



# Two polyphenol oxidases are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple<sup>☆</sup>

Joo Young Kim<sup>a</sup>, Young Sam Seo<sup>a</sup>, Jee Eun Kim<sup>a</sup>, Soon-Kee Sung<sup>b</sup>,  
Kwan Jeong Song<sup>c</sup>, Gynheung An<sup>d</sup>, Woo Taek Kim<sup>a,\*</sup>

<sup>a</sup> Department of Biology, College of Science, Yonsei University, Seoul 120-749, South Korea

<sup>b</sup> Hansol Institute of Science and Technology, Namyangju 472-850, South Korea

<sup>c</sup> Department of Fruit Breeding, National Horticultural Research Institute, RDA, Suwon 440-706, South Korea

<sup>d</sup> Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, South Korea

Received 17 May 2001; received in revised form 8 August 2001; accepted 13 August 2001

## Abstract

Polyphenol oxidase (PPO), a copper-containing metalloprotein, catalyzes the oxidation of phenolics to quinones which make brown pigments in wounded tissues. Because the phenomena decrease fruit quality, PPO has been regarded to be a critical enzyme in food technology. In the course of expressed sequence tags (ESTs) analysis of the Fuji apple (*Malus domestica* Borkh.), we identified two partial PPO cDNA clones; F114 corresponded to the previously isolated Granny Smith apple pAPO5 (Plant Mol. Biol. 27 (1995) 429), while F226 was a new clone. Using F226 as a probe, we isolated a full length PPO clone, pMD-PPO2, from a cDNA library prepared from young fruits of the Fuji apple. The deduced amino acid sequences of pMD-PPO2 and pAPO5 share 55% identity, and display a high degree of sequence identity (43–58%) with previously identified PPO from various species. RNA gel blot analysis using gene-specific probes showed that two apple PPO genes display unique patterns of expression in a tissue- and developmental-specific manner. In the process of flower development, the *APO5* transcript was detectable only at the post-anthesis stage. In contrast, *MD-PPO2* was expressed in all stages of flower development, with the abundance of mRNA being the highest at the pre-anthesis stage and then receding as the flower developed. Both genes are expressed in the early stages of fruit development. The expression was dramatically reduced as the fruit ripened. In leaf tissue, the *APO5* gene was highly expressed in young and immature leaves, while *MD-PPO2* was transcriptionally more active in both immature and mature leaves. Upon wounding, *APO5* was significantly induced in leaves and fruits, whereas the level of *MD-PPO2* mRNA was not affected by mechanical damage. Thus, it appears that two Fuji PPO genes are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple plants. The possible molecular mechanism of differential regulation of PPO gene expression in apple plant and its physiological significance are discussed. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** cDNAs; Differential gene expression; Fuji apple; Polyphenol oxidase

## 1. Introduction

Polyphenol oxidase (PPO; EC 1.14.18.1 or EC 1.10.3.2) is a nuclear-encoded copper-containing enzyme widely distributed in the plant species and catalyzes the oxidation of phenols to *o*-quinones. The *o*-quinones are highly reactive in plant cells and subsequently involved in the formation of polymeric dark-colored pigment deposits [1]. This enzymatic

<sup>☆</sup> The nucleotide sequence data reported in this paper have been deposited in the GenBank database under the accession number AF380300.

\* Corresponding author. Tel.: +82-2-2123-2661; fax: +82-2-312-5657.

E-mail address: wtkim@yonsei.ac.kr (W.T. Kim).

discoloration often results in the significant loss of post-harvest quality of commercially important fruits and vegetables [2]. PPO is localized in the plastids, while its phenolic substrates are mainly present in the vacuole [3]. Therefore, PPO-mediated browning reaction occurs only after a loss of this subcellular compartmentation, such as wounding. Although the exact physiological role of PPO in plant cells is not well established, PPO has been implicated to function in the formation of pigment [4], oxygen scavenging and pseudocyclic phosphorylation in chloroplast [4,5], and defense mechanism against insects and plant pathogens [6–9].

PPO is found to be encoded by a gene family. Recent molecular studies have shown that PPO gene family is differentially expressed in different organs, and at distinct developmental and physiological conditions. For example, seven PPO genes (PPOs A, A', B, C, D, E and F) were identified from tomato plants [10]. The expression of each member of gene family is differentially regulated in various vegetative and reproductive organs and in response to wounding in young leaves in tomato plants [8,11,12]. In potato plants, five distinct PPO cDNAs were isolated from developing tubers, and each gene was spatially and temporally expressed in a gene-specific manner in various tissues including tubers, roots, leaves, petioles and flowers [13,14]. The multi-gene family of PPO was also identified from faba bean leaves [15], pokeweed suspension culture [16], tobacco flower tissues [17] and apricot fruits [18]. Most recently, Constabel et al. [9] have isolated a PPO cDNA clone (PtdPPO) from hybrid poplar and described it as a gene family. In contrast, Dry and Robinson [19] reported the presence of only one PPO gene in grape berry.

