RESEARCH PAPER

Duplicate polyphenol oxidase genes on barley chromosome 2H and their functional differentiation in the phenol reaction of spikes and grains

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Abstract

Polyphenol oxidases (PPOs) are copper-containing metalloenzymes encoded in the nucleus and transported into the plastids. Reportedly, PPOs cause time-dependent discoloration (browning) of end-products of wheat and barley, which impairs their appearance quality. For this study, two barley PPO homologues were amplified using PCR with a primer pair designed in the copper binding domains of the wheat PPO genes. The full-lengths of the respective PPO genes were cloned using a BAC library, inverse-PCR, and 3’-RACE. Linkage analysis showed that the polymorphisms in PPO1 and PPO2 co-segregated with the phenol reaction phenotype of awns. Subsequent RT-PCR experiments showed that PPO1 was expressed in hulls and awns, and that PPO2 was expressed in the caryopses. Allelic variation of PPO1 and PPO2 was analysed in 51 barley accessions with the negative phenol reaction of awns. In PPO1, amino acid substitutions of five types affecting functionally important motif(s) or C-terminal region(s) were identified in 40 of the 51 accessions tested. In PPO2, only one mutant allele with a precocious stop codon resulting from an 8 bp insertion in the first exon was found in three of the 51 accessions tested. These observations demonstrate that PPO1 is the major determinant controlling the phenol reaction of awns. Comparisons of PPO1 single mutants and the PPO1PPO2 double mutant indicate that PPO2 controls the phenol reaction in the crease on the ventral side of caryopses. An insertion of a hAT-family transposon in the promoter region of PPO2 may be responsible for different expression patterns of the duplicate PPO genes in barley.

Key words: Gene duplication, grasses, Hordeum vulgare, mutant, phenol reaction, PPO.

Introduction

Polyphenol oxidases (PPOs) or tyrosinases catalyse oxidation of various phenolic substrates to quinones and initiate enzymatic browning in plant tissues. Although their role in metabolism remains largely unknown, PPOs are implicated in defence against insects and plant pathogens. In fact, PPOs are encoded by nuclear genes, but the enzyme is localized in the thylakoids of plastids without being an integral membrane protein. The enzyme is separated spatially from its phenolic substrates, which are located in the vacuole. The browning reaction therefore occurs only in the event that tissue damage degrades subcellular compartmentation. Plant PPO genes encode bi-copper metalloenzymes that possess two conserved copper-binding domains, CuA and CuB; at each site, a Cu ion is bound by conserved histidine residues. This pair of Cu ions is the site of interaction of PPOs with both oxygen molecule and phenolic substrates (Vaughn et al., 1988; van Gelder et al., 1997; Yoruk and Marshall, 2003; Marusek et al., 2006; Mayer, 2006).

To date, full-length sequences of PPO genes have been isolated in several dicotyledonous plant species including broad bean (Cary et al., 1992), potato (Hunt et al., 1993),...
The objective of this study was to isolate barley PPO genes using wheat PPO sequence information (Himi and Noda, 2004) because of their association with the quality of flour products (Demeke and Morris, 2002; Raman et al., 2005; Anderson et al., 2006; He et al., 2007; Massa et al., 2007). In addition, PPOs play an important role in the browning and discoloration of pasta (Simeone et al., 2002), steamed bread (Dexter et al., 1984), and Asian noodles (Fuerst et al., 2006). In rice, a PPO gene was recently proved to be responsible for the phenol reaction phenotype of grains and hulls, which is a diagnostic characteristic differentiating two subspecies; the phenol reaction is negative in all japonica subspecies lines, but it is positive in nearly all indica and wild progenitor lines. Three independent origins of negative phenol-reaction mutations were clarified in japonica rice (Yu et al., 2008). In barley, Takeda and Chang (1996) reported similar varietal differences in the phenol reaction of spikes and grains. Then they selected 51 (0.6%) phenol reaction-negative accessions from a screening of 8849 world collections. Phenol reaction-negative type is rare in barley, but is distributed in wide areas along the Silk Road, an ancient trade route, ranging from Spain through the Middle East to Korea. Although Takeda and Chang (1996) mapped the phenol reaction (Phr) gene to the long arm of barley chromosome 2H, molecular identity of the gene controlling the phenol reaction in awns remains unclarified.

