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ASSOCIATION OF MOLECULAR MARKERS WITH POLYPHENOL OXIDASE ACTIVITY IN SELECTED WHEAT GENOTYPES

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Abstract

Wheat (*Triticum aestivum L.*), a major staple food for the people of Pakistan and other Asian countries, is used as bread, chapatti, porridge, noodles and many other. It is established that color quality of wheat products depend on chemical and enzymatic factors especially the polyphenol oxidases (PPOs). These are copper containing enzymes which induce browning in wheat-based products. Various procedures for determining PPO activity available and differences in PPO activity among wheat genotypes have been documented. In present study, an attempt was made to establish the association of molecular markers with polyphenol oxidase activity in wheat genotypes having very high or very low PPO activities. Twelve pairs of markers were used out of which only three primer pairs viz. PPO43, PPO30 and WP2-2 yielded specific pattern discriminating high and low PPO genotypes. Cluster analysis for all 12 markers revealed that all the low PPO lline share the same sub cluster, but high PPO lines were dispersed in different clusters.

Key words: Molecular markers, Polyphenol oxidase, *Triticum aestivum*, Genomic DNA.

Introduction

Wheat is the second important staple crop after rice, in its importance as a source of energy and protein. In all products manufactured from wheat, color is the key quality factor in marketing, especially in noodles and pasta. During processing and/or storage of these products, browning may appear with the passage of time. Polyphenol oxidases have been implicated as major contributors of discoloration in wheat derived products especially noodles in an age dependent manner (Baik *et al.,* 1994; Kruger *et al.,* 1994; Feillet *et al.,* 2000). PPO is totally oxygen dependent and is responsible for conversion of phenolic compounds into quinones which either react with thiol groups and amines, or through self-polymerization develop dark gray or brown products (Mayer and Harel, 1979). PPO varies among different cultivars and breeding lines of wheat (Baik *et al.,* 1994; Park *et al.,* 1997). Caffeic acid, catechol, chlorogenic acid, L-tyrosine, methyl catechol, phenol, 3,4-dihydroxyphenyalanine (L-DOPA) are commonly used as substrates of PPO (Kihara *et al.,* 2005). Out of these, PPO shows the best activity with catechol and L-DOPA at pH 6.5 (Anderson and Morris, 2001).

PPO has been reported to exist in multiple isozymes in different plants species. In wheat, upto twelve isoforms of have been found at different development stages only in kernel tissues (Kruger, 1976). PPO isozymes studied in different plants differ in physical, chemical or enzymatic properties such as electrophoretic mobility, optimum temperature, pH and their substrate specificities. Jukanti *et al*. (2004) have held gene duplication responsible for evolution of PPO multigene family in wheat. Presence of isoforms of PPO has been ascribed to post-translational modifications, or to disproportionate expression of members of the multigene family (Yoruk & Marshall, 2003).

The flour browning is a commonly known phenomenon occurring in kneaded/ aged flour, which compromises the palatability and safety of the products. Although it is an important quality parameter, it has widely been ignored in developing countries mainly because the stress of breeding activity is directed solely towards yield enhancement. As food quality awareness will increase, developing wheat genotypes with reduced PPO activity will become more and more important goal in wheat breading programs in near future.

Wheat germplasm with very low PPO activity can be identified and utilized in breeding programs. Development and exploitation of different DNA markers associated with major quantitative trait loci (QTLs) governing PPO activity can shorten the selection process of cultivars with low PPO activity (Sun *et al*., 2005). Genes expressing PPO have been identified and sequenced from a number of plants and characterized. This is instrumental in understanding the molecular mechanism of wheat products darkening (Demeke & Morris, 2002; Jukanti *et al.,* 2004). Such studies have revealed that major QTLs related to the PPO activity are present on wheat homologous group 2 in different populations (Jimenez and Dubcovsky, 1999). Markers associated with high and low PPO activity have been reported by different researchers based on one or few wheat genotypes. These include *Xutv 1427-2A, Xcdo373, Xgwm294, and* PPO18 (Simeone *et al.*, 2002; Raman *et al.*, 2004; Sun *et al.*, 2005). The objective of this study was to verify the association of reported molecular markers with PPO activity in wheat lines with very high or very low PPO activity selected from an accession of more than 100 wheat lines (Naqvi *et al*., 2013), to establish reliable screening. By selecting the low PPO activity wheat genotypes and by omitting the high PPO genotypes, the product value can be increased and the post-harvest losses may be minimized.

