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Immunological and molecular comparison of polyphenol oxidase in Rosaceae fruit trees[1]

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Abstract

An antibody raised against apple polyphenol oxidase (PPO) cross-reacted with PPOs from Japanese pear (*Pyrus pyrifolia*), pear (*Pyrus communis*), peach (*Prunus persica*), Chinese quince (*Pseudocydonia sinensis*) and Japanese loquat (*Eriobotrya japonica*). Core fragments (681 bp) of the corresponding PPO genes were amplified and characterized. The deduced protein sequences showed identities of 85.3 to 97.5%. Chlorogenic acid oxidase activity of these PPOs showed higher activities when assayed at pH 4 than at pH 6. These results indicate that PPOs in Rosaceae plants are structurally and enzymatically similar.

Keywords: *Pyrus pyrifolia* (Japanese pear); *Pyrus communis* (pear); *Prunus persica* (peach); *Eriobotrya japonica* (Japanese loquat); *Pseudocydonia sinensis* (Chinese quince); Rosaceae; Polyphenol oxidase; Enzymatic browning

1. Introduction

In higher plants, browning of tissues is considered to be caused by oxidation of phenolic compounds, polymerization of resulting quinones and their interaction with other molecules. Plant phenolics are readily oxidized by the enzyme polyphenol oxidase (EC.1.10.3.1, PPO), most often following tissue damage. With regard to physiological function, PPO is thought to act as a defensive enzyme against insect herbivores (Felton, Workman, & Duffey, 1992). PPO is also an important enzyme in the food industry, due to its importance in browning reactions (Martinez & Whitaker, 1995).

PPO is a copper enzyme and two Cu ions at the active site catalyze the oxidation of phenolic compounds (Mayer & Harel, 1979). Recently, a number of plant PPO genes have been characterized (Lax & Cary, 1995; van Gelder, Flurkey, & Wichers, 1996) and six conserved histidine residues in each PPO have been identified as potential Cu binding sites (Solomon, Baldwin, & Lowery, 1996; Lerch, 1995), based on the similarity with hemocyanin, whose structure was confirmed with X-ray analysis (Geykema et al., 1984). The Cu-binding region of plant PPOs showed the greatest sequence conservation (Lax & Cary, 1995; van Gelder, Flurkey, & Wichers, 1996). As expected, PPO sequences from dicotyledons have greater similarity to each other than to PPOs from monocotyledons (i.e. sugarcane (Bucheli, Dry, & Robinson, 1996). In Solanaceous plants, such as tomato (Newman et al., 1993), potato (Hunt, Eannetta, Yu, Newman, & Steffens, 1993) and tobacco (Goldman, Seurinck, Marins, Goldman, & Mariani, 1998), very high sequence homologies were shown in comparison with other plant families. Furthermore, common antigenicity of PPO protein has been shown in beans, lettuce, spinach, and tobacco using PPO-specific antibodies (Lanker, Flurkey, & Hughes, 1988).

In fruit trees, PPO has been purified from apple (*Malus domestica*) (Murata, Kurokami, & Homma, 1992), Japanese pear (*Pyrus pyrifolia*) (Tono, Fujita, Kawasaki, & Li, 1986), pear (*Pyrus communis*) (Wissenmann & Montgomery, 1985), and peach (*Prunus persica*) (Flurkey, 1980). To obtain more information about PPOs in fruits of Rosaceae plants, antibodies were used to examine PPOs of Japanese pear, pear, peach, Japanese loquat (*Eriobotrya japonica*) and Chinese quince (*Pseudocydonia sinensis*).

Furthermore, we cloned gene fragments of their active

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the following accession numbers: AB011827, Japanese pear (*Pyrus pyrifolia* cv. Housui); AB011828, pear (*Pyrus communis* cv. la france); AB011829, peach (*Prunus persica*); AB011830, Japanese loquat (*Eriobotrya japonica*); AB011831, Chinese quince (*Pseudocydonia sinensis*).

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sites, and compared their primary structure. Here we discuss the similarity among these PPOs.

