Journal of Plant Biology, March 1999, 42(1) : 16-22

Characterization of PPO (Polyphenol Oxidase) cDNA in Sweet Persimmon (*Diospyros kaki*)

Sung Chul Bahn[1], Young Soo Chung[1], Jeong Sheop Shin[1]*, Chang Hoo Lee[2], and Nam In Hyung[3]

[1]Division of Plant Technology, Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[2]Department of Horticultural Science, Korea University, Seoul 136-701, Korea
[3]Department of Horticulture, Sangmyong University, Chunan 330-180, Korea

Polyphenol oxidases (PPOs) are copper metalloproteins which play a critical role in producing various derivatives of polyphenolic compounds. These enzymes are encoded in the nuclear genome and subsequently transported into the plastid. A partial cDNA clone encoding polyphenol oxidase was isolated from immature sweet persimmon leaves. Using two degenerate primers, a single band of PCR product was produced by the touchdown PCR method. In sequence analysis, this clone (define SPO; 1381 bp) revealed high similarities to the previously reported plant PPO genes. Moreover, this SPO clone was comprised of copper A and copper B-binding sites and a histidine-rich region, indicating that this clone contained the key conserved elements of PPO. To characterize the spatial and temporal expression of PPO, total RNAs were extracted from leaf and fruit peel tissues at different developmental stages. In northern analysis, transcripts of PPO were detected in all the samples tested, but the strongest expression levels were detected in mature fruit peels and leaves. Expression of PPO was inhibited in the transitional stage from chloroplast to chromoplast. In addition, the expression of PPO was highly induced in wounded immature fruit peel tissues.

Keywords: Copper metalloprotein, Degenerate primer, PPO (Polyphenol Oxidase), Sweet Persimmon (*Diospyros kaki*), Touchdown PCR

Polyphenol oxidases (PPOs) are nuclear-encoded, copper metalloproteins which play a critical role in producing various derivatives of polyphenolic compounds. These enzymes contain copper, which is essential for their activity (Delhize et al., 1985). Different PPOs catalyze a series of chemical reactions. In general, PPOs catalyze two apparent reactions; one is the hydroxylation of monophenols to *o*-diphenols resulting from the activities of cresolase, tyrosinase or monophenol monooxygenase, and the other is the dehydrogenation of *o*-dihydroxyphenols to *o*-quinones mediated by the activity of catecholase or diphenol oxygen oxidoreductase (Hunt et al., 1993). The *o*-quinones can react with amino acids and proteins to form melanin-like brown pigments, which often cause the loss of quality of fruits and vegetables.

PPOs are plastid-targeted proteins deduced from the existence of transit peptides in the immature polypeptides (Lawrence et al., 1993). Although reports of their occurrence in other cellular parts are abundant, these enzymes are generally localized in the plastid. They have been observed in plastid envelopes and in the thylakoid lumen (Sommer et al., 1994). Because phenolic substrates are located in the vacuole while PPO are in the plastids, the browning reaction only occurs when tissues are damaged and compartmentation is destabilized (Dry et al., 1994).

Several alternative views have been proposed for the function of PPO in plant-pest interactions. Because quinones produced by PPO modify the nucleophilic amino acids such as His, Cys, Met, Trp and Lys, the limitation of these amino acids results in a disturbance of insect growth on plant surfaces. Covalent modification of these essential amino acids in PPO-activated plants decreases their nutritional availability to herbivores and may result in decreased rates of insect growth and pathogen development (Hunt et al., 1993). In addition, direct toxicity of quinones against pathogens has also proposed (Mayer, 1987; Thipyapong et al., 1997).

Recently, Bachem et al. (1994) reported that antisense expression of potato PPO cDNA down-regulated PPO production of the tomato, resulting in pathogen hypersusceptibility without side effects on growth, development, or reproduction. This result confirmed that PPO is involved in defense mechanisms against invading pathogens or insect pests.

