

Cloning and expression analysis of ascorbate peroxidase gene from eggplant under flooding stress

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ABSTRACT. Previously, we found that flooding leads to an increase in APX enzymatic activity in the roots of eggplants. The objectives of this work were to clone the ascorbate peroxidase (APX) gene, and measure the regulation of APX gene expression in different tissues of eggplant under flooding stress conditions. Different tissues from eggplants displayed wide variations in their expression profiles using Real-Time PCR. The highest level of APX transcripts were detected in roots of EG117 at 72 h of flooding treatment. The differential expressions of each tissue and genotype were directly associated with flooding stress responses. After screening and comparing APX gene sequences at the NCBI database, the degenerate primer sets designed from tomato and potato were used to amplify the APX cDNA of eggplant with the reverse-transcription PCR method. The completion of a full-length of APX cDNA was performed using 5' and 3' rapid amplification of cDNA ends (RACE) technique. The open reading frame of cDNA clone was 753 base pair long encoding a cytosolic APX (cAPX). The sequence of eggplant APX gene had 96%, 95%, 93% and 91% homology to that from the potato, tomato, pepper and tobacco APX gene, respectively. A phylogenetic analysis of the deduced amino acid sequence of APX by Neighbor-Joining method indicated that the plant cAPXs diverge into two major clusters, and eggplant cDNA is more closely related to potato than to tomato. Southern blot analysis revealed that the eggplant gene encoding APX had two copies. These results indicate that the cAPX of eggplant may be involved in hydrogen peroxide-detoxification and thus helps overcome the stress induced by flooding.

Keywords: Ascorbate peroxidase; Eggplant; Flooding; Phylogenesis; Real-Time PCR.

INTRODUCTION

APX (EC 1.11.1.11) is a hydrogen peroxide-scavenging enzyme with a presumed function of protecting cells from hydrogen peroxide accumulation, particularly under stressful conditions. It catalyzes the reduction in hydrogen peroxide, using ascorbate as an electron donor, to yield water and oxidized ascorbate. The lack of suitable electron acceptors leads to the saturation of redox chains, the accumulation of NADPH, and a decline in ATP generation. The ascorbate-glutathione cycle has been shown to be of great importance in multiple stress reactions (Blockina et al., 2003). In flooded soil, oxygen limitation is one of the primary threats to plants. Excess production of reactive oxygen species (ROS)—superoxide radicals, hydrogen peroxide, singlet oxygen, and hydroxyl radicals—causes oxidative damage to cellular components, and their involvement in a number of biotic and abiotic stresses is well documented (Shigeoka et al., 2002). APX has been found in higher plants, algae, and some cyanobacteria, but

not in animals (Shigeoka et al., 2002). In higher plants, APX isozymes are distributed in at least four distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX), mitochondrial membrane-bound APX (mitAPX), and cytosolic APX (cAPX) (Kawakami et al., 2002). For example, in *Arabidopsis*, the APX gene family includes two cytosolic isoforms, APX1 and APX2, microsomal enzyme APX3, cAPX, sAPX, and tAPX (Jespersen et al., 1997). Heat stress induces oxidative stress and triggers the expression of APX1 and APX2 genes at the RNA level in *Arabidopsis*. Excessive light stress also induces APX1 expression (Karpinski et al., 1997; Panchuk et al., 2002).

Antioxidant enzymes and their corresponding genes have been studied in many species. We have already demonstrated that flooding stress raised ROS levels in plants and induced various kinds of antioxidative enzymes to overcome oxidative stress (Lin et al., 2004). APX was at its most active in eggplant roots under flooding stress and played an important role in detoxifying the H₂O₂ that flooding had generated in eggplant root. Saturation

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of soil fills air pockets with water, creating hypoxic conditions followed by anoxia. During flooding stress, many physiological changes occur that result in increased flooding tolerance. One of these may be increased expression of APX genes to protect against oxidative stress. cDNAs which encode APX of pea (Mittler and Zilinskas, 1992), tomato (Gadea et al., 1999; Zou et al., 2006), sweet potato (Tseng et al., 2002; Park et al., 2004; Lin et al., 2006), potato (Kawakami et al., 2002), *Arabidopsis* (Kubo et al., 1992; Santos et al., 1996), pumpkin (Yamaguchi et al., 1996), spinach (Webb and Allen, 1995; Ishikawa et al., 1996), maize (Bresagem et al., 1995), strawberry (Kim and Chung, 1998), tobacco (Ovar and Ellis, 1997), and barley (Shi et al., 2001) have been isolated. However, eggplant APX cDNA has not been cloned, and it is not known which form of APX efficiently detoxifies H₂O₂ in eggplant roots under flooding stress. Nothing is known about the response of APX gene expression and regulation under flooding stress. In this study, we investigate how APX gene expresses in different tissues of eggplants in response to flooding treatment. We also clone a full-length APX cDNA, and analyze evolution of the APX gene among plant species.