Apple is one of the most economically important woody plant species, widely cultured for its valuable fruits. The browning reaction of apple fruits in response to mechanical wounding reduces economic and nutritional values of apple fruits. This discoloration process is mediated by PPO activity, but information about the differential expression of apple PPO gene family is limited. Previously, Boss et al. [20] isolated a full-length PPO cDNA clone (pAPO5) from fruit peel cDNA library of Granny Smith apple and showed that the level of *APO5* transcript markedly increased in wounded fruit and leaf tissues. We have been interested in elucidating the differential expression pattern of PPO gene family in the Fuji apple, the most important and widely cultivated commercial fruit in East Asia. The particular aim of the present study was to isolate apple PPO gene family and identify whether different PPO genes were differentially expressed during development and by wounding in the Fuji apple plants. The results of this work show that the expression of two PPO genes is differentially regulated during vegetative and reproductive development and in response to wounding in the Fuji apple plants.

## 2. Materials and methods

### 2.1. Plant materials and RNA isolation

The apple (*Malus domestica* Borkh.) cv. Fuji was used in this study. Plant samples were obtained from the National Horticultural Research Institute, Suwon, South Korea. Leaves (1, 3, and 5 cm in length), floral buds (0.4–0.5 and 0.6–0.9 cm in length), whole flowers of pre-anthesis and post-anthesis, and fruits at different stages of growth and ripening process were harvested, immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until used. The young leaves and ripened fruits were detached from the plants and damaged by slicing with a razor blade into 0.2-cm-long and 1-cm-long segments, respectively. The wounded tissues were covered with wet paper towel and incubated for different time periods (1–48 h) at room temperature. The total RNAs were isolated from various tissues as described previously [21].

### 2.2. Construction of cDNA library

Poly (A)<sup>+</sup> RNA was purified from total RNA by oligo (dT) cellulose chromatography using a poly (A) Quick mRNA isolation kit (Stratagene, La Jolla, CA, USA). Young fruits at 0.5–1.5 cm in length were used for the construction of a unidirectional cDNA library according to the manufacturer's protocol (Stratagene). The initial plaque forming units were  $1.5 \times 10^6$  and average size of the inserts was about 1.7 kb. The library was amplified once on agar plates and stored in a 7% DMSO solution at  $-70^{\circ}\text{C}$ .

### 2.3. Screening of cDNA library and sequencing of DNA

The lambda uni-Zap II apple fruit cDNA library was screened using the expressed sequence tags (ESTs) cDNA fragment [22] as a probe by an established procedure [23]. The cDNA inserts containing putative apple PPO were subcloned into Bluescript SK plasmid by in vivo excision of pBluescript from Zap II vector as described in the protocols by Stratagene. Sequencing of DNA was performed using the Sequenase DNA sequencing kit according to the manufacturer's manual (US Biochemical, Cleveland, OH, USA). Sequence analysis was carried out using DNASIS computer software.

### 2.4. Isolation of genomic DNA and Southern blot analysis

Apple leaf genomic DNA was isolated as described previously [24]. Apple genomic DNA (10  $\mu\text{g}$  per lane) was digested with *EcoRI* or *HindIII*, separated by

electrophoresis on a 0.7% agarose gel, and blotted to a Hybond-N nylon membrane filter (Amersham, Buckinghamshire, UK). The filter was hybridized to  $^{32}\text{P}$ -labeled PPO cDNA clone as described previously by Jeong et al. [24].

### 2.5. RNA gel blot analysis

The total RNA was precipitated overnight at 4 °C by the addition of 0.3 volumes of 10 mM LiCl and then precipitated in ethanol. Total RNA (30 µg) was separated by electrophoresis on a 1% formaldehyde-agarose gel and blotted to a Hybond-N nylon membranes (Amersham). To ensure equal loading of RNA, the gel was stained with ethidium bromide after electrophoresis. In order to confirm complete transfer of RNA to membrane filter, both gel and membrane were viewed under UV light at the end of transferring. The filter was hybridized to  $^{32}\text{P}$ -labeled apple PPO gene-specific probes. The blots were washed as described previously [21] and visualized by autoradiography at -70 °C using Kodak XAR-5 film and intensifying screen.

### 2.6. PPO extraction and assay

PPO extraction and assay were carried out at 4 °C as described by Sherman et al. [25] with modifications. Wounded fruits were extracted into extraction buffer (100 mM  $\text{NaPO}_4$ , pH 7.2, 0.1% [w/v] SDS and 3 mM ascorbate) and centrifuged. The supernatant was assayed for PPO activity spectrophotometrically at 490 nm and 30 °C, to follow the conversion of L-dihydroxyphenylalanine (DOPA) to quinone polymers. The assay solution consisted of 1 ml of 10 mM DOPA in 100 mM  $\text{NaPO}_4$ , pH 4.5, 250 units of catalase (Sigma) and 50 µl of the enzyme extract. The specific activity of enzyme was defined as the change in absorbance ( $\Delta A$ ) per milligram protein per minute [26].