Molecular studies on PPO have been performed on a variety of crop plants with the intention of improving post-harvesting quality of agricultural product by suppression of gene expression. As yet, little is known

*Corresponding author; fax +82-2-927-9028
e-mail jsshin@kuccnx.korea.ac.kr

about the molecular biogenesis of sweet persimmon PPO. In order to characterize the molecular basis of this enzyme in sweet persimmon, PPO cDNA was isolated and characterized in this study.

MATERIALS AND METHODS

Plant Materials

Sweet persimmon (*Diospyros kaki*) cultivar "Fuyu" used in this study were obtained from the Kimhae Sweet-Persimmon Experimental Station of the Rural Development Administration, Gyeongnam. Leaves and peels of fruits were collected, frozen in liquid nitrogen and stored at −80°C prior to nucleic acid extraction.

RNA Extraction and Reverse Transcription

Total RNA was isolated from various developmental organs of sweet persimmon: young and mature leaves, and green, colour-turning, fully mature and wounded fruits. RNA was extracted as described by Manning (1991), using two 2-n-butoxyethanol differential precipitation steps. Poly(A)$^+$ RNA was isolated using Dynabeads Oligo(dT)$_{25}$ (Dynal, Norway).

The first strand of cDNA was prepared with 1 μg of poly(A)$^+$ RNA by MMLV (Moloney murine leukemia virus) reverse transcriptase in a final volume of 10 μL, using an anchor oligo (dT) primer containing two degenerate nucleotide positions at the 3' end (Mcpherson et al., 1995). The second strand was synthesized with a mixture of *Escherichia coli* DNA polymerase, RNase H and *E. coli* DNA ligase in a total volume of 80 μL (Clontech, USA), and then purified with phenol and stored at −20°C.

PCR Amplification of Internal PPO cDNA Fragment

Consensus sequences in the conserved regions of the previously published PPO sequences were used to design two degenerate oligonucleotides primers. Forward primer was (A/C)G(A/G)AG(A/G)AATGT(G/T)CTTCTTCG, and reverse primer was (C/T)A(A/T)A(A/G)CT(C/T)CC(T/G/C)GCAAACTC (Wada et al., 1992; Nakamura et al., 1997). These primers were used in the PCR reaction to amplify the internal partial PPO cDNA fragment.

As a template, the double stranded cDNA was diluted with 50 volumes of Tricine-EDTA buffer and denatured for 2 min at 95°C. PCR amplification was carried out with 5 μL of the denatured cDNA, 1 μL of 20 pmol primers, 5 μL of 10X *ExTaq* buffer, 250 μM dNTPs, 1U *ExTaq* DNA polymerase (TaKaRa, Japan) in a total volume of 50 μL. A PCR thermal cycler 480 (Perkin Elmer, USA) was set to denature for 1 min 30 s at 94°C, anneal for 1 min 30 s, and polymerize for 2 min at 72°C, followed by 10 min elongation at 72°C (Erlich et al., 1991). The annealing temperature of this reaction was decreased 1°C every second cycle from 65°C to a 'touchdown' at 50°C, at which temperature 10 cycles were carried out. (Don et al., 1991)

PCR products were separated by electrophoresis on 1% agarose gel. The amplified target DNA band was excised and purified with a PrepMate kit (Bioneer, Korea), and then cloned into pCR2.1 TOPO T/A cloning vector (Invitrogen, USA).

DNA Sequence Analysis

The cDNA insert was sequenced in both directions with M13 universal primers and gene specific primers, using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, USA). Electrophoresis was performed using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA). Nucleotide and amino acid sequences were analyzed using the PC/GENE program (IntelliGenetics, Switzerland).

RNA Gel Blot Analysis

Using High Prime^{TM} (BM, Germany) containing random oligonucleotides, the amplified partial PPO (SPO) was radiolabelled with ^{32}P. Approximately 20 μg of total RNA extracted from various samples were separated on the denatured 1% agarose gels containing 6% (v/v) formaldehyde and 1X Mops buffer (20 mM 4-morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and transferred onto Hybond-N$^+$ membrane (Amersham, England) for 5 h according to the method of Chomczynski (1992).