The objective of this study was to isolate barley PPO genes using wheat PPO sequence information (Himi and Noda, 2004) because of their association with the quality of flour products (Quinde-Axell and Baik, 2006) and putative involvement in the phenol reaction phenotype. Molecular cloning of two linked PPO genes from barley is described. The second objective was to clarify the role of the duplicate PPO genes of barley in the phenol reaction in spikes and grains. For this purpose, comparative phylogenetic analyses among grass species and analysis of phenol-reaction-negative mutations were carried out.

Materials and methods

Plant materials

For initial PCR amplification of partial sequences of the barley PPO genes, three barley cultivars were used as a template DNA source: Haruna Nijo (abbreviated hereafter as HA2, phenol reaction positive), Kodoku Shirazu (code I239, phenol reaction positive), Shogor 3 (I677, phenol reaction negative). Fifty-one barley accessions of negative phenol reaction in awns (Takeda and Chang, 1996) were obtained from Research Institute for Bioresources, Okayama University, Japan and used for allelic diversity studies of PPO genes. For gene expression analyses, an isogenic line of the Bowman barley cultivar carrying the naked Caryopsis gene (hereafter designated as nud-Bowman) was used. This line is phenol reaction positive. Representative phenol reaction-negative accessions were backcrossed with nud-Bowman, and near-isogenic lines (NILs) with regard to PPO1 variant alleles were developed following one or two backcrosses (BC1 or BC2). During backcrosses, plants that were homozygous for the recessive naked (nud) gene were selected using marker-assisted selection according to Taketa et al. (2008). The NILs with the nud gene allowed evaluation of PPO enzyme activity assay using whole naked caryopses because grains are easily separated from the hulls.

Cloning of barley PPO genes

For the amplification of PPO homologues from barley, a pair of wheat PPO primers (deg1LP and degRP) were used; the primers were designed on the conserved copper binding domains based on the registered sequences of three seed-specific PPO genes from the respective wheat genomes (A1, AB254803.1; B1, AB254804.1; and D1, AB254805.1; Himi and Noda, 2004). PCR-amplified bands were recovered individually from gels using a gel extraction kit (QIA quick; Qiagen Inc.) and cloned using a cloning kit (TOPO TA; Invitrogen Corporation). The bacterial artificial chromosome (BAC) library of HA2 (Saisho et al., 2007) was screened for clones containing PPO genes using a high-density replica membrane and an ECL kit (GE Healthcare, Buckinghamshire, UK). The BAC DNA extraction and pulsed field gel electrophoresis (PFGE) was done according to standard protocols. Inverse PCR was performed to isolate entire genomic DNA sequences of the PPO genes according to Ochman et al. (1988). PCR primer sequences are listed in Supplementary Table S1 at JXB online. The PPO sequences reported in this paper have been deposited in the EMBL/GenBank/DDBJ databases [accession no. AB549330 (PPO1) and AB549331 (PPO2)]. For genomic Southern hybridization, total DNA (10 μg) from HA2 was digested with BgIII, HindIII, or XhoI, electrophoresed in 0.7% agarose gels, transferred to a positively charged nylon membrane, and hybridized with a digoxigenin (Roche)-labelled probe synthesized from a full-length cDNA clone of barley PPO1. Washing and immunological detection was performed following the manufacturer's instructions.

Mapping

DNA was isolated according to the standard SDS-phenol method. Wheat (cv. Chinese Spring)–barley (cv. Betzes) disomic and ditelosomic chromosome addition lines (Islam et al., 1981; Islam, 1983) were used for chromosome arm assignment of the PPO genes. For molecular mapping of the PPO genes, 111 F2 plants derived from a cross between HA2 and Shogor 3 were used. An additional 171 F2 plants from the same cross combination were examined only for PPO1 and PPO2 markers and the phenol reaction. Molecular markers for barley chromosome 2H reported by Liu et al. (1996), Komatsuda et al. (1998), and Ramsay et al. (2000) were used.