## 3. Results and discussion

### 3.1. Isolation and sequence analysis of apple PPO cDNA clones

In our previous study, a total of 430 ESTs were generated from randomly selected clones of cDNA libraries prepared from young fruits, peels of mature fruits and carpels of the Fuji apple [22]. The results of the database alignment revealed that F114 and F226 cDNA clones (GenBank accession numbers AT000112 and AT000023) exhibited a sequence homology with PPO cDNA clones from various plant species; F114 corresponded to the previously isolated Granny Smith apple pAPO5 clone [20, GenBank accession number L29450], while F226 belonged to a new apple PPO

gene. The F226 cDNA clone of 427 bp in length was radioactively labeled and used as a probe to screen the cDNA library of young fruits of the Fuji apple [22]. Numerous putative PPO cDNA clones were isolated. Subsequent restriction enzyme mapping and DNA sequencing analyses indicated that these clones represented a single group of overlapping sequences. Fig. 1A shows the restriction map of F114 and F226 as well as pMD-PPO2 that contains the longest insert among isolated clones. The pMD-PPO2 is 1966 bp long comprising a 25 bp 5'-uncoding region, a 1758 bp coding region (encoding 586 amino acids) and a 183 bp 3'-uncoding region (Fig. 1B) (the sequence was deposited in GenBank, accession number AF380300). The predicted molecular mass of polypeptide encoded by pMD-PPO2 is 65.3 kDa which is similar to that of pAPO5 polypeptide (65.7 kDa) [20]. The overall nucleotide sequence identity between pMD-PPO2 and pAPO5 is 60%, while the coding region is 62% identical at the nucleotide level and 55% at the amino acid level. Both apple PPOs share 43–58% identity at the amino acid level with the other plant PPOs, including tomato [10,11], potato [13], faba bean [15], grape berry [19], apricot [18] and hybrid poplar [9]. These results confirm that higher plant PPOs are conserved. The apple PPOs share the higher sequence identity with PPOs from apricot (58%), grape berry (50%) and poplar (50%), three other woody plants. There exist several highly conserved amino acid sequences found in all these PPOs, such as two copper-binding domains containing typical His residues considered to be involved in copper binding (CuA and CuB, boxed in Fig. 1B). The CuA regions of Fuji PPOs are 88, 75 and 77% identical to their corresponding domains of apricot, grape berry and poplar, whereas the CuB regions are 80, 73 and 77% homologous, respectively, consistent with the essential role of copper in enzyme activity. An additional, less homologous His-rich putative copper-binding domain is found in the C-terminal region of apple PPOs, and similar amino acids sequences are also identified in other plant PPOs, including pokeweed, faba bean, potato and tomato [10,11,13,15,16] (CuC, boxed in Fig. 1B). As found in all plant PPOs sequenced to date, both APO5 and MD-PPO2 are synthesized as preproteins and contain putative plastid transit peptides of 89 and 93 amino acid residues, respectively, at the N-terminal region, which target the enzyme into chloroplast and thylakoid lumen (underlined in Fig. 1B). These two apple transit peptides are 47% identical and rich in the hydroxylated amino acid Ser and Thr and hydrophobic amino acids Ala and Val. The ends of both putative apple transit peptides are Ala-X-Ala consensus that is known to be recognized by the thylakoid processing peptidase [27]. These features indicate that the mature apple APO5 and MD-PPO2 proteins are composed of 504 and 493 amino acid residues, respectively, with predicted molecular masses of 56.3 and 55.4 kDa.