2. Results and discussion

Apple PPO was expressed in *E. coli* using an expression vector and this PPO protein was purified from inclusion bodies by SDS–PAGE. An antibody against the PPO was prepared. Murata & Kurokami et al. (1992) had previously prepared an antibodagainst native apple PPO; however, the antibody reacted only weakly with Japanese pear, and did not cross-react with other plants PPOs. In Western blots, PPOs from Rosaceae plants (Japanese pear, pear, peach, Japanese loquat and Chinese quince) cross-reacted with the antiserum prepared against apple PPO produced in *E. coli* (Fig. 1). Leaf and fruit extracts of persimmon (*Diospyros kaki*), not belonging to the Rosaceae family, did not show any bands by Western blot analysis. By contrast, one to three bands of PPO in fruits, and one to two bands of PPO in leaves, were detected in the other species analyzed. A band at ca. 65 kDa was the major band and a smaller size band at ca. 57 kDa was also found. In apple fruit, the 65 kDa band was the major band, while in apple leaf the 57 kDa band was more predominant. We previously demonstrated that apple PPO migrated at 65 kDa in SDS–PAGE, although its predicted molecular weight was 59 kDa (Haruta et al., 1998). It is not conclusively established if there are any relationships between the band patterns and tissues in which those two PPOs were localized. To make clear the relationship between these two bands (65 and 57 kDa bands), the mature form of apple PPO was expressed as an N-terminal fusion protein in *E. coli* and purified using a glutathione column (Pharmacia). In both Coomassie-stained protein gels (Fig. 1C, h) and Western blots (Fig. 1C, i), 65 and 57 kDa bands were again detected in a purified PPO fraction. This result suggests that apple PPO is proteolyzed in *E. coli*, as well as in apple fruit and leaves, at its C-terminal site, as is the case in *Vicia faba* (Robinson & Day, 1992). C-terminal cleavage has also been reported in grape PPO (Day & Robinson, 1994). However, it is also possible that the 65 and 57 kDa bands of apple fruit and leaves might be derived from different genes.

To establish how closely related the PPOs are from Rosaceae plants, partial PPO gene sequences containing Cu-binding regions were amplified from genomic DNA using the polymerase chain reaction (PCR). Two primers were used, each based on the apple PPO sequence. When primers encoding up- and down-stream sequences of a core region within PPO were used, DNA fragments with the expected size were amplified from all Rosaceae fruit tree plants. The amplified gene fragments were cloned and sequenced. All the gene fragments had the same size as the apple PPO gene (681 bp); they also had no gap or insertions, and no introns in this area as reported for other PPO genes (van Gelder, Flurkey, & Wichers, 1996). These clones encode partial protein sequences of 204 amino acids. As the alignment of core protein sequences shows, all gene sequences had six histidines in the predicted copper binding sites (Fig. 2). Comparison of the amino acid sequences shows that higher homologies (82.8–97.5%) were found among the Rosaceae plants than those of the other families (Table 1).

Since PPOs in these Rosaceae plants have similar amino acid sequences, PPO activities were measured in crude extracts to confirm PPO activity. Oxidation of chlorogenic acid, a common PPO substrate, showed higher rates in the crude extracts of all Rosaceae plants, when assayed at pH 4.0 rather than pH 6.0 (Table 2). The PPO activities were inhibited by kojic acid and were not influenced by catalase. The optimum pH of apple

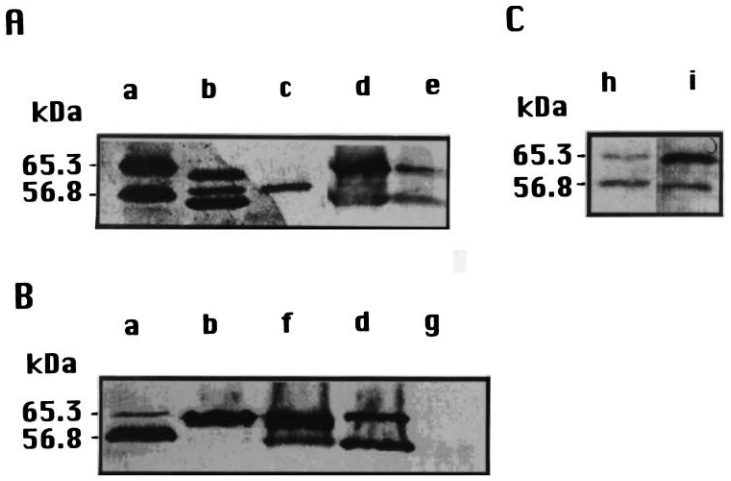

Fig. 1. SDS–PAGE (h) and Western blot analyses (a–g and i) of PPOs. (A) PPOs of fruits, (B) PPOs of leaves, (C) apple PPO expressed in *E. coli*. (a) apple; (b) Japanese pear; (c) pear; (d) Japanese loquat; (e) Chinese quince; (f) peach; (g) persimmon; (h) SDS–PAGE stained by Coomassie Brilliant Blue; (i) immuno-staining of (h).