Membranes were hybridized with the prepared probes at 65°C overnight, washed under high stringent conditions and exposed to X-ray film (Sambrook et al., 1989).

RESULTS AND DISCUSSION

Isolation of Internal cDNA Fragment of PPO

The touchdown PCR method was used in the amplification reaction with degenerate primers because

it gave rise to gene-specific amplification through its unique kinetics (Don et al., 1991). Initially, the regular 3-step PCR method with a stationary annealing temperature was used but initial trials failed to produce a clear single target band. Genomic DNA as well as double stranded cDNA was used as PCR template. Several non-specific bands were observed from the amplification with genomic DNA. Sequence analyses, however, revealed the putative fragments as false amplification (data not shown). To circumvent the generation of non-specific smaller fragments, the touchdown PCR method was used.

Using double stranded cDNA originated from a poly (A)$^+$ RNA mixture of various developmental leaves and fruits, touchdown PCR was performed with two degenerate primers deduced from the previously published PPO sequences of various plant species. As a result, a clear 1,381 bp fragment was amplified, which was thought to be an internal PPO gene fragment from its expected size (Fig. 1). In terms of multiplicity of the gene, PPO genes were known to be present as multigene families in most species (Thygesen et al., 1995), but single PPO genes had been identified in a few species (Dry et al., 1994).

The PCR amplified band was subcloned into a cloning vector and six colonies were chosen for sequencing to confirm the multiplicity of the gene. The sequence analysis of the amplified fragment revealed that all the colonies represented a single

Figure 1. Agarose gel electrophoresis of the amplified PPO cDNA fragment by RT-PCR. The ds cDNA was diluted with 50X Tricine EDTA buffer (Lane 1) and sterile water (Lane 2). Two degenerate primers, forward primer (A/C)C(A/G)AG(A/G)AATGT(G/T)CTTCTTCGG and reverse primer (C/T)A(A/T)A(A/G)C T(C/T)CC(T/G/C)CCAAACTC were used for touchdown PCR. Lane M is the Lambda/*Hin*dIII+*Eco*RI molecular weight marker.

gene, and the presence of two degenerate primers at both 5' and 3' ends was confirmed. Usage of the high fidelity enzyme, *LATaq* (Takara, Japan), known for its top performance in PCR amplification, seemed to provide consistent results as indicated in the sequence analysis. The sequences of the six clones were identified, suggesting that they all originated from a single gene (data not shown).

Sequence Analysis and Characterization of PPO cDNA Clones

The subcloned fragment was sequenced using the internal gene specific primers and analyzed. The comparison of the internal fragment of PPO with the various PPO sequences derived from other plants revealed that it contained almost all nucleotide sequences encoding an immature PPO except small parts of the 3' and 5' termini (Figs. 2 and 3). The unidentified 5' sequences seemed to include a transit peptide for plastid targeting (Joy et al., 1995). Nonetheless, the conserved cleavage site for mature PPO protein was confirmed at 70 bp downstream from the 5' end of the fragment (Hunt et al., 1993; Dry et al., 1994). It has been suggested that the C-terminal extension might be involved in targeting the protein to the chloroplast thylakoid membranes (Sommer et al., 1994). However, the function of the C-terminal extension was still not clear with the limited amount of information available. In this study, to obtain the 3' terminal sequence could not provide any information on the C-terminal processing.

Like grape PPO "GPO1" (Dry et al., 1994), apple PPO "APO5" (Boss et al., 1995), tomato PPO "TOM C" (Newman et al., 1993) and potato PPO "POT32" (Thygesen et al., 1995), the partial N-terminus of persimmon PPO contained a high content of the hydrophobic amino acids (Ala and Val) (Flurkey, 1989). The two copper-binding regions, A and B, which play a critical role in the activity of PPO were also found in the persimmon PPO sequence. These two domains are known to be highly conserved between and within plant species. Histidine residues predicted to be the copper-binding ligands for the activity of tyrosinase and haemocyanin (Cary et al., 1992; Shahar et al., 1992; Dry et al., 1994) showed complete homology in copper binding region.