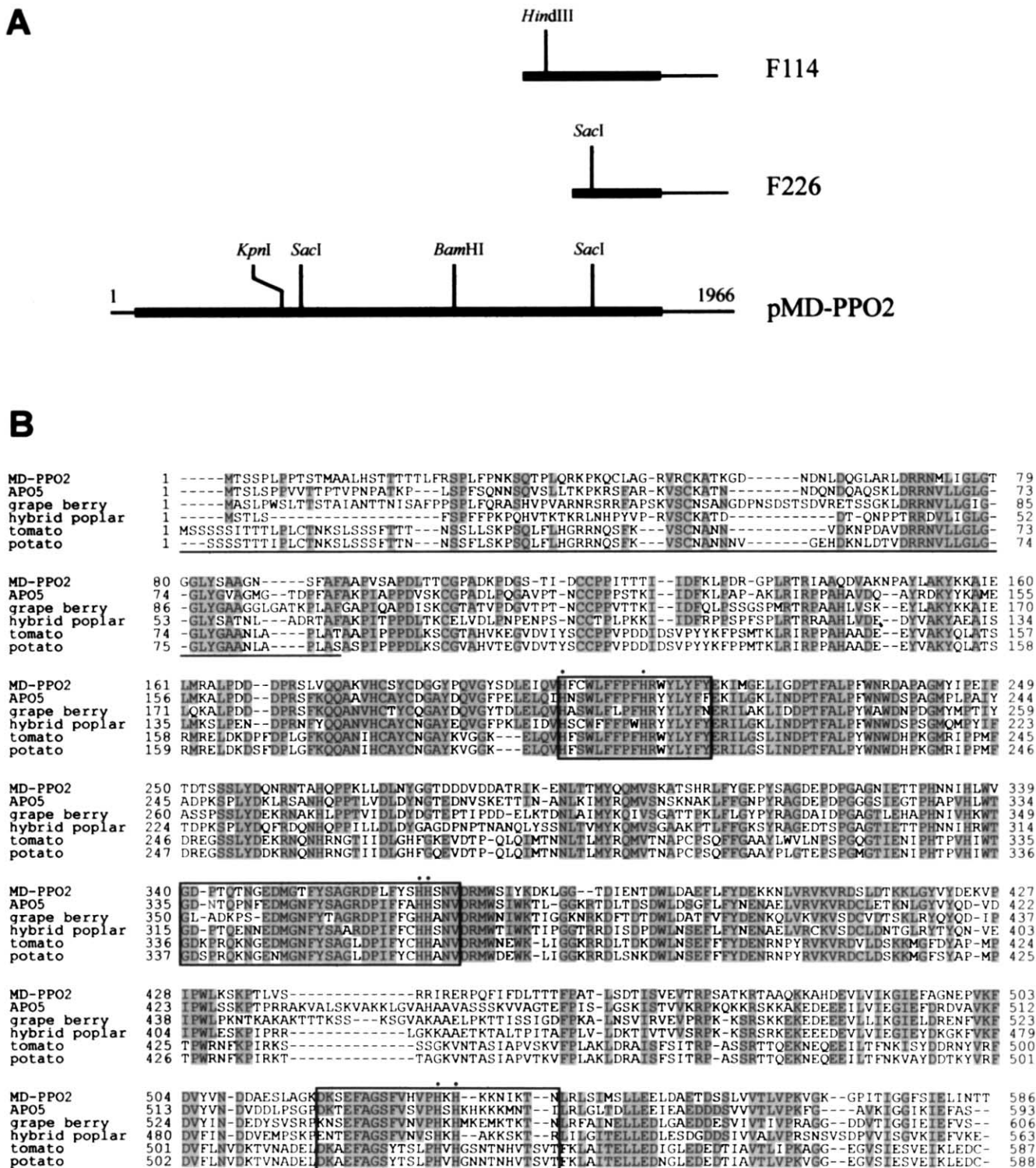


Fig. 1. (A) Restriction enzyme map analysis of Fuji apple PPO cDNA clones. Solid bars indicate the coding region. Solid lines present 5'- and 3'-untranslated regions. (B) Comparison of the derived amino acid sequences of the Fuji apple PPOs with the PPO proteins from grape berry, poplar, tomato and potato. Gaps were introduced to achieve maximum homology among the sequences. Amino acid residues conserved in at least four of the six sequences are shaded. Boxes depict consensus regions to the His-rich copper binding domains, CuA, CuB and CuC. Within the copper binding domains, His residues are marked by dots. The putative thylakoid transit peptide is underlined.

### 3.2. Genomic Southern blot analyses

Previous works from various species have shown that plant PPOs are encoded by a gene family. Boss et al. [20] carried out Southern blot analysis of Granny Smith apple genomic DNA using pAPO5 as a probe and suggested that there were at least four PPO genes on apple genome. In this study, Southern blot analyses were performed on Fuji apple genomic DNA digested with *EcoRI* or *HindIII*. When the filter was hybridized under normal stringent conditions to the <sup>32</sup>P-labeled probe derived from the entire sequence of pMD-PPO2, a complex pattern of hybridization was detected (Fig. 2A). However, when the filter was hybridized under high stringent conditions to the <sup>32</sup>P-labeled gene specific F226 probe (Fig. 1A), only one major band was detected (Fig. 2B). Because MD-PPO2 does not contain *EcoRI* and *HindIII* restriction sites (Fig. 1), and PPO genes are known to have no introns [10,19], these