Materials and Methods

Wheat seeds were germinated in pots at room temperature. Genomic DNA was isolated from young seedling leaves by CTAB method as described by Richards (1997). About 0.5-1g leaf samples were washed

with distilled water and homogenized in an autoclaved mortar and pestle using 3-4 ml of 2X CTAB buffer preheated at 65ºC. The homogenate was transferred to 1.5 ml eppendorf tube. The tubes were vortexed gently and incubated at 65°C in water bath for 40 minutes. The tubes were re-vortexed and centrifuged at 12,000 rpm for 10 minutes. Supernatants were shifted to fresh centrifuge tubes and chloroform was added in an equal volume to each tube. Contents were vortexed and centrifuged for 10 minutes at 12,000 rpm. This step was repeated twice and an equal volume of ice cold iso-propanol was added to clear supernatant obtained. The tubes were gently inverted few times for proper mixing and placed at -20°C for one hour followed by centrifugation at 14,000 rpm for 10 minutes. DNA was precipitated and aqueous phase was discarded. Pellets were washed with 70% ice cold ethanol and dissolved in 50µL nanopure water containing RNase A, after air drying.

Primer sequences for discrimination of high and low PPO genotypes were selected form previously published articles (Table 1). Amplification of the target fragment was attempted in 10 µL reaction mixture which included genomic DNA 2 µL, PCR buffer1 µL, each dNTPs 200 µM, Taq DNA Polymerase 1.5 units, $MgCl_2$ and 25 pM of each primer. PCR thermal profile for all the reactions included Pre-Amplification Denaturation (PAD) at 94°C for 3 minutes, primer annealing at the appropriate temperature for 2 min, and elongation at 72°C for 3 min. This Thermal cycling was continued for another 34 cycles of with same conditions except the time for boiling and elongation were decreased to 20 and 40 sec, respectively. Post-amplification extension was carried out at 72°C for 10 minutes. Amplicons were analized in 1.5% agarose in 40 mM TAE (pH 8.1). Ethidium bromide was used for staining of the agarose gels.

Results and Discussion

Association of molecular markers with PPO in wheat genotypes: Molecular markers offer fast and convenient rout for plant selection (Mohan *et al.,* 1997; Sohail *et al*., 2015; Rehman *et al*., 2015). It has also been reported that discovery and use of different molecular markers associated with major quantitative trait loci (QTLs) related with PPO activity can hasten the selection of cultivars with low PPO activity (Sun *et al*., 2005). For validation of the published markers for PPO, ten wheat genotypes (four low and six high PPO) as reported by (Farooq, 2010) were used. Twelve sets of molecular markers (Table 1) were employed to differentiate between high and low PPO cultivars as determined by their PPO activities. All the marker sets were successful in amplification in most of the genotypes, however the number and size of amplified fragments were different from those reported in the literature (Table 2).

The markers PPO33, PPO18, WMC170, BQ161439, BE443833, WP3-1, WP2-1, WP3-2and PPO16 yielded same banding pattern in high and low PPO cultivars (data not shown), and were not relevant for discrimination between low and high and low PPO genotypes studied.

Table 1. Molecular Markers selected from previously published articles for discrimination of high and low PPO varieties. In each primer pair, upper and lower sequences represent Forward and Reverse Primer respectively.

Markers	Primer sequences (5'-3')	Target gene	Annealing temp.	References
PPO18	AACTGCTGGCTCTTCTTCCCA AAGAAGTTGCCCATGTCCGC	Ppo-A1a and Ppo-A1b (2A)	60	Sun *et al*., 2005
PPO16	TGCTGACCGACCTTGACTCC CTCGTCACCGTCACCCGTAT	Ppo-D1a (2D)	59	He *et al*., 2007
PPO33	CCAGATACACAACTGCTGGC TGATCTTGAGGTTCTCGTCG	Ppo-A1a and Ppo-A1b (2A)	60	He *et al*., 2007
WMC 170	ACATCCACGTTTATGTTGTTGCAT TTGGTTGCTCAACGTTTACTTC	Xwmc170	60	Raman *et al.,* 2004
PPO30	Forward: CAGCCAGAAAGCAAACACCG Reverse: CATGGGAAGAAGAGCCAGCA	*Ppo-A1 (*2A)	60	He *et al.,* 2007
PPO43	Forward: GCAGCATGGAGAGCAGTCGC Reverse: GCAGTTGTGCACCTGGATCTCA	*Ppo-D1* (2D)	64	He *et al*., 2007
BQ161439	Forward: GGACGTGAACTT TGTCTGAAC Reverse: AGAGCGGCGTTCAGGATATC	XTc1 and XPPO-LDOPA (2A)	62	Raman *et al.,* 2007
BE443833	Forward: AATGAGAGCGAAGCTGGAGA Reverse: GCTGCGAAGTAATTTCCAAGA	2A	53	Raman *et al.,* 2007
WP2-1	Forward: ATCGACCAGAACCTCAACATCA Reverse: TCCTCCGCCTCCTCCTTCTC	TaPPO-2	60	Chang *et al.,* 2007
WP2-2	Forward: CGACGCTGAGGGAGACGGT Reverse: GTTCACCGTTCCGATTGTTCT	TaPPO-2	66	Chang *et al.,* 2007
WP3-1	Forward: ACTGCCAAACGCCCGACCT Reverse: CCGATGAGCTTGCCGAGGAT	TaPPO-3	64	Chang *et al.,* 2007
WP3-2	Forward: AGGTTCTACGTCTACTTCCAC Reverse: CCGCCGAGAAGAAGTTGC	TaPPO-3	58	Chang *et al.,* 2007