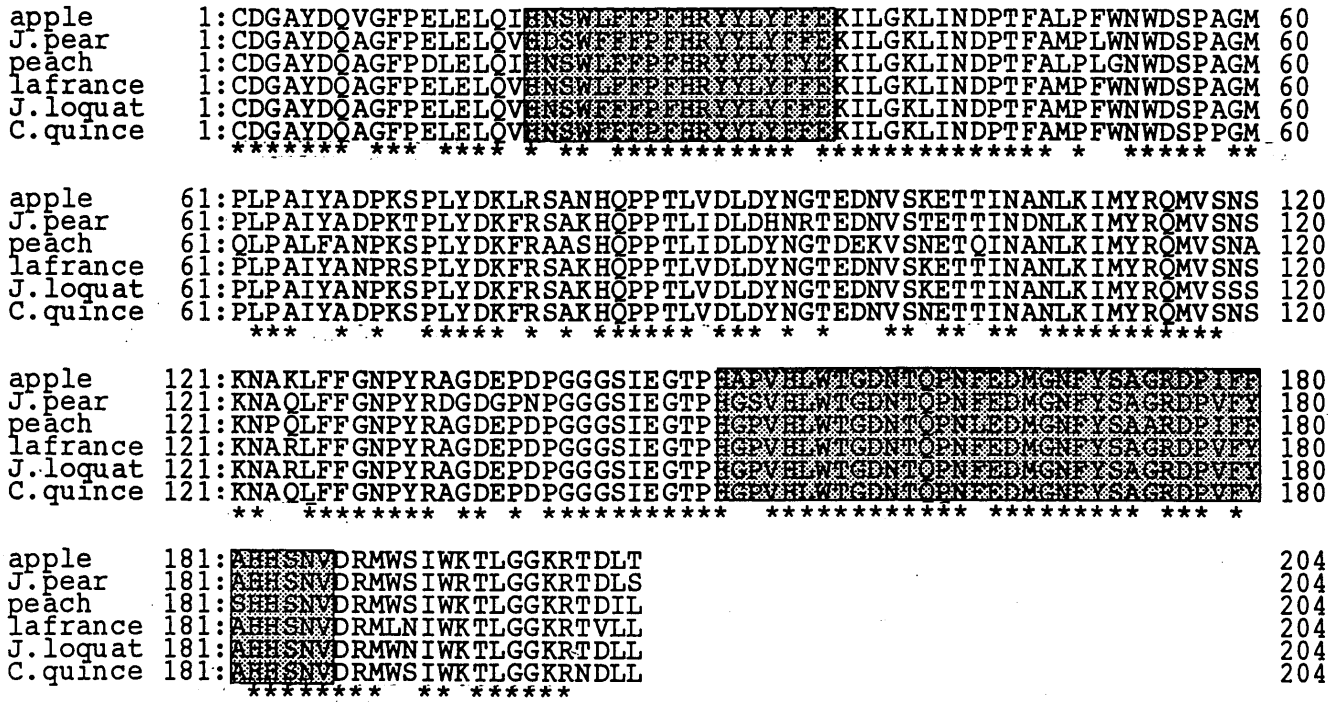

Fig. 2. Alignment of core PPO protein sequences of Rosaceae fruit plants. Shaded areas show the deduced copper binding sites (CuA and CuB). Asterisks indicate amino acid residues which are conserved among all sequences. J. pear, lafrance, J. loquat and C. quince show Japanese pear, pear, Japanese loquat and Chinese quince, respectively.