MATERIALS AND METHODS

Plant materials, cultivation and flooding treatment

'Pingtong Long Eggplant' (EG117) and eggplant 'EG203' were sown on April 2004 in the greenhouse of the Chinese Culture University. EG117 is more flood tolerant than EG203, and is used as rootstock for tomatoes in Taiwan (Lin et al., 2004). The seedlings were transplanted into 5-inch plastic pots containing a medium consisting of peat moss, loamy soil, and sand in a ratio of 2:1:1 (v/v/v) on May 2004. Plants were watered every other day, and an optimal amount of compound fertilizer solution (N-P₂O₅-K₂O, 20-20-20) was applied once a week. The two mentioned varieties (EG117 and EG203) and ten flooding treatments (0 min, 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h) were carried out on July, 2004, when the plants were 15 cm high. All pots for three replications of each flooding time for the treatment group were randomly placed in a 28 cm × 14 cm × 14 cm plastic bucket containing a tap water level 5cm above the medium surface. At different points in time following flooding, plants were taken out of the medium, and their roots were washed by rinsing with tap water. Roots, leaves and flowers from each plant were clipped and carried in an icebox to the laboratory in less than five min, and immediately frozen in liquid nitrogen. They were then stored in a -70°C freezer for subsequent analysis.

DNA sequence database analysis

Database searches were performed at the National Center of Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov>) with Entrez, BLAST. At

the time, no eggplant APX had been characterized and therefore a *Solanum* cDNA was used as a query. APX genes were identified by sequence similarities to known APX genes of tomato (Gene Bank accession number Y16773) and potato (Gene Bank accession number AB041343). Only full length APX sequences were included in the analysis. After homology searches using BLAST, sequence alignments were constructed with the Clustal-X program (Thompson et al., 1997). Specific primer sets for APX gene were designed using Vector NTI Suite 8.0 software within the manufacturer's default criteria (InforMax, Frederick, MD, USA). The designed sequences (20 mers) of APX 1, 2 and 3 were as follows: APX1, 5' ATGGGTAAGTGCTATCCCAC 3' (sense), APX2, 5' ATGGGTAAGTCCTATCCCAC 3' (antisense), APX3, 5' TTAAGCTTCAGCAAATCCCA 3' (antisense). These sense and antisense primers were custom synthesized by Bioengineer Biotech (Taipei, Taiwan) and designed to amplify a 753 bp region of the APX gene segment.

Amplification of eggplant cDNA using primer sets (APX1, APX2) and (APX1, APX3)

Total RNA was isolated from different flooding times from different parts of eggplants with a NucleoSpin RNA Plant Kit (Macherey Nagel, Duren, Germany) and quantified with GeneQuant (Amersham Biosciences, Buckinghamshire, UK) at 260 nm. First strand cDNA was synthesized from 1 µg of total RNA using AMV-reverse transcriptase with random hexamers according to the manufacture's instructions (Boehringer Mannheim). Paired primers (APX1, APX2) and (APX1, APX3) were used for amplification. PCR was carried out in an Eppendorf Mastercycler Gradient Thermal Cycler (Hamburg, Germany) with the following thermal program: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 47°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. PCR products were electrophoretically separated on a 1% agarose gel, and the predicted size of 753 bp was verified with a 100 bp DNA ladder of DNA marker (see Figure 1). The products were then sequenced using the same primer in combination with the Big Dye Terminator Cycle Sequencing Kit and ABI Prism 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). DNA sequences were compared with sequences deposited in the aforementioned BLAST program.