A phylogenetic analysis of the persimmon PPO with 12 deduced PPOs from 4 different plant species was used to assign the genetic relationship of SPO (AF039165) to others (Fig. 4). Three separate branches of PPOs were observed in the phylogenetic tree. Per-

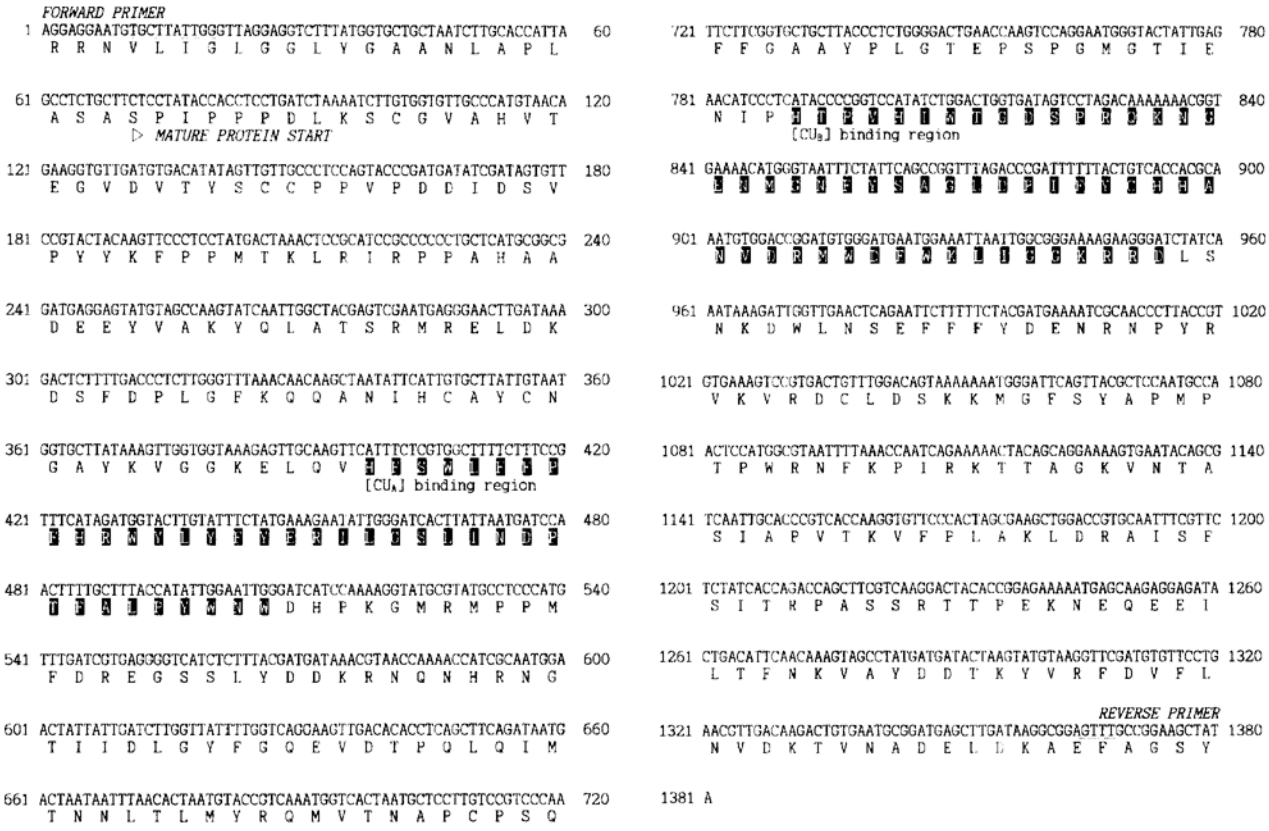

Figure 2. Translated amino acid sequence of the partial cDNA clone encoding persimmon PPO protein. The regions of the two degenerate primers used for synthesis are underlined. The N-terminal amino acid of the mature persimmon PPO protein is marked. Two putative copper binding regions are indicated by black highlighting and marked [CU_A] and [CU_B].

