RAPID COMMUNICATION

Evaluation of Wheat Polyphenol Oxidase Genes

Aravind K. Jukanti,[1] Phil L. Bruckner,[1] and Andreas M. Fischer[1,2]

Cereal Chem. 81(4):481–485

ABSTRACT

Polyphenol oxidases (PPOs) present in mature wheat (*Triticum aestivum* L.) kernels have been implicated in the undesirable darkening of cereal products such as Asian noodles. To accelerate the functional characterization of wheat PPOs and allow the identification of those PPO genes that are primarily involved in food biochemistry, several basic local alignment search tool (BLAST) searches of expressed sequence tag (EST) databases were performed using a known wheat PPO sequence as a search argument; identified ESTs were resequenced and aligned. Results from this study suggest the presence of at least six PPO genes in hexaploid wheat, falling into two clusters with three similar sequences each. Based on the tissues used for cDNA library preparation, three genes (all members of one cluster) are expressed during kernel development and may therefore influence cereal product quality; the remaining three genes (belonging to the second cluster) were isolated from nonkernel cDNA libraries and may not be expressed at high levels during grain development. Discovery of these genes represents an essential first step in the functional characterization of wheat PPOs.

Polyphenol oxidases (PPOs) are ubiquitous, copper-containing metalloproteins that have been found or characterized in numerous higher plants (Flurkey 1989; Cary et al 1992; Dry and Robinson 1994; Hind et al 1995; van Gelder et al 1997; Constabel et al 2000; Demeke and Morris 2002). These enzymes catalyze the hydroxylation of *o*-monophenols to *o*-diphenols (E.C. 1.14.18.1; monophenol mono-oxygenase, tyrosinase or cresolase activity) and the oxidation of *o*-dihydroxyphenols to *o*-quinones (E.C. 1.10.3.2; diphenol oxidase or catecholase activity) (Steffens et al 1994; van Gelder et al 1997). In subsequent reactions, the quinones react with amines and thiol groups or undergo self-polymerization to produce dark/brown products that are usually undesirable in fresh or processed food (Feillet et al 2000; Anderson and Morris 2001). The central domain of all PPOs consists of two Cu-binding sites (CuA and CuB); at each of these sites, a Cu ion is bound by conserved histidine residues. This pair of Cu ions is the site of interaction of PPOs with both molecular oxygen and phenolic substrates (van Gelder et al 1997). When comparing PPO sequences from a wide variety of organisms, the Cu-binding regions are the most conserved domains of the polypeptide, with 42% sequence identity between the CuA and CuB regions of bacterial and human PPO (van Gelder et al 1997). Several plant PPOs analyzed so far are associated with plastidial thylakoid membranes and contain N-terminal transit peptides needed for plastid import (Hind et al 1995; Jiménez and García-Carmona 1996; van Gelder et al 1997). Typical transit peptides consist of 80–100 amino acids; peptide cleavage during chloroplast import reduces the molecular mass of several plant PPOs from 65–70 kDa to <60 kDa for the mature protein (Cary et al 1992; Dry and Robinson 1994; van Gelder et al 1997).