DNA gel blot analysis

Leaves of EG117 and EG203 were ground to a fine powder with a mortar and pestle in liquid nitrogen. Genomic DNA was prepared essentially as previously described (Doyle and Doyle, 1990). Fifteen micrograms of DNA was digested with 30 units of EcoRI, BamHI and HindIII (Boehringer Mannheim, Germany), and then separated on 1% agarose gel. The separated DNA bands were transferred to a nylon membrane (Hybond-N⁺,

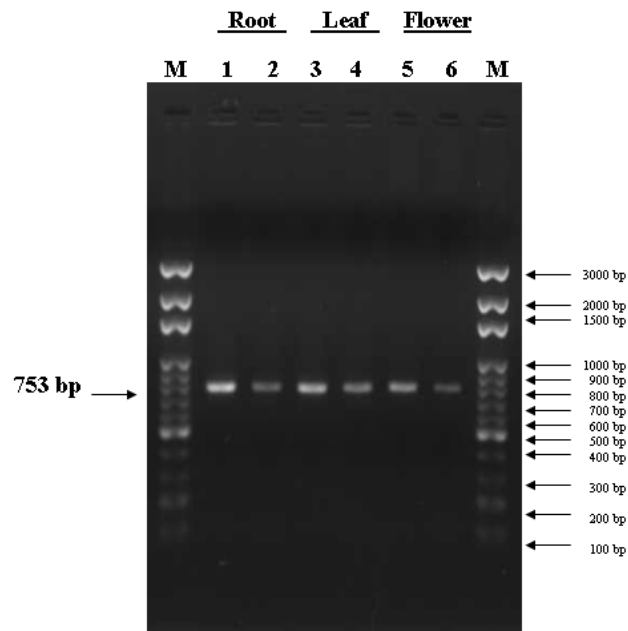


Figure 1. Paired primer sets APX1, APX2 and APX1, APX3 designed from tomato APX gene and potato APX gene, respectively, were used to amplify a 753-bp in length of APX cDNA in different tissues of eggplants. Lanes from 1 to 6 represent EG117 root, EG203 root, EG117 leaf, EG203 leaf, EG117 flower and EG203 flower, respectively. Reverse transcription PCR products from lanes 1, 3 and 5 were generated from APX1 and APX2 primers; furthermore, the bands of lanes 2, 4 and 6 were generated from APX1 and APX3 primers. M=100 bp ladder DNA marker (Gibco-BRL).

Amersham) by downward capillary alkaline transfer. The DNA was crosslinked to the membrane by UV crosslinker (SpectroLine, Westburg, NY, USA). The 753 bp product was amplified using the aforementioned 'Mastercycler gradient' in an annealing temperature of 47°C. APX probe (753 bp) was labeled with the DIG Probe Synthesis Kit (Roche, Mannheim, Germany) as recommended by the manufacturer. The membrane was incubated in 1% blocking agent for 30 min and in an antibody solution-diluted anti-DIG-AP conjugate for 30 min. After washing, the membrane was equilibrated and detected following the protocol of the CDP-Star Detection Kit (Roche). Autoradiography was carried out with Kodak Chemiluminescent Detection Film for 15 min, and the result is presented in Figure 2.

Rapid amplification of cDNA ends (RACE)

The amplification of the remaining part of the corresponding APX gene was obtained by RACE. For the isolation of the 5'-end of APX cDNA, the 5'-RACE technique was performed according to the protocol provided with the FirstChoice RLM-RACE Kit purchased from Ambion (Austin, TX, USA). For the completion of the 3'-end of the obtained cDNA fragments, the 3'-RACE method was also performed as described in the manufacturer's instructions. The

following oligonucleotide sequences were used as inner and outer primers of nested PCR for 5' and 3' RLM-RACE: CCAGGGTGAAAGGGAACA (5' RACE gene specific inner primer), TGTC AAAGATAAGGGGA TTGGTGGT (5' RACE gene specific outer primer), AAGCTGAGCAGCACATGG (3' RACE gene specific inner primer), and AGAGCACTCATTGCTGAAGAAAG AA (3' RACE gene specific outer primer). The PCR steps were: 3 min at 95°C, then 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 40 s, followed by 72°C for 7 min. The primer sets for 5' cDNA and 3' cDNA were designed to produce 950 bp and 500 bp fragments, respectively (see Figure 5).

