-assisted wheat breeding programmes targeting the development of wheat lines with low kernel PPO activity.

The *PPOA1b* allele has been documented to be associated with low kernel PPO activity (Sun *et al.,* 2005; He *et al.,* 2007). In corroboration to that, all except one of the wheat genotypes carrying the *PPOA1b* allele was found to have low kernel PPO activity (Fig. 4, Table 1). In a similar manner, all except one of the 14 wheat genotypes carrying the *PPOA1a* high *PPO* activity allele were observed to have high kernel PPO activity, irrespective of the allelic status at the *PPOD1* locus

(Fig. 4, Table 1).

The *PPOD1a* allele has been documented to be associated with low kernel PPO activity (He *et al.,* 2007). In the present study, all except one genotypes (WH 730) carrying *PPOD1a* allele, with *PPOA1b* allele (the low *PPO* allele at *PPOA1* locus) were observed to have low kernel PPO activity (Table 1). However, presence of the *PPOA1a* allele (the high PPO allele at *PPOA1* locus) was found to mask the effect of *PPOD1a* allele, as all the genotypes, except one (VL 900) carrying the *PPOD1a* allele with the *PPOA1a* allele were found to have high kernel PPO activity (Table 1). This observation is supported by previous studies (Raman *et al.,* 2007; Beecher and Skinner, 2011; Martin *et al.,* 2011; Nilthong *et al.,* 2013), where major role of *PPOA1* locus in governing kernel PPO activity in wheat has been documented. Moreover, a new gene family (*PPOA2, PPOB2* and *PPOD2*) located on chromosome 2 homeologoues (chromosome 2A, 2B and 2D) has been documented in recent past (Beecher and Skinner, 2011; Beecher *et al.,* 2012), where *PPOA2* and *PPOD2* along with *PPOA1* and *PPOD1* loci have been proposed as major regulators of kernel PPO activity in wheat. Furthermore, involvement of several other loci in governing kernel PPO activity has been proposed in a recent past (Zhai et al., 2016; Sadeque et al., 2018; Zhai et al., 2020). Examination of the allelic forms present at *PPOA2, PPOD2* and other loci in the wheat genotypes WH730 and VL900 will be helpful for further exploration of the genetics behind kernel PPO activity in wheat.

Conclusion

Molecular marker assisted selection (MAS) at *PPOA1* and *PPOD1* loci can efficiently evaluate the kernel PPO activity in wheat in a rapid, robust and reliable manner. Our strategy of using 3 primers in a single PCR for screening allelic status at *PPOD1* locus in co-dominant manner will help the wheat breeders in MAS of wheat genotypes in their breeding programme.

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Conflict of interest

The authors declare that they have no conflict of interest.

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