Evidence for the presence of several PPO isoforms or genes has been found in species for which a more detailed analysis was performed (e.g., wheat [*Triticum aestivum* L.], Kruger 1976; Demeke and Morris 2002; tomato [*Lycopersicon esculentum* L.], Thipyapong et al 1997; poplar [*Populus* spp.], Constabel et al 2000). At present, the physiological roles of these enzymes are not entirely clear, but available data point to a defense-related role of PPOs in higher plants (Constabel et al 1995, 2000; van Gelder et al 1997; Constabel and Ryan 1998; Mohammadi and Kazemi 2002). In this context, it is interesting that the highest PPO activities are usually associated with young tissues, which may be especially susceptible to challenges such as insect attack (Hunt et al 1993; Dry and Robinson 1994). From an applied point of view, PPOs have been implicated in enzymatic browning of vegetables, fruits, and cereal products (Feillet et al 2000; Nagai and Suzuki 2001). Specifically, it has been suggested that these enzymes play a major role in the darkening of noodles and other wheat products (Bhattacharya et al 1999; Feillet et al 2000). Because a bright color is usually preferred by consumers, especially for Asian noodles, it is one of the goals of many U.S. wheat breeding programs to develop and maintain cultivars with reasonably low PPO activities (and therefore higher commercial value) in mature grains. Interestingly, within the mature cereal grain, PPOs are mostly found in the aleurone layer, which is removed during the milling process; however, the remaining residual activity appears to be high enough to cause problems during food processing (Rani et al 2001). In spite of their commercial importance, the genetic, molecular and biochemical properties of wheat PPOs are not yet well understood. Data have been published on substrate preferences of (unpurified) wheat kernel PPOs (Anderson and Morris 2001). Additionally, it has been demonstrated that wheat kernel PPOs are activated to some extent by detergents and chaotropes (urea, guanidine.HCl) in crude extracts (Okot-Kotber et al 2002; Jukanti et al 2003). Different groups (Jiménez and Dubcovsky 1999; Demeke et al 2001) have also published data on the chromosome location of PPO genes or genes influencing PPO activity; these results may be useful for breeding and cultivar development. Biochemical (Kruger 1976; Anderson and Morris 2003) as well as molecular data (Demeke and Morris 2002) indicate the presence of several (3–12) PPO isoforms or genes in wheat, but only one (partial length) wheat PPO gene has been cloned so far (Demeke and Morris 2002), and one wheat kernel PPO was purified from bran of a high PPO cultivar (Anderson and Morris 2003). To accelerate the systematic functional characterization of wheat PPOs, especially of those involved in food biochemistry, the main goal of this research was the identification of all wheat PPO genes, utilizing bioinformatic tools and publicly accessible expressed sequence tag (EST) databases.

MATERIALS AND METHODS

To assess public databases for potential wheat PPO sequences, the sequence published by Demeke and Morris (2002; GenBank accession number AF507945) was utilized for a basic local align-

[1] Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717-3150.
[2] Corresponding author. Phone: 406-994-5908. Fax: 406-994-7600. E-mail: fischer@montana.edu

Publication no. C-2004-0519-02R.
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ment search tool (BLAST; Altschul et al 1990; http://www.ncbi.nlm.nih.gov/BLAST/) search against the National Center for Biotechnology Information (NCBI) nonhuman, nonmouse (i.e., including all plants) EST databases. All *Triticum* sequence "hits" were exported to the San Diego SuperComputer Center biology workbench (http://workbench.sdsc.edu/) for further analysis. Sequences were aligned using the CLUSTALW tool (Thompson et al 1994) either directly (not shown) or after translation to protein sequences (Fig. 1). To make sequence identities/similarities more easily visible, aligned sequences were shaded using the BOXSHADE tool (http://www.ch.embnet.org/software/ BOX_form.html). Phylogenetic relationships between sequences were calculated using PHYLIP (Felsenstein 1989) (Fig. 2).

All EST clones that could be obtained from the originating laboratories (GenBank accession numbers BQ839339, CA594200, CA716843, BQ246043, BQ239083, and BM136370) were resequenced using standard fluorescent chain-terminating dideoxynucleotides (full length of vector insert) (Prober et al 1987); deposited sequences were used for the previously published wheat PPO (Demeke and Morris 2002; GenBank accession AF507945) and for an EST clone with GenBank accession number CD935448.

RESULTS

To the best of our knowledge, only one wheat PPO DNA sequence has been published so far (Demeke and Morris 2002), and a few wheat PPO peptide sequences were obtained after purification and tryptic digestion of a kernel PPO (Anderson and Morris 2003). However, in the last two years, a large number of wheat EST sequences have been deposited by different labora-

Fig. 1. Sequence alignment of wheat PPO EST. EST sequences were identified through a NCBI BLAST search using GenBank accession #AF507945 (Demeke and Morris 2002) as search argument. Tentative PPO sequences were translated and aligned. Peptide sequences obtained after purification of a seed kernel PPO (Anderson and Morris 2003) are shown above the aligned sequences. Conserved copper binding regions (A and B [according to Demeke and Morris 2002]) are framed. Sequence #CD935448 was identical to #AF507945 but extended the 5'-end by 303 bp/101 N-terminal amino acids. The combined sequence is used as a reference sequence for this figure and labeled AF507945*. The start of the original sequence (AF507945) is indicated. Tentative N-terminus of mature polypeptides (obtained from alignment with other plant PPOs) is also shown.

tories. A detailed search of these resources as outlined above suggests the presence of a PPO multigene family with six members falling into two phylogenetic clusters in wheat (Figs. 1 and 2).