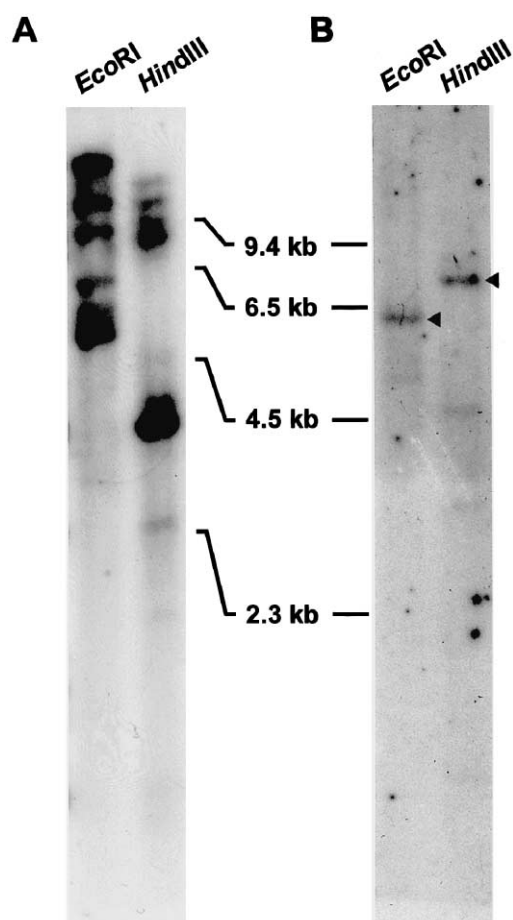


Fig. 2. Genomic Southern blot analysis of the Fuji apple PPO genes. The Fuji apple genomic DNA (10  $\mu$ g per lane) was isolated from leaf tissue, digested with either *EcoRI* or *HindIII*, and resolved on a 0.7% agarose gel. DNA on the gel was transferred to a nylon membrane filter. The filter was hybridized (A) to the <sup>32</sup>P-labeled pMD-PPO2 cDNA under the normal stringent condition or (B) to the gene specific probe F226 under the high stringent condition.

results are in line with the notion that apple PPO genes belong to the multigene family, and the *MD-PPO2* gene is present in single copy per haploid Fuji apple genome.

### 3.3. Tissue- and development-specific accumulation of two different PPO mRNAs

To assess the spatial and developmental expression pattern of two apple PPO genes, RNA gel blot analysis was performed using <sup>32</sup>P-labeled gene-specific F114 and F226 probes (Fig. 1A) with 30  $\mu$ g of total RNAs extracted from various tissues in different developmental stages of the Fuji apple plants. As a first step, the mRNA expression patterns of *APO5* and *MD-PPO2* were examined in flower buds. The development of flower buds was divided into four stages on the basis of the bud length and morphological events. In stage 1, the floral bud emerged on the flank of the branch and the bud length was 4–5 mm. In stage 2, ten days after stage 1, the length of bud became 6–9 mm. Stage 3 was at the mature flower stage and all the floral organs were morphologically differentiated. Finally, stage 4 was at the post-anthesis of the flower. At this stage, the anther wall was opened and stigma began to senesce. Fig. 3 reveals that both PPO cDNA probes hybridized to a single  $\sim$ 2.2 kb transcript in length and each PPO gene displays highly unique pattern of expression in a tissue- and development-specific manner. The *APO5* transcript was not detected in flower buds at stages 1–3, while the low level of mRNA was observed at the post-anthesis stage (Fig. 3A). By contrast, the *MD-PPO2* mRNA was detected at all four stages of flower development, with the intensity of the mRNA band being the strongest at stage 3 and then receding as the flower developed (Fig. 3A). These results indicate that the *MD-PPO2* gene is flower-predominant gene. To understand the role of PPO genes in fruit development, we have performed RNA gel blot analysis using apple fruit total RNAs. The fruit development was also divided into four stages depending on the size of fruits. Stage 1 was very young fruit stage and fruit size was 0.5–1 cm, whereas the size of fruit at stages 2 and 3 was 1–2 and 3–5 cm, respectively. The fruits at stage 4 were fully ripened. As shown in Fig. 3B, both apple PPO genes were expressed in the early stages of fruit development. The *APO5* transcript was present only at stage 1, while the *MD-PPO2* gene was expressed at stages 1 and 2, with the maximum mRNA level being detected at stage 2 (Fig. 3B). The level of both *APO5* and *MD-PPO2* transcripts was dramatically reduced as the fruit ripened. These results are consistent with recent finding by Chevalier et al. [18], in which the *PA-PPO* transcript markedly accumulated at the immature-green stage of apricot fruit development and then totally disappeared early in the ripening process of fruit. We