simmon PPO was not clustered with the PPOs from grape and apple which are thought to be closely related by morphology, but was clustered in the same subgroup with TOM E, one of the tomato PPOs. It seems that tomato PPOs have most genetic diversity while apple PPOs are genetically narrow. Two potato PPOs are clustered within the same subgroup but they are also separated by tomato PPOs. These results indicate that low levels of sequence homology even in the same species was prevalently exist, and that the amino acid sequences of PPOs was not corresponded to the phylogenetic or morphological relatedness among plants.

Expression of PPO in Persimmon Tissues

In order to investigate the spatial and temporal expression of PPO, total RNAs isolated from various organs of sweet persimmon were characterized by northern blot analysis.

The presence of PPO transcripts was examined in fruits and leaves at different developmental stages (Fig. 5). Recently, many studies of PPO expression have been reported in various plants. Dry et al. (1994) showed that the PPO mRNA was highly expressed in young developing tissues of grape berries and leaves but not detected in other developing tissue. Similar developmental expression patterns were shown in tomato (Shahar et al., 1992) and potato (Hunt et al., 1993). However, the results of this study revealed that the PPO gene of sweet persimmon was expressed in fruits and leaves in all developmental stages. Especially high expression levels were detected in fully mature and developed fruits and leaves. An investigation of plastid-targeted proteins has revealed that their expression stops 5 to 10 days before the fruits are fully matured (Lawrence, 1993). The relatively weak signal from fruits at the color-turning stage appeared to result from the transition of chloroplast to chromoplast when the expression of PPO was reduced; corresponding with the existence of its transit peptide and localization in plastid. The expression pattern of PPO in various organs and at various times indicates that the gene expression is