Table 1
Homologies of core amino acid sequences of PPO (%)

	Apple[a]	J. pear[a]	Pear[a]	C. quince[a]	J. loquat[a]	Peach[a]	Bean	Grape	Tomato	Potato
Apple	100	88.2	93.1	93.6	93.6	87.2	68.6	63.6	58.5	57.1
J. pear		100	90.1	92.6	91.6	82.8	64.7	59.0	55.1	55.1
Pear			100	95.1	97.5	85.3	67.5	62.3	56.9	55.9
C. quince				100	96.6	86.3	67.0	61.3	56.9	56.4
J. loquat					100	85.8	66.5	62.3	56.9	55.9
Peach						100	68.5	62.3	57.8	56.9
Bean							100	61.0	55.6	53.7
Grape								100	57.9	55.9
Tomato									100	97.0
Potato										100

[a] Rosaceae plants. J. pear, C. quince, J. loquat show Japanese pear, Chinese quince and Japanese loquat, respectively.

Table 2
PPO activity of Rosaceae fruits

	PPO activity	
Plants	pH 4.0	pH 6.0
Pear	2.8	0.29
Japanese loquat	6.0	0.83
Chinese quince	44.3	0.20
Apple	12.7	0.44
Japanese pear	31.7	13.5

(U/mg protein).

PPO is 4.0 (Murata, Kurokami, & Homma, 1992). The optimum pH for PPO activity in Rosaceae plants seems to be more acidic compared to vegetable plants, where the optimum pH for PPO activity is somewhat higher, for example in carrot (pH 8.0) (Söderhäll, 1995), spinach (pH 7.0) (Sheptovitsky & Brudvig, 1996) and lettuce (pH 5.0–8.0) (Heimdal, Larsen, & Poll, 1994).

In addition, Southern blot analysis was performed in order to know how many PPO genes are present in the genomes of the six species of Rosaceae plants (Fig. 3). The genomic DNA of these plants was digested with *Bam*HI, whose recognition site is not in the probe sequences, and was probed with a mixture of the five

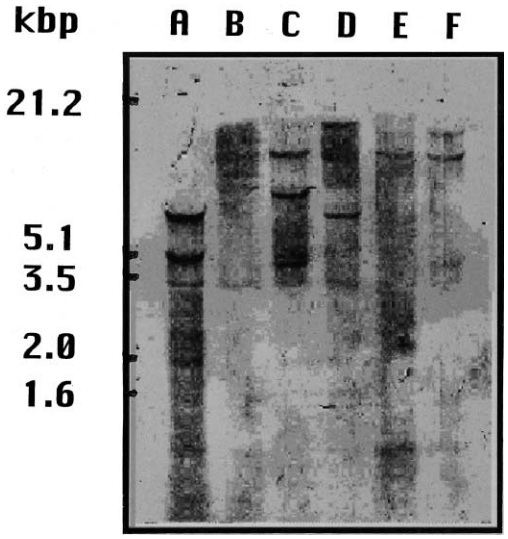

Fig. 3. Southern blot analysis of Rosaceae plants. (A) Japanese pear; (B) Japanese loquat; (C) apple; (D) peach; (E) Chinese quince; (F) strawberry.

DIG-labeled PPO gene fragments. Two strongly hybridizing bands and one or two weak bands were detected in apple and Japanese pear. In peach, Japanese loquat, and Chinese quince, only one band could be detected. In Japanese pear, an almost full length PPO gene was amplified by PCR with primers based on the apple PPO sequence, where the 3′ primer used corresponded to the nucleotide sequence downstream from the stop codon (data not shown). This suggests that PPO genes of apple and Japanese pear are very similar. The pear PPO gene also contained no intron, as for the apple gene. In strawberry, which belongs to the Rosaceae, but which is not a fruit tree, hybridization signals were detected only weakly, and a PPO gene fragment could not be amplified by PCR using the apple primers.

PPOs in fruit trees have been previously studied enzymatically (Tono et al., 1986; Wissermann & Montgomery, 1985; Flurkey, 1980), and our laboratory has focused on apple PPO (Murata et al., 1992, 1993, 1995; Haruta et al., 1998). However, a detailed characterization of fruit PPOs at the molecular level has not been carried out. Here PPOs of fruit trees were analyzed immunologically, and partial PPO gene sequences were isolated and sequenced from Japanese pear, peach, pear, Chinese quince and Japanese loquat. It is concluded that PPOs in Rosaceae fruit plants are very similar from both molecular and immunological criteria.

