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 line 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 (Triticum aestivum L.), a major staple food 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; 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 isoforms in different plants species. In wheat, upto twelve isoforms of PPO have been found at different development stages only in kernel tissues (Kruger, 1976). PPO isoforms 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 breeding 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 65°C. 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 isopropanol was added to clear supernatant obtained. The tubes were gently inverted few times for proper mixing and placed at -20 ºC 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% isopropanol and dissolved in 50µL nanopure water containing RNase A, after air drying.

Primer sequences for discrimination of high and low PPO genotypes were selected from previously published articles (Table 1). Amplification of the target fragment was attempted in 10 µL reaction mixture which included genomic DNA 2 µL, PCR buffer 1 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 analyzed 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 route 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 and PPO16 yielded same banding pattern in high and low PPO cultivars (data not shown), and were not relevant for discrimination between low and high PPO genotypes studied.

### Table 1. Molecular Markers selected from previously published articles for discrimination of high and low PPO varieties.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Primer sequences (5'-3')</th>
<th>Target gene</th>
<th>Annealing temp.</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO18</td>
<td>AACTGCTGGCTTCTTCTCCA AAGAAGTTGCCCATGTCGCC</td>
<td>Ppo-A1a and Ppo-A1b (2A)</td>
<td>60</td>
<td>Sun et al., 2005</td>
</tr>
<tr>
<td>PPO16</td>
<td>TGTGACCGACCTTGACTCC CTCGTCACCGTACCGGTAT</td>
<td>Ppo-D1a (2D)</td>
<td>59</td>
<td>He et al., 2007</td>
</tr>
<tr>
<td>PPO33</td>
<td>CCAGATACAAACTGCTGGCC AGATCTTGAGGTTCTCGTCG</td>
<td>Ppo-A1a and Ppo-A1b (2A)</td>
<td>60</td>
<td>He et al., 2007</td>
</tr>
<tr>
<td>WMC 170</td>
<td>ACATCCACGTTTATGTTGTGGCAT TGTGTTCCACTAAGTITCTTC</td>
<td>Xwmc170</td>
<td>60</td>
<td>Raman et al., 2004</td>
</tr>
<tr>
<td>PPO30</td>
<td>Forward: CAACCCAAAGCAAACACCG Reverse: CATGGGAAGAAGGCCAGCA</td>
<td>Ppo-A1 (2A)</td>
<td>60</td>
<td>He et al., 2007</td>
</tr>
<tr>
<td>PPO43</td>
<td>Forward: GCAGCATGGAGAGCAGTCGC Reverse: GCAGTTGTCACCTGACTTC</td>
<td>Ppo-D1 (2D)</td>
<td>60</td>
<td>He et al., 2007</td>
</tr>
<tr>
<td>BQ161439</td>
<td>Forward: GAGCGTGAACCTTGTCTGAAC Reverse: AGACCGGCTCAGGATATC</td>
<td>XTc1 and XPO-Pollo (2A)</td>
<td>62</td>
<td>Raman et al., 2007</td>
</tr>
<tr>
<td>BE443833</td>
<td>Forward: AATGAGACCGACGAGCTTGGAGA Reverse: GCTTGCAGAAATTTTCCAAAGA</td>
<td>2A</td>
<td>53</td>
<td>Raman et al., 2007</td>
</tr>
<tr>
<td>WP2-1</td>
<td>Forward: ATCGACGACGACCTCACACTCA Reverse: TCCTCGCCCTCTCCTCTTC</td>
<td>TaPPO-2</td>
<td>60</td>
<td>Chang et al., 2007</td>
</tr>
<tr>
<td>WP2-2</td>
<td>Forward: CGACGCTGAGGAGACCGT Reverse: GTTCCACGGTCCCGGTTCGTC</td>
<td>TaPPO-2</td>
<td>60</td>
<td>Chang et al., 2007</td>
</tr>
<tr>
<td>WP3-1</td>
<td>Forward: ACTGCCCCAGGGCCGACCT Reverse: CCAGTAGACGTGGCCGAGGAT</td>
<td>TaPPO-3</td>
<td>64</td>
<td>Chang et al., 2007</td>
</tr>
<tr>
<td>WP3-2</td>
<td>Forward: AGGTTCACGTCTACTTCCAC Reverse: CCGCCGAGAAGAGTTGC</td>
<td>TaPPO-3</td>
<td>58</td>
<td>Chang et al., 2007</td>
</tr>
</tbody>
</table>
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 TaPPO-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, PPO30 and WP2-2 produced differential banding pattern with high and low PPO cultivars. The number in parentheses indicates PPO activity.
Fig. 2. Dendrogram based on UPGMA analysis of genetic similarity of different genotypes from molecular marker data. **Cluster A**: 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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References


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