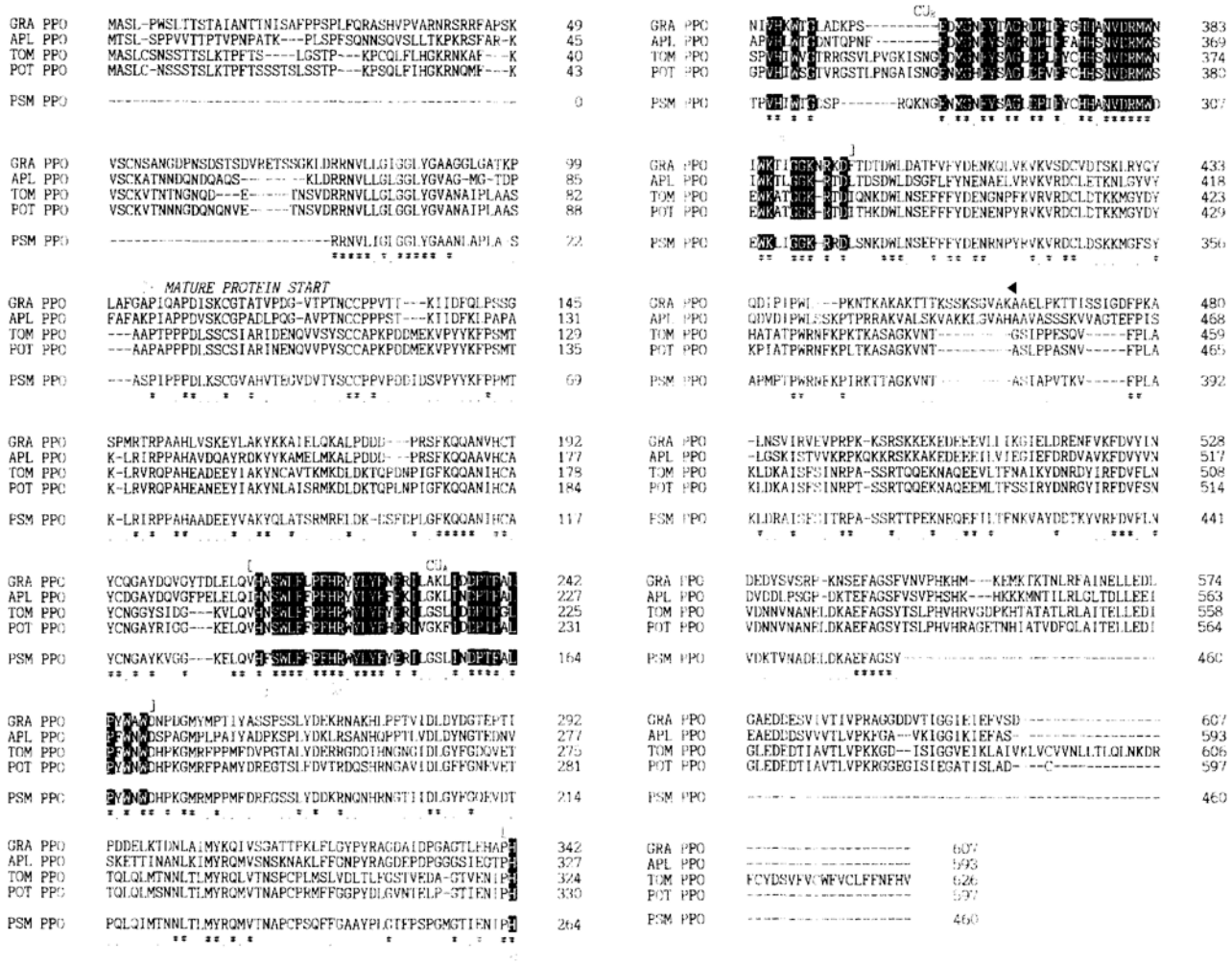

Figure 3. Comparison of the deduced amino acid sequence of sweet persimmon PPO cDNA with sequences of previously published PPOs from other plants. Conserved amino acid residues are indicated by '*' and similar amino acid residues by '.'. Identical amino acid residues in the putative copper binding region[CU_A] and [CU_B] of other known PPO are shown with black highlighting. The N-terminal amino acid of the mature persimmon PPO protein is marked by '↑' and the predicted site of C-terminal cleavage of grape PPO is marked by '◀'. A gap(-) was introduced in the PPO sequences for alignment purposes. The histidine residues known to be involved in copper binding in tyrosinase and haemocyanin are marked by '↖'.

nearly constitutive; probably due to its important role in defense mechanisms.

For a more detailed study on the induced expression of persimmon PPO during fruit-tissue browning after wounding, total RNA was isolated from wounded fruits at the color-turning stage, at 0, 6, 12, and 24 h, respectively (Fig. 6). High levels of PPO mRNA accumulation were detected in wounded fruit tissues. The expression of PPO was maximized at 6 h and maintained until 24 h after wounding. This observation revealed that the induction of PPO expression culminates quickly in sweet persimmon. However, the detection of PPO by northern analysis, especially at high levels, seemed not to be frequent case. Other researches often failed to detect the PPO activity by northern analysis even in fruit tissue without wounding, though the gene was detected by RT-PCR in the same tissue (Boss et al., 1995). The wound-inducible gene expression of persimmon also revealed that the expression of PPO was controlled in mRNA levels, and specifically up-regulated in this case.

In conclusion, although the possible roles of PPO in sweet persimmon have not yet been defined, its unique mechanisms of enzymatic function lie in the defense of the cell when the cell structure is collapsed by mechanical or biological damage.

