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

Sung Chul Bahn¹, Young Soo Chung¹, Jeong Sheop Shin^{1*}, Chang Hoo Lee², and Nam In Hyung³

¹Division of Plant Technology, Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea ²Department of Horticultural Science, Korea University, Seoul 136-701, Korea ³Department of Horticulture, Sangmyong University, Chuban 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 PPOactivated 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 iovading 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 μ g of poly(A)⁺ RNA by MMLV (Moloney murine leukemia virus) reverse transcriptase in a final volume of 10 μ L, using an anchor oligo (dT) primer containing two degenerate nucleotide positions at the 3^e 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 μ L (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)CTTCTTGG, 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 μ L of the denatured cDNA, 1 μ L of

20 pmol primers, 5 μ L of 10X *ExTaq* buffer, 250 μ M dNTPs, 1U *ExTaq* DNA polymerase (TaKaRa, Japan) in a total volume of 50 μ L. 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 PrimeTM (BM, Germany) containing random oligonucleotides, the amplified partial PPO (SPO) was radiolabelled with ³²P. 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 1381 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 tragment 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)G(A/G)AG(A/G)AATGT(G/T)CTTCTTGG and reverse primer (C/T)A(A/T) A(A/G)CT(C/T)CC(T/G/C)GCAAACTC were used for touch-down PCR. Lane M is the Lambda/HindIII+FcoRt 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 tragment 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 (lov 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 (Flunt 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^t 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-

1	FORWARD PRIMER AGGAGGAATGTGCTTATTGGGTTAGGAGGTCTTTATGGTGCTGCTAATCTTGCACCATTA R R N V L I G L G G L Y G A A N L A P L	60	721 TTCTTCGGTGCTGCTTACCCTCTGGGGACTGAACCAAGTCCAGGAATGGGTACTATTGAG 780 FFGAAYPLGTEPSPGMGTIE					
61	GCCTCTGCTTCTCCCTATACCACCCCCTGATCTAAAATCTTGTGGTGTTGCCCATGTAACA A S A S P I P P P D L K S C G V A H V T \triangleright MATURE PROTEIN START	120	781 AACATCCCTCATACCCCCGGTCCATATCTGGACTGGTGGTGGTGGTGGTGGTGGCGACAAAAAACGGT 840 N I P II					
121	GAAGGTGTTGATGTGACATATAGTTGTTGCCCTCCAGTACCCGATGATATCGATAGTGTT E G V D V T Y S C C P P V P D D I D S V	180	841 GAAAACAIGGGTAATTICTATICAGCCGGITTAGACCCGAITTITIACTGICACCACGCA 900 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9					
181	$ \begin{array}{c} \texttt{CCGTACTACAAGTTCCCTCCTATGACTAAACTCCGCCATCCGCCCCCTGCTCATGCGGCG} \\ \texttt{P} & \texttt{Y} & \texttt{K} & \texttt{F} & \texttt{P} & \texttt{M} & \texttt{T} & \texttt{K} & \texttt{L} & \texttt{R} & \texttt{P} & \texttt{P} & \texttt{A} & \texttt{H} & \texttt{A} & \texttt{A} \end{array} $	2 4 0	901 AATGTGGACCGGATGTGGGATGAATGGAAATTAATTGGCGGGAAAAGAAGGGATCTATCA 960 N N N N N N N N N N N N N N N N N N N					
241	GATGAGGAGTATGTAGCCAAGTATCAATTGGCTACGAGTCGAATGAGGGAACTTGATAAA D E E Y V A K Y Q L A T S R M R E L D K	300	961 AATAAAGATIGGTTGAACTCAGAATICTITTICTACGATGAAAATCOCAACCCTTACCGT 1020 N K D W L N S E F F F Y D E N R N P Y R					
301	GACTCTTTTGACCCTCTTGGGTTTAAACAACAAGCTAATATTCATTGTGCTTATTGTAAT D S F D P L G F K Q Q A N I H C A Y C N	360	1021 GTGAAAGTCCGTGACTGTTTGGACAGTAAAAAAATGGGATTCAGTTACGCTCCAATGCCA 1080 V K V R D C L D S K K M G F S Y A P M P					
361	GGTGCTTATAAAGTTGGTGGTAAAGAGTTGCAAGTTCATTTCTGGTGGCTTTTCTTTC	420	1081 ACTOCATGGCGTAATTTTAAACCAATCAGAAAAACTACAGCAGGAAAAGTGAATACAGCG 1140 T P W R N F K P I R K T T A G K V N T A					
421		480	1141 TCAATTGCACCCGTCACCAAGGIGTTCCCCACTAGCGAAGCTGGACCGTGCAATTTCGTTC 1200 S I A P V T K V F P L A K L D R A I S F					
481	ACTITIGCTITACCATATTGGAAITGGGATCATCCAAAAGGTATGCGTATGCCTCCCATG	540	1201 TCTATCACCAGACCAGCTTOGTCAAGGACTACACCGGAGAAAATGAGCAAGAGGAGATA 1260 S I T R P A S S R T T P E K N E Q E E 1					
541	TTTGATCGTGAGGGGTCATCTCTTTACGATGATAAACGTAACCAAAACCATCGCAATGGA F D R E G S S I. Y D D K R N Q N H R N G	600	1261 CTGACATTCAACAAAGTAGCCTATGATGATACTAAGTATGTAAGGTTCGATGTGTTCCTG 1320 L T F N K V A Y D D T K Y V R F D V F L					
601	ACTATTATTGATCTTGGTTATTTTGGTCAGGAAGTTGACACCACCTCAGCTTCAGATAATG T I I D L G Y F G Q E V D T P Q L Q I M	660	REVERSE PRIMER 1321 AACGTTGACAAGACTGTGAATGCGGATGAGCTTGATAAGGCGGAGTTTGCCGGAAGCTAT 1380 N V D K T V N A D E L D K A E F A G S Y					
661	ACTAATAATTTAACACTAATGTACCGTCAAATGGTCACTAATGCTCCTTGTCCGTCC	720	1381 A					
Figure 2. Translated amino acid sequence of the partial cDNA clone encoding persimmon PPO protein. The regions of the two								