Table 2. PCR amplified bands in High and Low PPO wheat genotypes with different molecular markers.

Marker	Band sizes (bp) (previous studies)		Band sizes (bp) (current study)		71	70	64	55	P90	R90	KN83	75	46	91
	Low	High	Low	High										
PPO 33	481	290	400	400	1	1	1	1	1	1	1	1	1	1
PPO18	876	685	300	300	1	1	1	1	1	1	1	1	1	1
PPO16	713	No band	1250	1250	1	1	1	1	1	1	1	0	1	1
PPO30	615		600	600, 950	1	1	1	1	2	2	2	2	1	1
PPO 43	578		No band	600	0	0	0	0	1	1	0	1	0	1
WMC170	245	232	232	232, 245	1	1	1	1	1	1	2	1	2	1
BQ161439	135		500	500	1	1	1	1	1	1	1	1	1	1
BE443833	650		650, 1000	650	1	1	1	2	1	1	1	1	1	1
WP2-1	Data not available		500, 600, 700, 900, 1000	600, 700,900, 1100	3	4	3	5	4	3	4	2	4	5
WP2-2	Data not available		350, 600,750	600, 750	3	3	3	3	2	2	2	2	2	2
WP3-1	Data not available		500, 600,750	500, 600,750	3	3	3	3	3	3	3	3	0	3
WP3-2	550		450, 550, 800	450, 550, 800	3	3	3	3	2	2	2	3	3	3

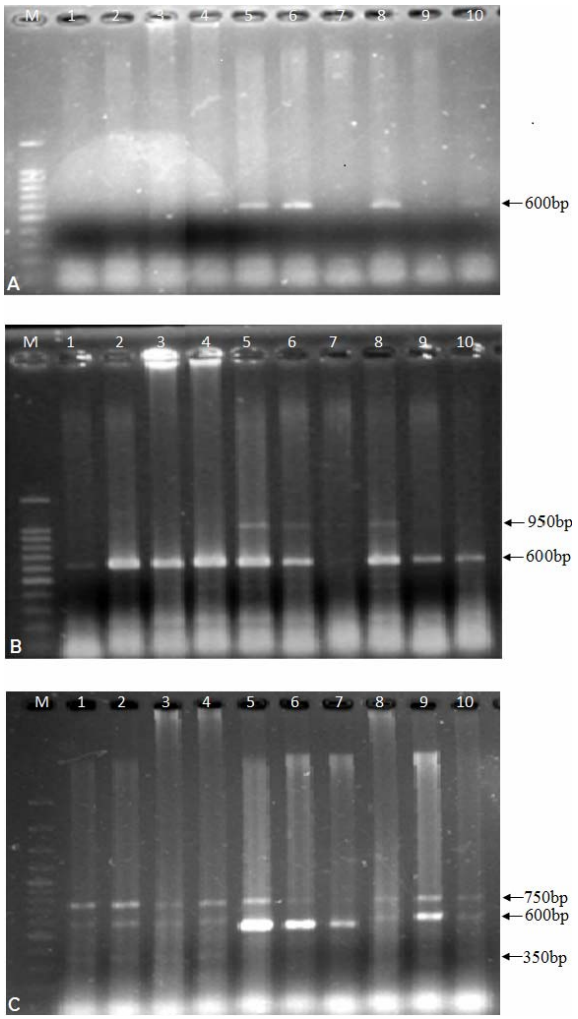

Fig. 1. Polymorphic test of PCR fragments amplified by (A) PPO43, (B)PPO30, (C) WP2-2 in four cultivars with low PPO activity and six cultivars with high PPO activity. Lane M: 100 bp DNA marker; lane 1: 011171 (7.268); lane 2: 011170 (9.288); lane 3: 011164 (15.339); lane 4: 011155 (40.18); lane 5: Pasban 90 (121.444); lane 6: Rohtas 90 (122.457); lane 7: KNoor 83 (152.185); lane 8: 011175(155.381); lane 9: 011146 (193.199); lane 10: 011191 (217.486); The number in parentheses indicates PPO activity.