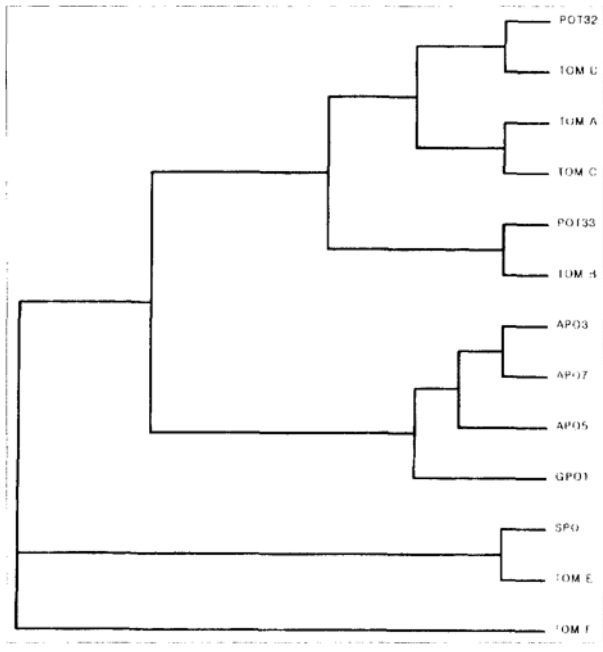

Figure 4. Phylogenetic analysis based on the deduced amino acid sequence from SPO (AF039165) and other PPOs like grape PPO "GPO1" (Dry et al., 1994), apple PPO ["APO5" (Boss et al., 1995), "APO3" (D87669) and "APO7" (D87670)], tomato PPO ["TOM A(A')", "TOM B", "TOM C", "TOM D", "TOM E", and "TOM F" (Newman et al., 1993)] and potato PPO ["POT32" and "POT33" (Thygesen et al., 1995)].

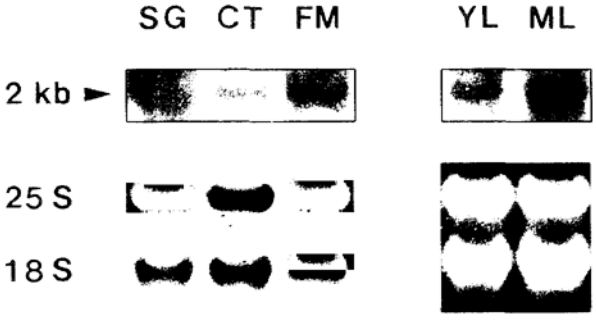

Figure 5. RNA northern hybridization analysis of the induced PPO mRNA by ripening in persimmon. For each sample, 20 μg of total RNAs was loaded, from small green fruits (5 cm in diameter)(SG), color-turning fruits (CT), fully mature fruits (FM), immature small leaves without wax (YL), and mature leaves coated by wax (ML). Gels were ethidium bromide-stained to ensure equal loading of total RNA.

ACKNOWLEDGEMENT

We are grateful to the Kimhae Sweet-Persimmon Experimental Station of the Rural Development Administration for providing us with sweet persimmon material. This research was supported by a grant from the Korea Science and Engineering Foundation (project No. 95-04-02-10-01-3).

Received October 2, 1998; accepted January 8, 1999.

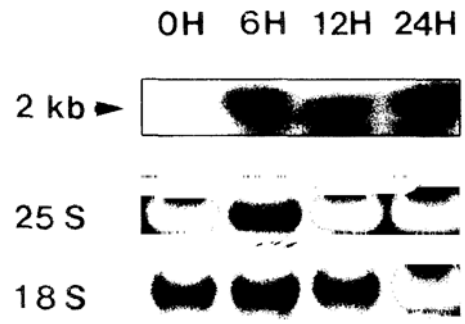

Figure 6. RNA northern hybridization analysis of the induced PPO mRNA after wounding in immature persimmon fruits. Each total RNA was isolated from persimmon peel samples, while the superficial wound was developed after being cut with a sterile knife. Each peel samples were obtained from fruit tissue in 0, 6, 12, 24 h after wounding. Gels were ethidium bromide-stained to ensure equal loading of total RNA.