Phenotyping for the phenol reaction

For mapping, the phenol reaction was phenotyped in awns by staining with a 1% phenol solution because the reaction was sharpest (Takeda and Chang, 1996). In the NILs and their parental accessions, the phenol reaction of various tissues, such as awns, hulls, grains, and rachises, was tested at maturity. The phenol reaction of grains was evaluated by placing samples in a Petri dish on the filter paper moistened with a 1% phenol solution for 24 h.

cDNA cloning and expression analysis

Tissues for RNA extraction were collected from nud-Bowman plants grown in a growth cabinet with a constant temperature of 15 °C and natural sunlight. Individual spikes were tagged daily to mark the flowering date, and total RNA was extracted from hulls (including palea and glume), caryopses, flag leaf, and awns, at 7 d intervals for 3 weeks starting from the date of flowering. For caryopses, endosperm parts were removed by pinching with
Phylogenetic analysis

The deduced amino acid sequences for PPO1 and PPO2 in barley are compared with PPO proteins of wheat as well as those of four grass species having known genome sequences (rice, maize, sorghum, and Brachypodium distachyon). For analysis, partial peptide sequences corresponding to motifs 1, 4, 10, 3, 7, and 2 were extracted and aligned using SALAD (Mihara et al., 2010). Motifs used for tree construction were extracted from the following 19 sequences: barley (AB549330, AB549331), wheat (AB254803.1, AB254804.1, AB254805.1, EF070147.1, EF070148.1, EF070149.1, and EF070150.1), rice (LOC_Os04g53300 and LOC_Os01g58100), maize (EU956830.1 and EU963699.1), sorghum (Sb06g025570, Sb06g025580, Sb03g035850, Sb03g036870, and Sb10g024220), and B. distachyon (Brad1g252260). The evolutionary history was inferred using the Neighbor–Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed, according to Felsenstein (1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch length in the same units as the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). In all, the final dataset included 194 positions. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Allelic diversity of PPO1 and PPO2 genes

For sequencing of the PPO1 and PPO2 genes, 10 accessions (7 phenol reaction negative and 3 phenol reaction positive) were selected based on their geographical distribution and morphological characteristics. The sequenced accessions were I677, I750, K095, I341, U004, C2-60, C1-76, HA2, F11 (Bowman), and J239, of which the last three are phenol reaction positive and the others are phenol reaction negative. For these 10 accessions, genomic sequences from the start codon to stop codon were determined for both PPO genes. The DNA sequence was determined using the ABI310 sequencer, and both strands were sequenced. Phylogenetic trees based on nucleotide sequences of PPO1 and PPO2 from start to stop codon were constructed using the Neighbor–Joining method (Saitou and Nei, 1987). Based on DNA sequences of PPO1 and PPO2 alleles, polymorphic markers were developed. These markers allow genotyping of each allele specifically as CAPS or dCAPS markers. The primers and restriction enzymes used are summarized in Supplementary Table S1 at JXB online.

PPO enzyme activity assay

The PPO enzyme activity of whole grains in nud-Bowman and its NILs for five mutant PPO1 alleles was measured according to Anderson and Morris (2001). 3,4-Dihydroxy-L-phenylalanine (L-DOPA) was used as a substrate; absorbance at 475 nm was recorded. Experiments were conducted in six replications and each replication contained five grains. Data were analysed statistically using t-tests.