Cloning of APX gene

The amplified PCR products were separated on 1% agarose gel, purified with the High Pure Product Purification Kit (Roche) and cloned into yT&A vector (Yeastern Biotech. Coop., Taipei, Taiwan, see Figure 6), following the protocol supplied. Transformations were carried out using DH-5 α competent cells (Invitrogen, San Diego, CA, USA) and plated on LB plates with ampicillin (50 μ g/ml), 5-bromo-4-chloro-3-indolyl-B-D-galactosidase (0.4%) and isopropyl-B-D-thiogalactopyranoside (0.1 mM). White colonies were selected for plasmid isolation, and digested *Bam*HI and *Hind*III were used to release the insert. Sequencing was performed by ABI Prism 310 Genetic Analyzer as described earlier. DNA sequences were compared with sequences deposited in the GeneBank database using the BLAST program.

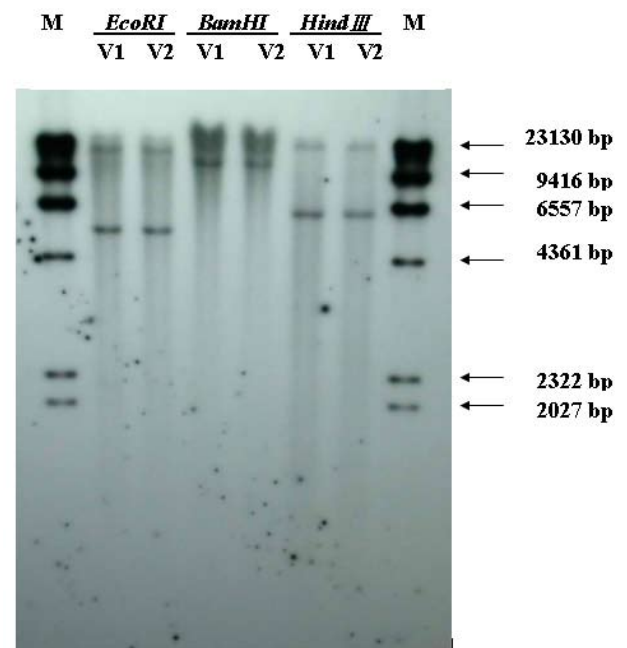


Figure 2. Southern blot hybridization of the PCR product for analysis of the APX gene. Eggplant genomic DNA (15 μ g/lane) was digested with restriction enzymes and electrophoresed through 1% agarose gel. V1=EG117, V2=EG203. M=Molecular Weight Marker II DIG-labeled (Roche).

Real-Time PCR (RT-qPCR) and quantification of RNA levels

The expression levels of APX gene at various flooding times were determined by RT-qPCR using a Roter-Gene G3000 Detection System (Corbett Research, Sydney, Australia). Ubiquitin gene was used as a reference gene. After screening the database for *Solanum* sequences, specific primer sets for ubiquitin and APX gene were designed using the mentioned Vector NTI Suite 8.0 software. The primers for ubiquitin (Hoffman et al., 1991) and APX were: qUBI1, 5' ATGCAGATCTTCGTGAAAAC 3'; qUBI2, 5' AGCACCGCACTCAGCATT 3'; qAPX1, 5' AAGCTGAGCAAGCACATGG 3'; qAPX2, 5' CCAGGGTGAAAGGGAACA 3'. Amplification of PCR products was monitored via intercalation of SYBR-Green (Molecular Probes; 1:1,000 dilution of 10,000x stock solution). The following program was applied: initial polymerase activation: 95°C, 5 min; then 40 cycles at 95°C, 10 s; 58°C, 10 s; 72°C, 20 s. The Roter-Gene software, Version 6.0 was used for threshold selection and standard curve interpolation to derive RNA concentrations relative to the RNA standard. These relative RNA quantities of RNA samples are presented as 'Expression' values in Figures 3 and 4 and allowed the comparison of relative RNA amounts among treatments.

Phylogenetic relationship analysis

The deduced amino acid sequence of the eggplant APX was aligned and compared with other plant APX using the Cluster-W software (Thompson et al., 1994) with the default settings. Phylogenetic trees were inferred using MEGA, Version 3.0 (Kumar et al., 2001). The neighbor joining, minimum evolution and maximum parsimony methods of tree generation were used to assess evolutionary relationships (Rzhetsky and Nei, 1992; Saitou and Nei, 1987). The significance of clustering was evaluated by bootstrap with 1,000 replications.