LITERATURE CITED

Bachem CWB, Speckmann GJ, Linde PCG, Verheggen FTM, Hunt MD, Steffens JC, Zabeau M (1994) Antisense Expression of Polyphenol Oxidase Genes Inhibits Enzymatic Browning in Potato Tubers. Bio Technology **12**: 1101-1105

Boss PK, Gardner RC, Janssen BJ, Ross GS (1995) An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. Plant Mol Biol **27**: 429-433

Cary JW, Lax AR, Flurkey WH (1992) Cloning and characterization of cDNAs coding for *Vicia faba* Polyphenol Oxidase. Plant Mol Biol **20**: 245-253

Chomczynski P (1992) One-Hour Downward Alkaline Capillary Transfer for Blotting of DNA and RNA. Anal Biochem **201**: 134-139

Delhaize E, Lonergan JF, Webb J (1985) Development of three copper metalloenzymes in clover leaves. Plant Physiol **78**: 4-7

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res **19**: 4008

Dry IB, Robinson SP (1994) Molecular cloning and characterisation of grape berry polyphenol oxidase. Plant Mol Biol **26**: 495-502

Erlich HA, Gelfand D, Sninsky JJ (1991) Recent Advances in the Polymerase Chain Reaction. Science **252**: 1643-1650

Flurkey WH (1989) Polypeptide Composition and Amino-

Terminal Sequence of Broad Bean Polyphenoloxidase. Plant Physiol **91**: 481-483

Hunt MD, Eannetta NT, Yu H, Newman SM, Steffens JC (1993) cDNA cloning and expression of potato polyphenol oxidase. Plant Mol Biol **21**: 59-68

Joy RW, Sugiyama M, Fukuda H, Komamine A (1995) Cloning and Characterization of Polyphenol Oxidase cDNAs of *Phytolacca americana*. Plant Physiol **21**: 59-68

Lawrence SD, Cline K, Moore GA (1993) Chromoplast-Targeted Proteins in Tomato (*Lycopersicon esculentum Mill.*) Fruit. Plant Physiol **102**: 789-794

Manning K (1991) Isolation of Nucleic Acids from Plants by Differential Solvent Precipitation. Anal Biochem **195**: 45-50

Mayer AM (1987) Polyphenol oxidase in plants-recent progress Phytochemistry **26**: 11-20

Mcpherson MJ, Hames BD, Taylor GR (1995) PCR2: A Practical Approach. IRL Press, Oxford

Nakamura Y, Gojobori T, Ikemura T (1997) Codon usage tabulated from the international DNA sequence database. Nucleic Acids Res **25**: 244-245

Newman SM, Eannetta NT, Yu H, Prince JP, Vicence MCD, Tanksley ED, Steffens JC (1993) Organisation of the tomato polyphenol oxidase gene family. Plant Mol Biol **21**: 1035-1051

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Shahar T, Hennig N, Guffinger T, Hareven D, Lifschitz E (1992) The Tomato 66.3-kD Polyphenoloxidase Gene: Molecular Identification and Developmental Expression. The Plant Cell **4**: 135-147

Sommer A, Ne'eman E, Steffens JC, Mayer AM, Harel E (1994) Import, Targeting, and Processing of a Plant Polyphenol Oxidase. Plant Physiol **105**: 1301-1311

Thipyapong P, Joel DM, Steffens JC (1997) Differential Expression and Turnover of the Tomato Polyphenol Oxidase Gene Family during Vegetative and Reproductive Development. Plant Physiol **113**: 707-718

Thygesen PW, Dry IB, Robinson SP (1995) Polyphenol Oxidase in Potato. (A Multigene Family That Exhibits Differential Expression Patterns). Plant Physiol **109**: 525-531

Wada KN, Wada Y, Ishibashi F, Gojobori T, Ikemura T (1992) Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res **20**: 2111-2118