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

				CU*	
GRA PPO APL PPO TOM PPO POT PPO	MASL-PWSLITSTAIANTINISAFPPSPLFQRASHVPVARNRSRRFAPSK MTSL-SPPVVTTPTVPNPATKPLSPFSONNSOVSLITKPKRSPARK MASLCSNSSTISLKTPFTSLGSTPKPSOLPTHGKRNOMFK MASLC-NSSSTSLKTPFTSSSTSLSSTPKPSOLPTHGKRNOMFK	49 45 40 43	GRA PPO APL PPO TOM PPO POT PPO	NI WKAT DI ADKPSDX-NY YIAFRUTI PERITANUTRAN APPLIAT DATORAF	383 369 374 380
PSM PPO		0	PSM ⊴PO	TPEED TO BESPROKING NAME NO BED NO TRANSPORT	307
GRA PPO APL PPO TOM PPO POT PPO	VSCNSANGDPNSDSTSDVRETSSGKLDRRNVLLGIGGLYGAAGGLGATKP VSCKATNNDONDOACSKLDRRNVLLGLGGLYGVAG-MG-TDP VSCKVTNTNGNQDETNSVDRRNVLLGLGGLYGVANAIPLAAS VSCKVTNNNGDQNQNVETNSVDRRNVLLGLGGLYGVANAIPLAAS	99 85 82 88	GRA PPO APL PPO TOM PPO POT PPO	J I AKTI TOKNENG DETOTTUWLDATEVEYDENKGLVKVKVSDCVDTSKI.RYCY I AKTL TOK EDDITDSDWLDSGELEYNENAELVRVKVRDCLETKNI.GYVY EXKATANK EDDITONDWLNSEFFFYDENGNPFKVRVRDCLDTKKMGYDY EXKATANK EDDITHKDWLNSEFFFYDENENPYRVKVRDCLDTKKMGYDY	433 418 423 429
PSM PPO	RNVLIGIGGLYGAANLAPLA S	22	PSM PPC	EXILI CER ROLSNKIMLNSEFFFYDENRNPYFVKVRDCLDSKKMGFSY	356
GRA PPO APL PPO TOM PPO FOT PPO	MATURE PROTEIN START LAFGAPIOAPDISKCGTATVPDG-VTPTNCCPPVTTKIIDFOLPSSG FAFAKPIAPPDVSKCGPADLPOG-AVPTNCCPPPSTKIIDFKIPAPAAAPTPPPDLSSCSIARIDENQVVSYSCCAPKPDDMEKVPYYKFPSMTAAPAPPPDLSSCSIARIDENQVVPYSCCAPKPDDMEKVPYYKFPSMT	145 131 129 135	GRA PPO APL PPO TOM PPO POT PPO	QD:PIPHIPKNTKAKAKTITKSSKSUVAGLPKTTISSIGDFPKA QDVDIPHISSKPTPRAKVALSKVAKKLUVAHAAVASSSKVVAGTEFPIS HATATPHRNFKPKTKASAGKVNTGSIPPESUVFPLA KPIATPHRNFKPLTKASAGKVNTASLPPASNVFPLA	480 468 459 465
PSM PPO	ASPIPPPDLKSCGVAHVTEGVDVTYSCCPPVPDDIDSVPYYKFPPMT	69	PSM PPO	APMPTPWRNEKPIRKTTAGKVNTEPLA	392
GRA PPO APL PPO TOM PPO POT PPC	SPMRTRPAAHLVSKEYLAKYKKAIELQKALPDDUPRSFKQQANVHCT K-LRIRPPAHAVDQAYROKYYKAMELMKALPDDUPRSFKQQAAVHCA K-LRVROPAHEADEEYIAKYNCAVTKMKDLDKTQPDNPIGFKQQANIHCA K-LRVRQPAHEANEEYIAKYNLAISRMKDLDKTQPLNPIGFKQQANIHCA	192 177 178 184	GRA PPO APL PPO TOM PPO POT PPO	-LNSVIRVEVPRPK-KSRSKKEKEDEEEVLIIKGIELDRENFVKFDVYIN -LOSKISTVVRPEKOKKRSKAKEDEEETIVIEGIEFDRDVAVKEDVYN KLDKAISFSINRPA-SSRTGOEKNAGEEVLTFNAIKVDNRDYIRFDVFLN KLDKAISFSINRPT-SSRTGOEKNAGEEMLTFSSIRVDNRGYIRFDVFSN	528 517 508 514