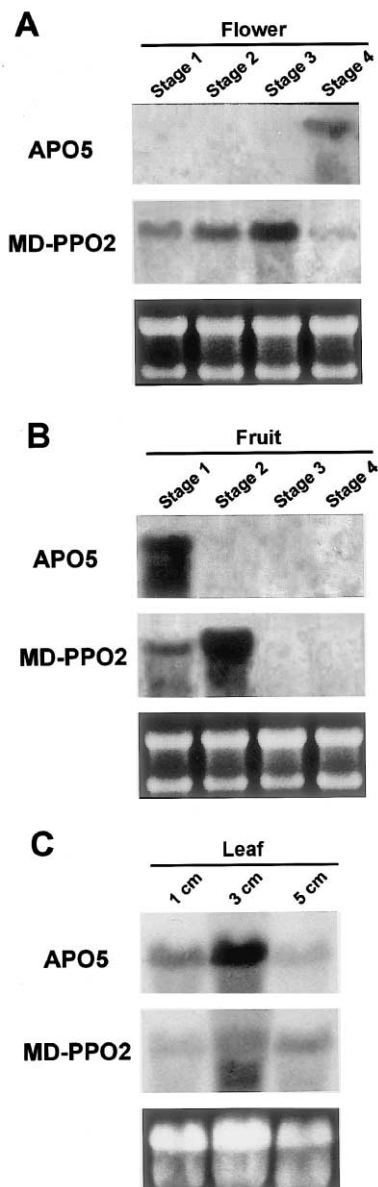


Fig. 3. Tissue specific expression of two Fuji apple PPO mRNAs. Total RNAs were isolated from the different developmental stages of (A) flower, (B) fruit and (C) leaf tissues as indicated. Total RNAs (30  $\mu$ g per each lane) were resolved on a 1% agarose-formaldehyde gel and blotted onto nylon membrane filters. The blots were hybridized to  $^{32}$ P-labeled gene specific probe for pAPO5 (probe F114) or pMD-PPO2 (probe F226) under the high stringent condition. The equivalence of RNA loading among lanes of agarose gels was demonstrated by ethidium bromide staining of RNA on the gel.

next investigated the level of apple PPO transcripts in leaf tissue. The expression of both PPO genes was also temporally regulated in a gene-specific manner during leaf development. The level of *APO5* mRNA was higher in young (1 cm) and immature (3 cm) leaves than in mature (5 cm) tissue. On the other hand, *MD-PPO2* was transcriptionally more active in both immature and mature leaves compared to young leaves (Fig. 3C). Taken together, it is conceivable to suggest

that the expression of *APO5* and *MD-PPO2* is spatially and temporally regulated in the Fuji apple plants.

### 3.4. Differential expression of PPO mRNAs in response to mechanical wounding

The wound inducibility of PPO genes has been documented and it has been indicated that PPO plays a critical role in plant defense mechanism [6–9]. Boss et al. [20] showed that Granny Smith apple *APO5* mRNA was induced by wounding in leaf and fruit tissues. Therefore, we considered the possibility that two Fuji apple PPO genes are differentially expressed in response to mechanical damage. Previously, it was shown that PPO mRNA was not induced by wounding in fully grown mature leaves in potato, tomato and tobacco plants [7,8,13,17]. In addition, young leaves exhibited a much greater responsiveness to tissue damage compared to mature leaves of hybrid poplar [9]. Thus, we chose the young Fuji leaf tissue (1 cm in length) for our wound-induction experiment. The immature apple leaves were damaged by slicing with a razor blade into 0.2-cm-long segments, and total RNAs were isolated after different time periods of physical wounding and then analyzed by RNA gel blotting. As shown in Fig. 4A, a significant increase in the *APO5* mRNA level was observed at 3 and 6 h after wounding of young leaves. The level of *MD-PPO2* transcript, however, was not affected by physical damage in this tissue. To examine the expression of PPO mRNAs during the tissue browning reaction of the Fuji apple, the ripened fruits were excised into 1-cm-long segments and incubated for various time periods. A time course study depicted that a slight enhanced level of *APO5* mRNA was detected at 6 h, and the level reached a maximum after 24 h, and subsequently declined (Fig. 4B). As in the case of young leaves, the expression of *MD-PPO2* was not increased at all by wounding in ripened apple fruits (Fig. 4B). It should be noted that among seven PPO gene family members in tomato plant, only one gene is wound-inducible, while the others are developmentally regulated [8,12]. When the ripened apple fruits were wounded, the tissue browning occurred immediately (data not shown). In addition, PPO enzyme activity increased rapidly at 1 h after wounding in the ripened fruits (Fig. 5). The increase in PPO activity continued until 24 h after wounding, and subsequently began to decline. By contrast, the level of *APO5* mRNA increased only slightly between 6 and 12 h (Fig. 4B). Thus, it is possible to assume that the expression of *APO5* is regulated at the post-transcriptional level for the efficient translation to produce the *APO5* enzyme rapidly or there might be another PPO gene(s) whose expression is inducible by wounding in the ripened fruits (Fig. 2A). Alternatively, the rapid wound-induced browning reaction of ripened fruits was mediated by PPO enzyme

synthesized and stored in the early stage of fruit development (see Fig. 3). In apricot fruits, PPO protein and its enzyme activity were present whatever the fruit age, although the *PA-PPO* gene was transcriptionally active only in the early immature-green fruits [18]. Thus, we propose that Fuji apple PPO is a rather stable protein.