RESULTS

Primer design for APX gene in eggplant

Degenerate paired primers (APX1, APX2 and APX1, APX3) were designed by comparing the APX cDNA conserved sequences from *Solanum lycopersicum* and *Solanum tuberosum*, respectively. The paired oligonucleotides were used to amplify a cDNA fragment (753 bp) from a single-strand cDNA made from RNA extracted from different tissues (root, leaf and flower) of each genotype (EG117 and EG203). The reverse transcription-PCR products were shown in Figure 1. The bands (lanes 1, 3 and 5) generated from APX1 and APX3 primers appeared higher in intensity and brightness than the bands (lanes 2, 4 and 6) generated from APX1 and APX2 primers. This indicates that the APX1 and APX3 primer set had a higher specificity to eggplant DNA than the APX1 and APX2 primer set, and it was therefore used

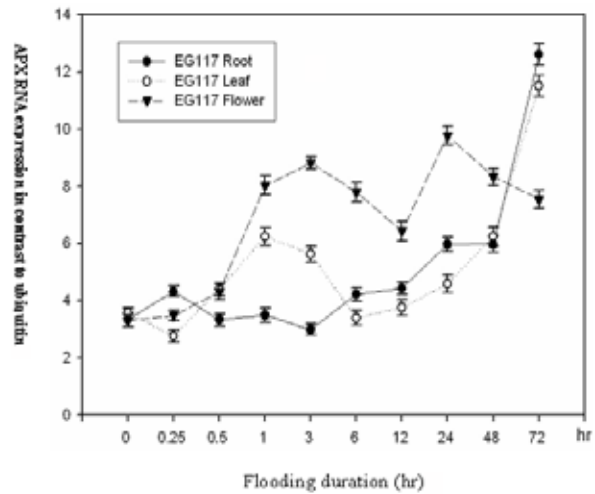


Figure 3. Real-Time PCR of APX transcripts response from root, leaf and flower of EG117 exposed to 0- to 72-h flooding. Relative amounts were calculated and normalized with respect to ubiquitin RNA.

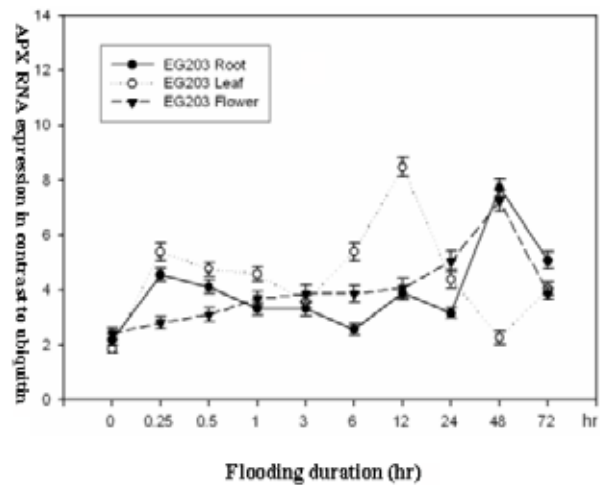


Figure 4. Real-Time PCR of APX transcripts response from root, leaf and flower of EG203 exposed to 0- to 72-h flooding. Relative amounts were calculated and normalized with respect to ubiquitin RNA.

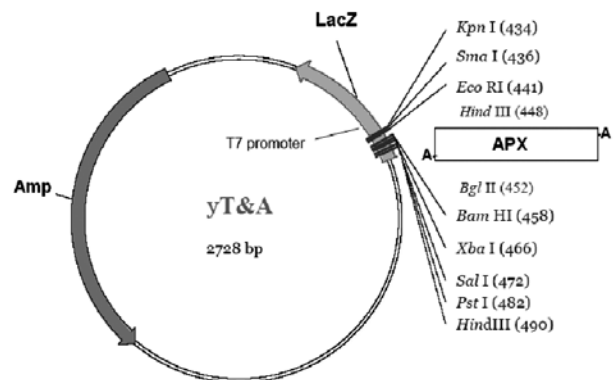


Figure 5. yT&A vector with multiple cloning sites. Amp= ampicilline gene.