PSM PPO	K-LRIRPPAHAADEEYVAKYQLATSRMRFIDK-ESFEPLGFKQQANIHCA	117	ESM PPO	KLDRAISESTTRPA-SSRTTPEKNEGEFTLTFNKVAYDDTKYVREDVFLN	441
GRA PPC APL PPO TOM PPO POT PPO	CUA VCQGAYDOVGYTDLELOVAASHTELPFHRVITYINERLAKLIOOPTEAL VCDGAYDQVGFPELELOVAASHTEPFHRVITYINEKLAKLIOOPTEAL VCNGGYSIDGKVLOVANSALTEPHHRVITYINELGSLIOOPTIGI VCNGAYRIGGKELOVENSALTEPPHRVITYINETRVGKFIOOPTIAL	242 227 225 231	GRA FPG APL FPO TOM FPO FOT FPO	DEDYSVSRP-KNSEFAGSFVNVPHKHMKEMKIKTNLRFAINELLEDL DVDDLPSCP-DKTEFAGSFVSVPHSHKHKKKMNTILRLGLTDLLEEI VDNNVNANELDKAEFAGSYTSLPHVHRVGDPKHTATATLRLAITELLEDI VDNNVNANELDKAEFAGSYTSLPHVHRAGETNHIATVDFGLAITELLEDI	574 563 558 564
PSM PPO	YCNGAYKVGGKELOV JFSMIBFDTHDWYINTYDROL GSL ONDETBAD	164	PSM PPO	VDKTVNADELDKAEFAGSY	46C
GRA PPO APL PPO TOM PPO POT PPO] EYAK KONPUGMYMPTIYASSESSLYDEKRNAKHLPPTVIDLDYDGTEPTI EFAN DSPAGMPLPAIYADPKSPLYDKLRSANHOPPTLVDLDYNGTEDNV EFAN DHEKGMEPPMPDVPGTALYDERNGDDINNGNGIDLGVFGDOVET EYAN DHEKGMEPPAMYDREGTSLFDVTRDQSHRNGAVIDLGFFGNEVET	292 277 275 281	GRA PPO APL PPO TOM PPO POT PPO	GAEDEESVIVTIVPRAGGDUVTIGGIEIEFVSD EAEDDDSVVVTLVPKFGAVKIGGIKIEFAS- GLEDEDTIAVTLVPKKGDISIGGVEIKLAIVKLVCVVNLLTLOINKDR GLEDEDTIAVTLVPKRGGEGISIEGATISLADC	607 593 606 597
PSM PPC	BY BNB DHPKGMRMPPMF DREGSSLYDDKRNGNHRNGT I I DLGYFCOEVDT	214	PSM PPO		460
GRA PPO APL PPO TOM PPO POT PPO	PDDELKTUNLAIMYKOIVSSATTFKLFLGYPYRACDAIDPGAGTLFHA SKETTINANLKIMYROMVSNSKNAKLFFCNPYRAGDEPDFGGSIEGTF TQLDIMTNLTLMYROMVTNAPCPRMFFGGPYDLGVNTELP-STIENIP TQLOLMSNNLTIMYROMVTNAPCPRMFFGGPYDLGVNTELP-STIENIP	342 327 324 330	GRA PPO APL PPO TOM PPO POT PPO	607 593 FCYDSVFVCWFVCLFFNFHV 526 597	
PSM PPO	PQLQIMTNNLTI.MYRQMVTNAPCPSQFFGAAYPI.CIFPSPGMGTIFNIP	264	PSM PPO	460	