Results

Molecular cloning and mapping of barley PPO homologues

Using a wheat PPO primer pair, PCR amplified two bands in all three barley accessions that were tested (Fig. 1A). A stronger band of about 700 bp was designated as PPO1; a weaker band of about 800 bp was named PPO2. After cloning and sequencing, primer pairs that specifically amplify either PPO1 or PPO2 and detect polymorphisms by restriction enzyme digestion were designed. Mapping using wheat–barley chromosome addition lines and molecular markers showed that both PPO1 and PPO2 were located on the long arm of chromosome 2H (see Supplementary Fig. S1 at JXB online) and that no recombination among PPO1, PPO2, and the phenol reaction phenotype were found in 111 F2 plants (Fig. 1B). An additional 171 F2 plants were tested, but no recombination was found among them. Consequently, in 282 F2 plants, PPO1 and PPO2 co-segregated with Phr. It is therefore suggested that the
phenol reaction of awns is likely to be controlled by either 
PPO1 or PPO2.

**Gene structure of PPO1 and PPO2 in barley**

To recover entire PPO sequences from barley, BAC clones containing the PCR-amplified PPO1 and PPO2 fragments were screened from the HA2 BAC library. No BAC clone carrying both PPO1 and PPO2 fragments was identified. One BAC clone each containing either PPO1 (clone name HNB164B24) or PPO2 (HNB630O21) was selected and analysed in detail. The BAC clones differed considerably in PFGE patterns after digestion with three eight-base cutters (Fig. 2A). The insert sizes of PPO1-containing and PPO2-containing BAC clones were estimated, respectively, as about 110 kb and 120 kb. Using primer pairs designed based on BAC-end sequences of the respective clones, PCR showed that the PPO1-containing and PPO2-containing BAC clones did not mutually overlap (data not shown). These observations indicate that PPO1 and PPO2 genes are physically separated on the 2HL.

To select restriction enzymes suitable for cloning of the full-length PPO genes by inverse PCR, BAC DNA was digested using various six-base cutters and analysed using Southern hybridization. Actually, KpnI was selected for PPO1 (Fig. 2B), and XhoI was selected for PPO2 (data not shown) because these enzymes showed a single band in BAC DNA Southern hybridization using respective PPO gene fragments as a probe. In the case of PPO1, clones with the expected 6 kb insert size were selected using colony PCR. Unfortunately, sequencing to the direction of 3’-UTR region revealed a precocious KpnI site. Thus, the 3’-UTR region of the PPO1 gene is not fully recovered in the clones obtained using inverse PCR. To recover the entire PPO1 coding sequence, 3’-RACE was performed. The 3’-UTR of the PPO1 was 162 bp long; a typical polyadenylation signal (AATAAA) was found. In PPO2, the expected 4.5 kb insert size was recovered. The 4.5 kb clones covered the entire PPO2 gene; 3’-RACE showed that the length of the 3’-UTR was 177 bp without a canonical polyadenylation signal. About 3.6 kb of the sequence upstream from the PPO2 translation start codon was also obtained by ‘walking’ along the BAC clone. Genomic Southern hybridization using a full-length cDNA clone of PPO1 as a probe detected two bands for BglII or HindIII digest and three bands for XhoI digest (data not shown), confirming the presence of at least two PPO genes in the barley genome.

To know the intron–exon structure, genomic sequences and cDNA sequences were compared. Figure 3 shows the gene structure of PPO1 and PPO2. Both PPO genes harbour two introns, one within the CuA domain and the other between CuA and CuB. The PPO1 gene is 1946 bp long and has two introns of 96 bp and 122 bp. The PPO2 gene is 2026 bp long and has two introns of 205 bp and 90 bp. The thylakoid translocation domain and two copper binding domains are highly conserved in both genes (see Supplementary Fig. S2 at JXB online). Predicted amino acid lengths are 575 aa for PPO1 and 576 aa for PPO2. Apparently, PPO1 and PPO2 encode 64.1 kDa and 62.8 kDa precursor proteins, respectively, and those are processed into mature PPO of 55.8 kDa and 54.0 kDa after removal of the amino-terminal signal peptide. The sequence identity between PPO1 and PPO2 was 82% at the nucleotide level and 73% at the amino acid level. Similar gene structure and high sequence identity suggest that PPO1 and PPO2 are duplicate genes.