Utilizing the published sequence (Demeke and Morris 2002; GenBank accession number AF507945) as a search argument for a NCBI BLAST search, six “hits” with sequence identities of >60% in overlaps of at least 200 base pairs (bp) were obtained. Interestingly, among these, based on 100.0% sequence identity in a 260-bp overlap, one EST (GenBank accession number CD935448) was identical to the gene used as a search argument, but extended its sequence by 303 bp at the 5’-end. Based on sequence comparisons (not shown) of the combined sequence (from GenBank accession numbers AF507945 and CD935448) with PPO from potato (*Solanum tuberosum* L., Q06355), tomato (*Lycopersicon esculentum* L., Q08307), tobacco (*Nicotiana tabacum* L., CAA73103), fava bean (*Vicia faba* L., Q06215), poplar (*Populus* sp., AF263611), grapevine (*Vitis vinifera* L., P43311), and spinach (*Spinacia oleracea* L., P43310), this sequence represents the first wheat PPO cDNA that is almost (no N-terminal methionine residue) full-length, including a transit peptide (needed for plastid import). Consequently, this combined sequence (designated as AF507945* in Fig. 1) was used as a reference for comparisons with all other wheat PPOs, both at the cDNA and, after translation, at the protein level (Fig. 1). Based on sequence alignments with the other higher plant PPOs mentioned, the tentative start of the mature polypeptide chain (after cleavage of the transit peptide) is also shown in Fig. 1.

An EST with GenBank accession number CA594200 appears to represent a second, novel PPO gene. This clone was resequenced in our laboratory; it is 5’-truncated but contains a complete 3’-end, including a poly-A tail. This clone demonstrates ≈86% sequence identity with the reference sequence over its full length, including the 3’-untranslated region (UTR). A clone with accession number CA594195 may represent the same gene, but sequence quality is poor; additional controls by the authors of this sequence indicated potential problems with this clone (S. Tingey, DuPont, *personal communication*).

A third interesting clone has accession number BQ839339, with 84.6% sequence identity to the reference sequence in a 610-bp overlap. This clone was also resequenced in our laboratory; while it is, most likely, still 5’-truncated (missing part of the sequence information corresponding to a tentative transit peptide), it is the longest of all currently available wheat PPO ESTs/cDNAs (Fig. 1). This clone may represent a third wheat PPO gene, closely related to both the reference sequence and to clone CA594200.

A clone with accession number CA716843 clearly represents an additional wheat PPO gene. This clone was resequenced in our laboratory; it demonstrated ≈56% sequence identity with the reference sequence over its full length (it is 5’-truncated but contains a complete 3’-UTR). While this gene was more different from the reference sequence than those mentioned above, it still appears to be a PPO gene, based on sequence comparison at and around the Cu-binding regions (Fig. 1). Furthermore, Anderson and Morris (2003) reported several peptide sequences obtained after purification, tryptic digestion, and microsequencing of one kernel PPO, and sequences of peptides #3 and 5 are exact matches with CA716843; one amino acid was different in peptide #4 (Fig. 1). Based on this finding, and on the fact that this EST was isolated from a cDNA library prepared from developing kernels at 14 days past anthesis, clone CA716843 most likely encodes a PPO actively expressed in developing kernels.

A last, tentative wheat PPO EST from this first BLAST search has GenBank accession number BQ239083. Again, this clone was resequenced in our laboratory. Comparably to CA716843, it demonstrates ≈55% sequence identity with the reference sequence but is closely related to CA716843. As this clone was also isolated from a kernel cDNA library (mRNA isolated at 5 days post anthesis), it may represent a second wheat kernel PPO.

Research in our laboratory strives to understand the functional role of kernel PPOs in the discoloration of wheat products. Because results from the laboratory of C. Morris (Demeke and Morris 2002; Anderson and Morris 2003) indicate that several PPOs might be present in developing kernels, two more BLAST searches were performed using the tentative kernel PPOs (clones CA716843 and BQ239083) as search arguments. Four additional sequences (not discovered in the first search, with >80% sequence identity in >100-bp overlaps) were found by this approach. Two of these (GenBank accession numbers BQ246043 and BM136370) also showed significant sequence identity with the original reference sequence. The others, while clearly related to BQ239083, are more distant from the original reference sequence and may not represent functional PPO genes. Resequencing of BQ246043 in our laboratory (again including the 3’-UTR) demonstrated that this clone is identical to CA716843 (cDNAs derived from the same gene). On the other hand, resequencing clone BM136370 suggested that this EST is derived from an additional wheat PPO gene that is different from all others mentioned above, but closely related to CA716843 and BQ239083 (Fig. 1). This EST was isolated from a cDNA library prepared from developing spikes.