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 's' and similar amino acid residues by 'c', 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 $-\frac{1}{2}$, and the predicted site of C-terminal cleavage of grape PPO is marked by $-\frac{1}{2}$. 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 $-\frac{1}{2}$.

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 truit 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 detense of the cell when the cell structure is collapsed by mechanical or biological damage.

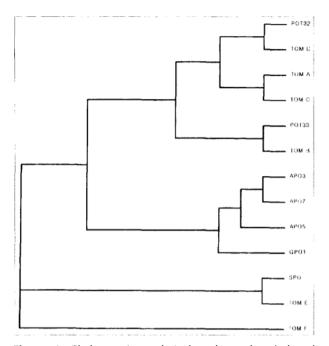


Figure 4. Phylogenetic analysis based on the deduced amino acid sequence from SPO (AF039165) and other PPOs like grape PPO "GPO1" (Dry et al., 1994), apple PPO ["APO5" (Boss et al., 1995), "APO3" (D87669) and "APO7" (D87670)], tomato PPO ["TOM A(A')", "TOM B", "TOM C", "TOM D", "TOM E", and "TOM F" (Newman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

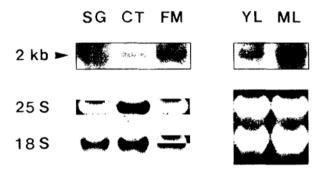


Figure 5. RNA northern hybridization analysis of the induced PPO mRNA by ripening in persimmon. For each sample, 20 μ g of total RNAs was loaded, from small green fruits (5 cm in diameter)(SG), color-turning fruits (CT), fully mature fruits (FM), immature small leaves without wax (YL), and mature leaves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

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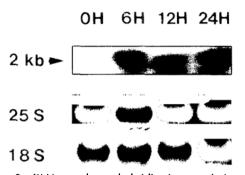


Figure 6. RNA northern hybridization analysis of the induced PPO mRNA after wounding in immature persimmon fruits. Each total RNA was isolated from persimmon peel samples, while the superficial wound was developed after being cut with a sterile knife. Each peel samples were obtained from fruit tissue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

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