**Phylogenetic analysis of PPO in grasses**

Phylogenetic analysis was performed based on predicted amino acid sequences of the PPO genes from six grass species (Fig. 4A). Two barley PPO protein sequences from

![Fig. 2. Analysis of barley BAC clones containing either the PPO1 or the PPO2 gene. (A) After digestion with eight-base cutters, BAC DNA was separated by pulsed-field gel electrophoresis. (B) BAC DNA containing the PPO1 gene was digested with various six-base cutters and separated using electrophoresis on a 1% agarose gel (left). Southern blot was probed with the PPO1 fragment (right). M1: DNA molecular weight marker III (Roche). M2: λ HindIII digest.](image-url)
were selected and used for analyses (Fig. 4B). Accession with a common central domain of tyrosinase (Pfam00264) using the SALAD database, only PPO peptide sequences selected through a database search. Based on motif analysis (wheat, rice, maize, sorghum, and the deduced PPO amino acid sequences of five other species cultivar HA2 (HvPPO1 and HvPPO2) were compared with the barley and wheat clade. It remains unknown whether this study represent true PPO validate that the two barley

Allelic diversity of PPO1 and PPO2 genes

To know which of PPO1 or PPO2 is responsible for the phenol reaction phenotype in awns, seven phenol reaction-negative (I677, I750, K095, I341, U004, C2-60, and C1-76) and three phenol reaction-positive (HA2, FI1, and J239) accessions of diverse geographic origin were sequenced for both PPO1 and PPO2 genes. Seven alleles in PPO1 and four alleles in PPO2 were found (see Supplementary Tables S2 and S3 at JXB online). In PPO1, HA2 and FI1 (both phenol reaction positive) had an identical sequence, and another phenol reaction-positive accession, J239, had a sequence identical to those of two phenol reaction-negative accessions (C2-60 or C1-76). However, the remaining five phenol reaction-negative accessions had nucleotide changes that are predicted to cause an amino acid substitution(s) or premature stop codon affecting functionally important motifs compared with that of the standard accession HA2 (Fig. 3). In fact, I341 (type I) had a single amino acid substitution within the thylakoid translocation domain. Also, U004 (type II), I677 (type III), and K095 (type V) had a premature stop codon at amino acid positions 161, 248, and 528, respectively; I750 (type IV) had a single amino acid substitution within a copper binding domain (CuB). Types I–IV are estimated to affect either or both of the copper binding domains of the PPO1 gene, whereas type V had a truncated carboxyl terminus. Some PPO studies of grape have shown that the C-terminal extension must be removed for activation (Dry and Robinson, 1994); thereby, type V barley is likely to be affected in PPO activity. On the other hand, in PPO2, predicted amino acid sequences of three types were found, but only one type was specifically found in the phenol reaction-negative accessions tested. Compared with the standard accession HA2, I677 had an 8 bp insertion in the first exon, causing a frame shift and a premature stop codon at amino acid position 147. The 8 bp insertion in PPO2 was found in three out of 51 phenol reaction-negative accessions. A single amino acid deletion of the 125th alanine (A) as a result of an in-frame 3 bp deletion in the first exon was found both in phenol reaction-negative accessions (I750, K095, C2-60, and C1-76) and in a phenol reaction-positive accession (J239), suggesting that the missing amino acid residue 125A is dispensable for PPO2 function. Available results indicate that PPO1, rather than PPO2, controls the phenol reaction in awns.

Neighbor-Joining trees were constructed based on PPO1 and PPO2 nucleotide sequences including both exons and introns in 10 accessions (Fig. 5). In PPO1, five phenol reaction-negative accessions (types I–V) occupied unique positions separated from the three phenol reaction-positive accessions. In the PPO2 gene, ambiguous separation of phenol reaction-positive and reaction-negative accessions was observed, which also supports the conclusion that PPO1 is the major determinant of the phenol reaction in awns. Although I677 (a type III Pakistani accession) and U004 (a type II Spanish accession) share the short-haired rachilla trait in common, they are distantly related.