All PPO sequences have been visualized after translation and alignment (Fig. 1). As this figure suggests the presence of two distinct sequence groups, phylogenetic relationships between the six wheat PPOs were determined using PHYLIP (Felsenstein 1989). This analysis (Fig. 2) confirms that known wheat PPOs fall into two distinct clusters of three sequences each, and that members of each cluster are closely related.

Novel sequence information obtained from this research (full-length sequencing of EST clones) has been submitted to GenBank. New GenBank accession numbers AY596266–AY596270 correspond to EST clones BQ839339, CA594200, CA716843, BQ239083, and BM136370 (in this order). Additionally, a sequence with accession number AY515506 has been submitted by the laboratory of J. Anderson (USDA, Fargo, ND) to GenBank while this publi-

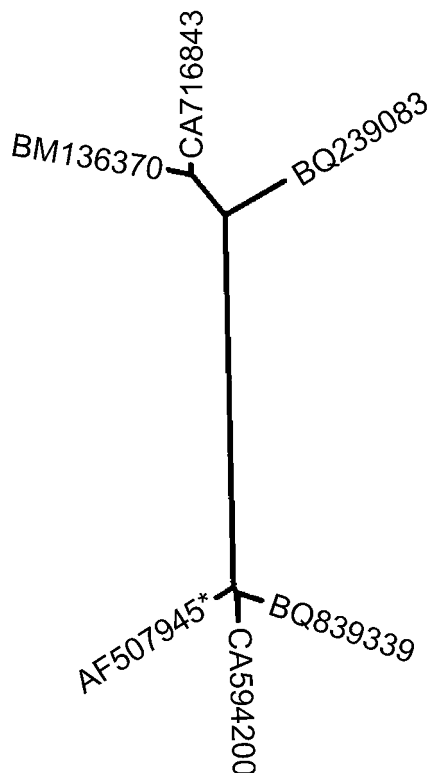

Fig. 2. Phylogenetic relationships of wheat PPOs shown in Fig. 1.

cation was under review; it appears to be derived from the same gene as EST clone BM136370 (new accession number AY596270) utilized for this manuscript, but contains additional 5'-sequence information.

DISCUSSION

Higher plant PPOs have been intensely studied during the last few years, both because of their potential roles in defense against pathogens and insects (Constabel et al 1995; Mohammadi and Kazemi 2002) and because of their involvement in postharvest food biochemistry (Feillet et al 2000; Nagai and Suzuki 2001). Specifically, wheat PPOs have been investigated by a number of research groups due to their role in the undesirable darkening of Asian noodles produced from high-PPO germplasm (Feillet et al 2000; Anderson and Morris 2001). Investigating the biological basis of the high- vs. low-PPO activity trait in mature kernels of established or new wheat lines (as needed for cultivar improvement) has proven difficult, mostly due to a lack of information on the number of PPO genes actively expressed during grain development.

Based on the analysis of publicly accessible EST data, our research has identified six different wheat PPO DNA sequences, falling into two clusters with three similar sequences each (Figs. 1 and 2). As these ESTs were, in part, isolated from different wheat cultivars, some of these differences might be due to allelic variation. However, the following points strongly argue against this interpretation.

1) Southern blots performed by Demeke and Morris (2002) on DNA extracted from the wheat cultivar Penawawa using a probe derived from GenBank accession AF507945 detected 2–4 bands (depending on the restriction enzymes used for DNA digestion). Three bands each were detected in DNA from a number of additional wheat cultivars digested with *Hind*III, but some differences were detected in the banding pattern between low- and high-PPO cultivars. Considering the sequence differences shown in Fig. 1, it is reasonable to assume that the probe used in this study detected all members of the lower but not of the upper cluster presented in Fig. 2, suggesting that this cluster contains three (or possibly more) different sequences (i.e., genes) in each cultivar.