From the results described above, it is most likely that two Fuji PPO genes are differentially expressed during vegetative and reproductive development and in response to wounding in the apple plants. These results indicate that different Fuji PPOs may have distinct biochemical properties and *in vivo* functions. This would permit the Fuji apple plant to fine-tune its responses to various developmental and environmental cues. The selective activation of individual genes under different conditions may reflect the existence of distinct signal transduction pathway and *trans*-acting factors to turn on the different PPO genes. To identify the occurrence of PPO isoforms and characterize their biochemical parameters, the individual PPOs encoded by pAPO5 and pMD-PPO2 were expressed in *E. coli* cells as a

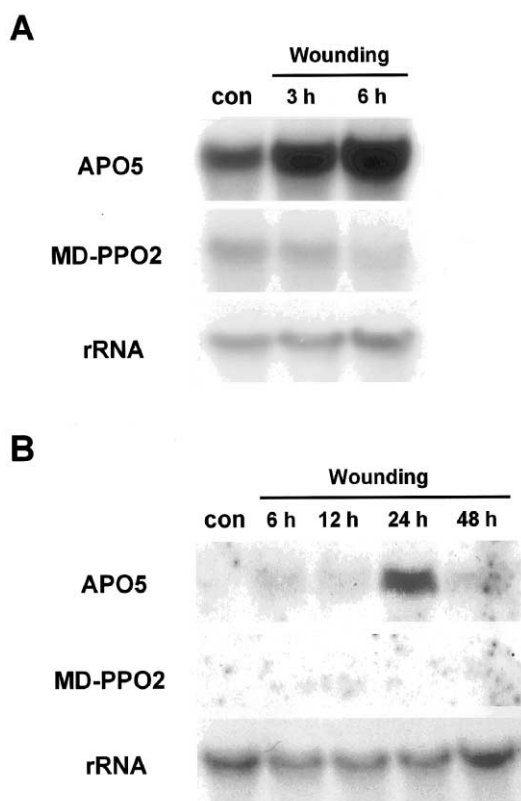


Fig. 4. Differential expression of the Fuji apple PPO genes in response to wounding. Young leaves (A) and ripened fruits (B) were subjected to wounding as described in Section 2 for different time periods. Total RNAs (30  $\mu$ g per lane) were isolated from treated tissues at the indicated time points and resolved on a 1% agarose-formaldehyde gel. The gel was blotted onto membrane filters and the blots were hybridized to  $^{32}$ P-labeled gene specific probe for pAPO5 (probe F114) or pMD-PPO2 (probe F226), or 18S rRNA gene under the high stringent condition.

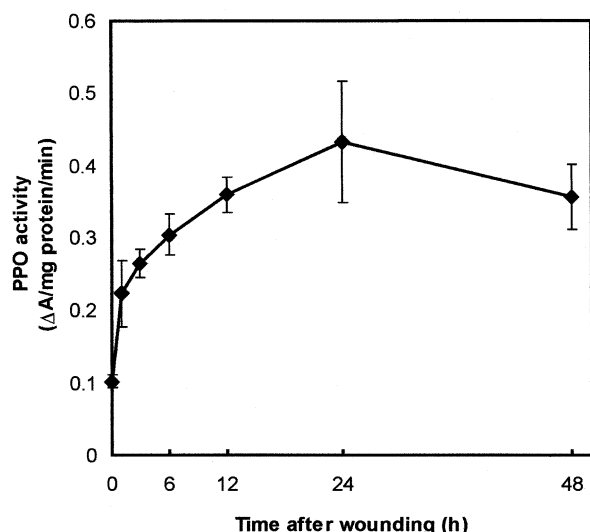


Fig. 5. Changes in PPO enzyme activity in response to wounding in ripened Fuji apple fruits. Ripened fruits were wounded and harvested at the indicated time points for PPO enzyme assays. The values are means  $\pm$  SD ( $n = 3$ ) determined independently.

product of fusion with glutathione *S*-transferase (data not shown). However, the expressed proteins did not show any detectable PPO enzyme activity. This would be due to the fact that numerous higher plant PPOs are latent and require post-translational modification for their full activation, such as intramolecular disulfide bridges, glycosylation or binding by phenolic glucosides [18,28]. We are currently attempting to express the individual PPOs in yeast cells and to study the mode of action of the PPO isoforms.

#### Acknowledgements

This work supported in part by grants from the MAF (a special grant research program), MOST (Plant Diversity Research Center project No. PF 003105-01) and KOSEF (Plant Metabolism Research Center) to W.T.K.

#### References

- [1] J. Nicolas, F.C. Richard-Forget, P.M. Goupy, M.J. Amiot, S.Y. Aubert, Enzymatic browning reactions in apple and apple products, *Crit. Rev. Food Sci. Nutr.* 34 (1994) 109–157.
- [2] A.M. Mayer, E. Harel, Phenoloxidases and their significance in fruit and vegetables, in: F.P. Fox (Ed.), *Food Enzymology*, Elsevier, New York, 1991, pp. 373–398.
- [3] K.C. Vaughn, A.R. Lax, S.O. Duke, Polyphenol oxidase: the chloroplast oxidase with no established function, *Physiol. Plant.* 72 (1988) 659–665.
- [4] K.C. Vaughn, S.O. Duke, Tentoxin stops the processing of polyphenol oxidase into an active protein, *Physiol. Plant.* 60 (1984) 257–261.