The PCR primers that specifically distinguish mutant alleles in the PPO1 locus were designed; all 51 phenol
reaction-negative accessions were genotyped. Figure 6 portrays the geographical distribution of mutant \textit{PPO1} genes that were found in domesticated barley accessions with a negative phenol-reaction of awns. Of 51, 40 accessions were classified into one of the five non-functional types found by sequencing. However, the remaining 11 accessions were indistinguishable from phenol reaction-positive accessions by marker analyses and are classified as others. These unclassified accessions, which were collected in India, China, and Nepal, require further sequencing in the promoter region. Type IV was most prevalent and was distributed in wide areas ranging from Turkey, Iran, and Afghanistan, to Xinjiang. This distribution pattern indicates that type IV was disseminated by human activities along the Silk Road. Types I and III were locally distributed in Pakistan; type I accessions are all naked caryopsis (\textit{nud}), and type III accessions all have short rachilla hair (\textit{srh}). Type II was only found in Spain and this type carried short rachilla hair (\textit{srh}). Type V was found only in Korea. These results show that loss-of-function mutations of the \textit{PPO1} gene occurred independently as barley spread from the two centres of origin, namely the Near East and southern areas.
Expression analysis

Results show that \( PPO1 \) is most strongly expressed in awns followed by in hulls, but \( PPO2 \) is weakly expressed in caryopses; expression of \( PPO1 \) in awns and hulls peaked at 2–3 weeks after flowering, but the peak of \( PPO2 \) expression in caryopses occurred at 2 weeks after flowering (Fig. 7). This expression pattern coincides with the present conclusion—based on allele diversity analysis—that \( PPO1 \) is responsible for the phenol reaction of awns. In the proximity of the \( PPO2 \) promoter region, an insertion of a hAT-family transposon was found (Fig. 3). Such a transposon insertion might be responsible for the differential expression patterns of \( PPO1 \) and \( PPO2 \) genes.

The phenol reaction of the representative phenol reaction-negative barley accessions and NILs were tested. The results showed that \( PPO1 \) mutants are not stained in awns (Fig. 8A), grains (Fig. 8B), hulls or rachises, although these tissues are strongly stained in the phenol reaction-positive control. It is particularly interesting that a closer examination of grains revealed that the \( PPO1 \)PPO2 double mutant (type III) showed no staining in the crease on the ventral side of grains, although other \( PPO1 \) single mutants and the control show strong coloration in the crease (Fig. 8B). This phenol reaction pattern suggests that \( PPO2 \) expression is localized to the crease of grains, whereas \( PPO1 \) appears to govern the phenol reaction of overall spike tissues including awns, hulls (lemma and palea), rachises, and caryopsis surface except for the crease on the ventral side.

The PPO activity assay in whole grains showed that NILs of the five \( PPO \) mutant alleles had a reduced level of PPO activity compared with the control (Fig. 9). The NIL of the...
**Discussion**

**Allele diversity of PPO genes, and phenol reaction phenotypes in barley**

In this study, duplicate barley PPO genes that are closely linked on the long arm of chromosome 2H were isolated. Both PPO1 and PPO2 co-segregated with Phr controlling the phenol reaction of the awns. Allele variation in PPO1 was closely associated with the phenol reaction of the awns: in 40 (78.4%) out of 51 barley accessions having negative phenol reaction of awns, five amino acid variants affecting functionally relevant motif(s) or C-terminal region(s) were found. Such a strong association was not found for PPO2. Thus, the available results indicated that PPO1, rather than PPO2, is the major determinant controlling the phenol reaction of awns. PPO1 also appears to control the phenol reaction of hulls, caryopses, and rachises because these parts were stained in normal barley but were not stained in PPO1 single mutants. In the PPO1PPO2 double mutant, staining of the crease on the ventral side of caryopses was absent. This indicates that PPO1 controls the phenol reaction of the crease of caryopses. Subsequent RT-PCR experiments revealed that PPO1 is expressed in awns and hulls, and that PPO2 was weakly expressed only in caryopses among the tissues tested. Consequently, the observed pattern of phenol staining of caryopses is slightly contradictory to the gene expression pattern. This contradiction might be explained by assuming that PPO1 is also weakly expressed in caryopses under the detection limit by RT-PCR and that PPO2 is expressed specifically in the increase of the ventral side of caryopses.