2) Two EST situated in the upper cluster in Fig. 2 (BQ246043 and BQ239083) were isolated from cDNA libraries prepared from the same cultivar (Glenlea). BQ246043 is not shown in Fig. 1, as it is essentially identical to clone CA716843. This suggests the presence of at least two different PPO sequences (i.e., genes) within this cultivar.

3) Careful comparative analysis of all sequences presented in this manuscript at the DNA level, both in the coding region and 3'-UTR (not shown, but accessible through GenBank) indicates too important differences between sequences in each of the two clusters to be explained by allelic variation. Additionally, it has to be kept in mind that PPOs are ubiquitous enzymes; it is reasonable to assume that such genes are present on each of the three (A, B, and D) genomes of hexaploid wheat. In this context, Demeke et al (2001) found quantitative trait loci (QTL) influencing PPO activity on chromosomes 2A, 2B, 3D, and 6B. Therefore, our data are best explained by postulating a PPO multigene family with at least six members for hexaploid wheat.

Considering the tissues from which RNA for cDNA library preparation was extracted, it appears that three genes (members of the upper cluster in Fig. 2 GenBank accession numbers CA716843, BQ239083, and BM136370) are expressed at some point during kernel development, and may therefore be important for food biochemistry. Members of the lower cluster (AF507945, BQ839339, and CA594200) were isolated from other tissues and were not present in kernel cDNA libraries. Therefore, these genes are probably not expressed at high levels during kernel development and are not likely to contribute to undesirable food darkening processes. Interestingly, an affinity-purified antipeptide antibody developed in our laboratory against the sequence GTAGRDPVFYSHHAN (derived from the CuB-binding region of sequence AF507945, but identical in BQ839339 and CA594200) (compare Fig. 1) reacted only weakly with proteins extracted from bran of the high-PPO winter wheat cultivar Rampart (data not shown), again suggesting that these PPOs are not present, or only present at low levels, in kernels. Additionally, the PPO isoform purified by Anderson and Morris (2003) clearly belongs to the first cluster, as demonstrated by the alignment of obtained peptide sequences with translated EST in Fig. 1. The single amino acid difference between the peptide #4 sequence obtained by these authors and clones CA716843, BQ239083, and BM136370 (serine instead of glycine in the penultimate position of the peptide) appears intriguing. Considering the fact that the sequences of peptides #3 and 5 are in complete agreement with the sequence of clone CA716843, this might be explained by allelic variation in this gene. However, at this point, the presence of an additional wheat PPO, which would be very closely related to clone CA716843, cannot be excluded. Among other higher plant PPOs, serine residues (instead of alanine or glycine) are present in this position in genes from potato (*Solanum tuberosum* L., U22921), fava bean (*Vicia fava* L., Q06215), poplar (*Populus* sp., AF263611), and spinach (*Spinacia oleracea* L., P43310).

Data presented in this manuscript represent a necessary and essential first step toward the systematic functional characterization of wheat PPOs. They will allow the identification of those PPO genes that are principally involved in food biochemical processes such as darkening of Asian noodle preparations. At the same time, this data set will also initiate research leading to an improved understanding of the (potential) role of wheat PPOs in defense against pests or pathogens. The development of new germplasm, improving end product quality without making new cultivars more susceptible to known insects or diseases, will be the final goal of these efforts. To work toward this goal, isolation of full-length cDNA and genomic clones, studies of expression patterns of the different genes in developing kernels of low- and high-PPO germplasm, and subcloning into appropriate vector systems for protein expression (allowing antibody production and biochemical analysis) have been initiated. Additionally, using nulli-tetrasomic lines (Sears 1954), it will be possible to determine on which chromosome of the A, B, and D genomes the different genes are present. Comparison of these data with previous mapping efforts (Jiménez and Dubcovsky 1999; Demeke et al 2001) will yield additional data for the identification of those genes associated with the high-PPO activity trait.

CONCLUSIONS

Data presented in this manuscript suggest for the first time that wheat PPOs are organized in a multigene family with at least six members, falling into two clusters with three similar sequences each. Members of one cluster are expressed in developing kernels and may therefore be involved in undesirable food darkening processes.

ACKNOWLEDGMENTS

We would like to thank Sylvie Cloutier (Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, Canada), Scott Tingey (DuPont, Wilmington, DE) and Olin Anderson (USDA, Albany, CA) for making available wheat EST clones for this study.

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[Received February 17, 2004. Accepted April 19, 2004.]