- [5] A. Trebst, B. Depka, Polyphenol oxidase and photosynthesis research, *Photosynth. Res.* 46 (1995) 41–44.
- [6] C.P. Constabel, D. Bergey, C.A. Ryan, Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway, *Proc. Natl. Acad. Sci. USA* 92 (1995) 407–411.
- [7] P. Thipyapong, M. Hunt, J. Steffens, Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase, *Phytochemistry* 40 (1995) 673–676.
- [8] P. Thipyapong, J. Steffens, Tomato polyphenol oxidase: differential response of the polyphenol oxidase F promoter to injuries and wound signals, *Plant Physiol.* 115 (1997) 409–418.
- [9] C.P. Constabel, Y. Lynn, J.J. Patton, M.E. Christopher, Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory, *Plant Physiol.* 124 (2000) 285–295.
- [10] S.M. Newmann, N.T. Eannetta, H. Yu, J.P. Prince, C.M. de Vicente, S.D. Tanksley, J.C. Steffens, Organization of the tomato polyphenol oxidase gene family, *Plant Mol. Biol.* 21 (1993) 1035–1051.
- [11] T. Shahar, N. Henning, T. Gutfinger, D. Hareven, E. Lifschitz, The tomato 66.3-kD polyphenoloxidase gene: molecular identification and developmental expression, *Plant Cell* 4 (1992) 135–147.
- [12] P. Thipyapong, D. Joel, J. Steffens, Differential expression and turnover of the tomato polyphenol oxidase gene family during vegetative and reproductive development, *Plant Physiol.* 113 (1997) 707–718.
- [13] M.D. Hunt, N.T. Eannetta, H. Yu, S.M. Newmann, J.C. Steffens, cDNA cloning and expression of potato polyphenol oxidase, *Plant Mol. Biol.* 21 (1993) 59–68.
- [14] P.W. Thygesen, I.B. Dry, S.P. Robinson, Polyphenol oxidase in potato. A multigene family that exhibits differential expression pattern, *Plant Physiol.* 109 (1995) 525–531.
- [15] J.W. Cary, A.R. Lax, W.H. Flurkey, Cloning and characterization of a cDNA coding for *Vicia faba* polyphenoloxidase, *Plant Mol. Biol.* 20 (1992) 245–253.
- [16] R.W. Joy, M. Sugiyama, H. Fukuda, A. Komamine, Cloning and characterization of polyphenol oxidase cDNAs of *Phytolacca americana*, *Plant Physiol.* 107 (1995) 1083–1089.
- [17] M.H.S. Goldman, J. Seurinck, M. Marins, G.H. Goldman, C. Mariani, A tobacco flower-specific gene encodes a polyphenol oxidase, *Plant Mol. Biol.* 36 (1998) 479–485.
- [18] T. Chevalier, D. de Rigal, D. Mbeguie-A-Mbeguie, F. Gauillard, F. Richard-Forget, B.R. Fils-Lycaon, Molecular cloning and characterization of apricot fruit polyphenol oxidase, *Plant Physiol.* 119 (1999) 1261–1269.
- [19] I. Dry, S. Robinson, Molecular cloning and characterization of grape berry polyphenol oxidase, *Plant Mol. Biol.* 26 (1994) 495–502.
- [20] P.K. Boss, R.C. Gardner, B.-J. Janssen, G.S. Ross, An apple polyphenol oxidase cDNA is up-regulated in wounded tissues, *Plant Mol. Biol.* 27 (1995) 429–433.
- [21] S.-K. Sung, G.-H. Yu, G. An, Characterization of MdMADs2, a member of the SQUAMOSA subfamily of genes, in apple, *Plant Physiol.* 120 (1999) 969–978.
- [22] S.-K. Sung, D.-H. Jeong, J. Nam, S.-H. Kim, S.-R. Kim, G. An, Expressed sequence tags of fruits, peels, and carpels and analysis of mRNA expression levels of the tagged cDNAs of fruits from the Fuji apple, *Mol. Cell* 8 (1998) 565–577.
- [23] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
- [24] D.-H. Jeong, S.-K. Sung, G. An, Molecular cloning and characterization of CONSTANS-like cDNA clones of the Fuji apple, *J. Plant Biol.* 42 (1999) 23–31.
- [25] T.D. Sherman, K.C. Vaughn, S.O. Duke, A limited survey of the phylogenetic distribution of polyphenol oxidase, *Phytochemistry* 30 (1991) 2499–2506.
- [26] H. Laukkanen, H. Haggman, S. Kontunen-Soppela, A. Hohtola, Tissue browning of in vitro cultures of Scots pine: role of peroxidase and polyphenol oxidase, *Physiol. Plant.* 106 (1999) 337–343.
- [27] C. Robinson, A. Mant, Targeting of proteins into and across the thylakoid membrane, *Trends Plant Sci.* 2 (1997) 431–437.
- [28] M. Jimenez, F. Garcia-Carmona, The effect of sodium dodecyl sulphate on polyphenol oxidase, *Phytochemistry* 42 (1996) 1503–1509.