The present study revealed that two genes (PPO1 and PPO2) are responsible for the phenol reaction phenotypes in barley. This is in sharp contrast to the case in rice, where Phr1 on syntenic rice chromosome 4 is the sole PPO gene controlling the phenol reaction in hulls and grains (Yu et al., 2008).

**Phylogenetic analysis of PPO in grasses**

The phylogenetic tree shows that, in the grass family, two groups of PPO genes exist (Fig. 4). The two barley PPO genes on 2HL isolated here were included in the first group (group A in Fig. 4A) together with wheat PPO genes on homoeologous group 2 chromosomes (Himi and Noda, 2004; He et al., 2007) and rice chromosome 4 PPO gene (Phr1) (Yu et al., 2008). Triticeae group 2 chromosomes and rice chromosome 4 are known to be syntenic (Devos and Gale, 2000), and therefore an orthologous relationship is suggested. Another active PPO gene on rice chromosome 1 (Yu et al., 2008) was included in the second group (group B). Presumably the rice chromosome 1 PPO gene is functionally differentiated from the rice chromosome 4 PPO gene because the former is predominantly expressed in vegetative organs such as the leaf blade and leaf sheath; the latter is strongly expressed in panicles (Y Nagamura et al., personal communication). Based on phylogenetic analysis of partial PPO sequences of wheat, Jukanti et al. (2004) classified wheat PPO genes into two groups: one group is expressed in seeds; the other group is expressed in vegetative organs. However, this classification is not fully confirmed because of the absence of full-length cDNA sequences and unknown chromosome assignment of the latter group. Using maize PPO gene as a probe. Southern hybridization detected PPO genes on a minimum of five chromosomes in wheat—5B, 7D, 6A, 6B, and 6D (Li et al., 1999). Quantitative trait loci for PPO activity were detected on wheat chromosomes 2A, 2B, 3B, 3D, and 6D (Demeke et al., 2001). A syntenic relationship of wheat homoeologous group 3 chromosomes and rice chromosome 1 was reported (Devos and Gale, 2000). To obtain the whole picture of PPO genes in barley and wheat, identification of full-length PPO genes that are preferentially expressed in vegetative tissues is necessary.

**Gene duplication and functional differentiation in barley PPOs**

In many plant species, PPOs are encoded by a multigene family. In tomato, a seven-member gene family is clustered within a 165 kb locus on chromosome 8. They are differentially expressed in different stages and tissues (Newman et al., 1993; Thipyapong et al., 1997). In red clover, six PPOs are clustered over an approximately 510 kb region (Winters et al., 2009). In rice, two pseudogenes are clustered in the vicinity of an active PPO gene (Phr1) on chromosome 4, and another PPO-like gene is present on chromosome 1 (Yu et al., 2008). The PPO genes that control the phenol reaction phenotypes in barley are similarly clustered as described above. The PPO gene family appears to have been generated through the duplication of an ancestral single-copy gene by unequal crossing over or other mechanisms. Results of the present expression analysis suggest

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**Fig. 9.** PPO activities of grains of near-isogenic lines assayed using the whole grain method: C, nud-Bowman; I–V, near-isogenic lines for PPO1 mutant alleles—III is a double mutant for PPO1 and PPO2. Asterisks (*) indicate that activity was significantly different (P < 0.01) from the control (C) according to the t test. Bars represent standard deviations (n=6).
functional differentiation between PPO1 and PPO2 in barley. Functional diversification of duplicate PPO genes in barley might be mediated by an insertion of a hAT-family transposon in the proximity of the PPO2 promoter because the transposon insertion in the promoter region was reported to modulate gene expression (Lee et al., 2009; Hayashi and Yoshida, 2009).