He *et al.* (2007) designed two STS markers PPO30 and PPO43 from the sequence of a putative PPO gene by blast search of AY596268 and AY515506 against the Genbank wheat ESTs database and amplified 615 and 578bp product respectively, but without any discrimination between cultivars exhibiting either high or low PPO activity. In the current study PPO43 showed polymorphism with a 600bp fragment amplified in cultivars showing high PPO activity, while no band was amplified in lines with low PPO activity (Fig. 1A). PPO30 showed positive results for discrimination of the contrasting genotypes by yielding two bands i.e. about 600 and 950 bp in case of high and only one band of 600 bp in low PPO genotypes (Fig. 1B).

Chang *et al*., 2007 designed a primer pair WP2-2 from the mRNA sequence of *Ta*PPO-2 but could not detect any variation among different wheat genotypes on the basis of PPO. In the current study, this marker was successful at revealing variation among high and low PPO lines. Two bands were amplified in high PPO varieties and three bands in low PPO varieties (Fig. 1C).

Among all the markers used in this study only three makers PPO43, PPO30 and WP2-2 produced differential banding pattern with high and low PPO cultivars.

To analyze the relationship among the genotypes showing different PPO activities cluster analysis was performed using the scoring data for all molecular markers obtained in the form of gel bands following PCR amplification, and the dendrogram was constructed by unweighted pair group method Arithmetic mean (UPGMA). The genotypes initially splitted into two clusters A and B (Fig. 2). Cluster B had only one line (high PPO) in it while all others fell in cluster A, which further split into three sub clusters. All low PPO genotypes were located in cluster Ai while the other two sub-cluster Aii, and Aiii were each comprised of a high PPO genotype.

In conclusion, different molecular markers were tested on selected genotypes. Three primer pairs PPO 43, PPO 30 and WP2-2 yielded specific pattern for wheat lines that could discriminate between high and low PPO genotypes. Ultimately the selection of wheat genotypes with low PPO activity or exclusion of high PPO genotypes will assist in developing wheat cultivars with better market acceptability.

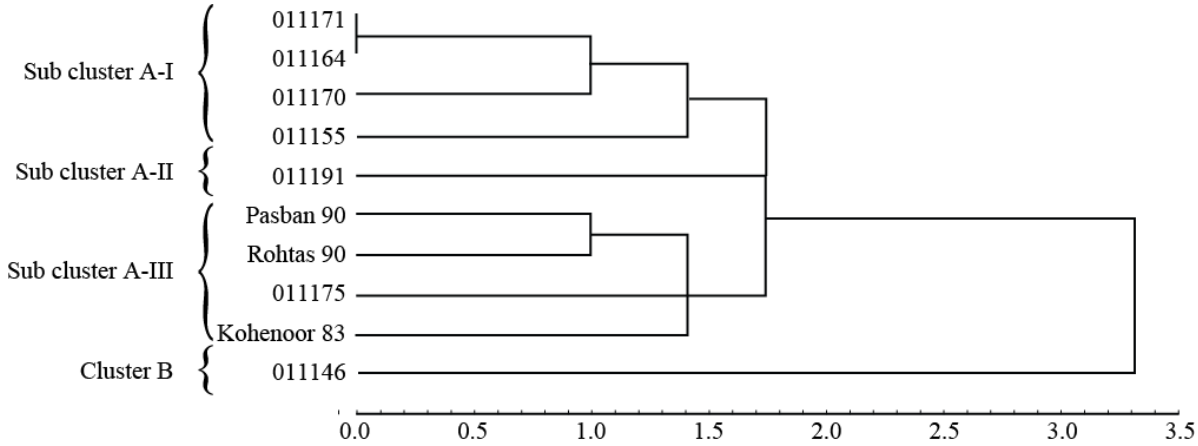

Fig. 2. Dendrogram based on UPGMA analysis of genetic similarity of different genotypes from molecular marker data. **Cluster Ai:** includes four low PPO wheat genotypes (Acc. 011171, 011170, 011164 and 011155). **Cluster Aii:** includes one high PPO wheat genotype (Acc. 011191). **Cluster Aiii:** includes all four high PPO wheat genotypes (Acc. Pasban 90, Rohtas 90, KNoor83 and 011175). and **Cluster B**: includes one high PPO wheat genotype (Acc. 011146).

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