3. Experimental

3.1. Plant material

Fruits of apple (*Malus domestica* cv. Fuji), Japanese pear (*Pyrus pyrifolia* cv. Housui), pear (*Pyrus communis* cv. la france), Japanese loquat ('biwa') (*Eriobotrya japonica*) and Chinese quince ('karin') (*Pseudocydonia sinensis*), strawberry (*Fragaria* × *ananassa*) and persimmon (*Diospyros kaki*) were purchased from local markets in Tokyo. Leaves of apple, pear Japanese loquat, and persimmon were obtained from the University garden. Leaves of Japanese pear were obtained from an orchard in Chiba prefecture.

3.2. Preparation of apple PPO antibody

DNA manipulation methods were carried out as described by Sambrook, Fritsch, & Maniatis (1989). An apple PPO gene (Haruta et al., 1998) coding for mature PPO, was inserted into pET23d(+) (Novagen) at *Nco*I and *Bam*HI sites. *E. coli* strain BL21(DE3) carrying the constructed plasmid was induced with 1 mM isopropyl-*β*-D-thiolactopyranoside, the cells were harvested by centrifugation and broken by sonication in phosphate buffered saline (pH 7.5) (PBS) containing 3% Triton X-100. Inclusion bodies containing expressed PPO were pelleted by centrifugation, and the proteins were separated by SDS–PAGE. After protein staining and decolorizing, the PPO band was excised from the gel. The gel slice was crushed with an equal volume of PBS. Three female ddY mice were each injected with 25 μg of the protein solution emulsified with an equal volume of Freund's complete adjuvant. Two ybooster injections of 25 μg of protein each were given at intervals of two weeks.

3.3. Protein extraction and Western blot analysis

Fruit or leaves were ground in a mortar in two volumes of cold extraction buffer, 10 mM K–Pi buffer (pH 7.0) containing 3% Triton X-100 and 10% polyclar AT. Soluble protein was extracted with the buffer. Protein concentrations were measured by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as a standard. Proteins were separated by SDS–PAGE. SDS–PAGE and Western blot analyses were carried out as described previously (Murata et al., 1993).

3.4. DNA extraction, cloning and sequencing of PPO gene

Chromosomal DNA was isolated from leaf or fruit tissue using the CTAB method (Murray & Thomson, 1980). Two oligonucleotide primers corresponding to amino acid residues, 168–178 (5′-TTCAAGCAACAGG CAGCCGTGCATTGCGCTTAT-3′, sense) and 384–394 (5′-GTAAAACAAGAATCCGGAGTCCAACC AGTCCGA-3′, antisense) of apple PPO were synthesized (Pharmacia) and used for PCR (94°C 2 min and 30 cycles of 94°C 1 min, 60°C 1 min, 70°C 1.5 min). One hundred nanograms of DNA from Japanese pear, pear, peach, Japanese loquat and Chinese quince were used as

templates. PCR products with the predicted size were cloned into pT7 Blue vector (Novagen) and sequenced with an Applied Biosystems model 373S DNA sequencer (Perkin Elmer) according to the manufacturer's instructions. Genetyx-Mac 7.3 was used to analyze sequence data.

3.5. Southern blot analysis

Ten micrograms of each plant DNA was digested with *Bam*HI, separated on 0.8% agarose gel, and then transferred to a nylon Hybond-N membrane (Amersham). Hybridization was performed at 68°C with DIG-labeled probes, with detection performed according to the kit manufacture's instruction (Boehringer, Mannheim).

3.6. PPO assays

PPO activity was measured by the spectrophotometric method at 325 nm to detect the decrease of chlorogenic acid as a substrate (Murata et al., 1992). The reaction solution consisted of 0.8 ml of McIlvaine buffer (pH 4.0 or 6.0), 0.1 ml of 0.5 mM chlorogenic acid and 0.1 ml of the enzyme solution. The specificity of PPO activity was verified by performing parallel assays with 1 mM kojic acid or 280 unit/ml catalase. A decrease in absorbance of 0.1 per min at 30°C is defined as 1 unit of PPO activity (Murata et al., 1992).

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