Origin of PPO1 and PPO2 mutant alleles in barley

Takeda and Chang (1996) proposed a monophyletic origin for the phenol reaction-negative accessions in barley based on a very low frequency of occurrence (0.6%) and restricted distribution along the Silk Road. However, the present molecular characterization of the PPO1 gene demonstrated multiple independent origins of phenol reaction-negative mutations in barley. In barley, five types of non-functional PPO1 alleles were detected. They were distributed in different regions of the world with clear localization. The fact that the most prevalent PPO1 type-IV allele distributed along the Silk Road indicates that domesticated barley was transported eastward through this trade route by human activities. The low frequency of mutant accessions probably reflects their recent origin. Multiple independent origins of phenol reaction-negative accessions were also observed in rice, but their frequency was much higher because all japonica subspecies carries one of the three types of loss-of-function alleles (Yu et al., 2008). Fixation of dysfunctional PPO genes in japonica rice was probably caused by positive selection for reduced browning of grains during storage (Yu et al., 2008). Plant PPOs are reportedly associated with disease resistance (Thipyapong et al., 1995; Li and Steffens, 2002). Yu et al. (2008) argued that the retention of phenol reaction activity in indica rice, in contrast to its ubiquitous loss in japonica, is as follows: Phr1 activities are necessary for indica rice to maintain disease resistance in warmer climates. The independent occurrence of five non-functional PPO1 alleles in different regions of barley cultivation might indicate that such mutants do not pose serious problems in maintaining disease resistance in the case of barley, which grows in temperate climates.

The PPO2 gene is also of particular evolutionary interest, although the magnitude of its genetic diversity is lower than that of the PPO1 gene and a weaker contribution to the phenol-reaction phenotype in the ventral side of the crest was observed. However, a unique hAT family transposon insertion in the proximity of the promoter sequence makes this gene an attractive model for duplicate gene evolution.

PPO mutants in barley research

In rice, PPO activity of phenol reaction-negative accessions was almost completely lost (Yu et al., 2008), but barley PPO mutants—either single PPO1 mutants or a PPO1PPO2 double mutant—retained about half the PPO activity of the normal control. This difference in the level of residual PPO activity in PPO mutants between rice and barley might be explained as follows. Polyphenol content in barley grains is about twice that in rice (Hodzic et al., 2009). Therefore, auto-oxidation of phenolic compounds might occur rapidly in barley and preclude accurate PPO activity measurements. Alternatively, oxidation might be mediated by non-PPO oxidase enzymes such as dioxygenases or mono-oxygenases (cytochrome P450). Another possibility is that an unidentified PPO gene(s) might still be present in the barley genome because genomic Southern analysis suggests the possible occurrence of a third PPO in barley. Such a PPO might have diverged sequences and could have escaped the PCR-oriented cloning approach. It is not certain whether manipulation of the PPO genes might be effective in reducing the discoloration of barley products. However, allele variants of PPO1 and PPO2 identified in this study should be useful to test the relation of PPOs to disease resistance in barley.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Chromosome assignment of the PPO1 gene using wheat–barley disomic and ditelosomic chromosome addition lines. PCR primers of Hvdeg1LP1 and degRP21 were used for amplification. Barley chromosomes added to wheat are indicated above the lane. M: 100-bp ladder, W: wheat cv. Chinese Spring, B: barley cv. Betzes. An arrow indicates 500 bp.

Figure S2. Alignment of two barley PPO protein sequences of Haruna Nijo. Copper binding domains and a putative thylakoid translocation signal sequence are highlighted respectively in red and green. An arrow indicates the predicted cleavage site of N-terminal signal peptide, and triangles show the intron positions.

Table S1. PCR primers used in this experiment.

Table S2. Natural allelic variation in the PPO1 gene using Haruna Nijo (HA2) as a standard.

Table S3. Natural allelic variation in the PPO2 gene using Haruna Nijo (HA2) as a standard.

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