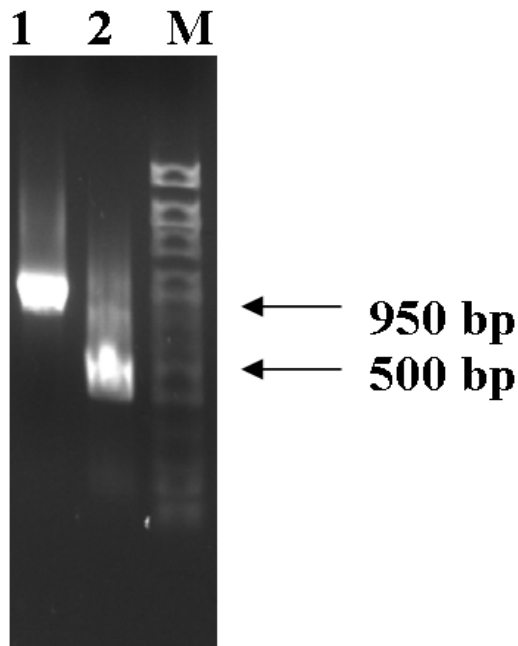


Figure 6. Nested RT-PCR for 5' RLM-RACE product (lane 1) and nested RT-PCR for 3' RLM-RACE product (lane 2). M= 100 bp ladder DNA marker (Gibco-BRL).

for Southern hybridization, RT-qPCR and RACE.

Southern blot analysis

To prove the existence of APX DNA in the EG117 and EG203, genomic Southern hybridization was carried out using the 753 bp fragment as a probe. Figure 2 presents those two hybridization bands for DNA digested with *ECoRI*, *BamHI*, and *HindIII* enzymes. This result indicates that two copies of APX gene exist in the eggplant genome. In addition, no recognition sites of *ECoRI*, *BamHI* and *HindIII* were found within the cDNA of the APX (see Figure 7).

Changes in the RNA level of APX gene in different tissues under flooding treatments

Quantification of the RNA levels of APX gene was performed using RT-qPCR of reverse transcripts of RNA from root, leaf, and flower of EG117 and EG203 that had been subjected to flooding from 0 h to 72 h (Figures 3 and 4). The data were normalized with respect to the RNA level of ubiquitin, a housekeeping gene that is constantly expressed in plants. The trend and level of the increase in APX RNA expression over time were different in EG

M13 universal primer →

T7 promoter



Figure 7. Nucleotide sequence of the full-length APX clone from eggplant (892 bp). APX cDNA contains an open reading frame (753 bp), 5' untranslated region (18 bp), 3' untranslated region (21 bp), and the locations of each primer (APX1, APX3 and M13), T7 promoter and restriction enzymes (*BamHI* and *HindIII*). *ATG, start codon. →TAA, stop codon. GGATCC, *BamHI* site. AAGCTT, *HindIII* site.

117 (Figure 3). The RNA level of APX gene in the root of EG117 was accumulated at different rates from 0 h (3.34) to 72 h (12.6) of flooding. APX RNA expression in EG117 leaf increased from 0 h (3.57) to 1 h (6.52) of flooding, fell to 3.51 at 6 h of flooding, and then peaked (11.5) after 72 h of flooding treatment. The transcript of the APX gene for EG117 flower initially increases up to 3 h of flooding (8.81) and then begins to decrease to 6.44 at 12 h of flooding. At 24 h of flooding, the level peaked at 9.77 and began to drop thereafter. Waterlog stress over time caused a change in APX transcript, which was found to be more abundant in the root and leaf compared to flowers at 72 h of flooding.

Figure 4 presents the effect of flooding time on the expression of APX gene in EG 203. The level of APX gene in EG203 root showed irregular changes. The maximal increase (7.83) was found at 48 h of flooding treatment and was threefold the level at 0 h flooding time (2.09). APX gene transcript of EG203 leaf was affected during the time course of flooding, and the highest (8.48) and lowest (1.85) levels were observed at 12 h and 0 h of waterlog treatment, respectively. A slight increase in APX transcript was noted in EG203 flower as the flooding time was extended, with the exception of a decreased level from 7.25 (48 h) to 3.88 (72 h). The APX gene was more expressed in the root (5.18) than in the leaf (4.00) and flower (3.88) at 72 h of flooding.

Cloning of APX cDNA from RACE

The inner and outer fragments (nested primers) were used to amplify a full-length of APX cDNA, using the 5' and 3'-RACE method. Figure 5 shows the 5' and 3' reverse transcription PCR products of 950 bp (lane 1) and 500 bp (lane 2), respectively. These PCR products were purified, cloned and sequenced, and the sequences were compared with sequences deposited in the GeneBank database of NCBI BLASTN. The full length clone contains an eggplant APX cDNA that is 892 bp in length and has an open reading frame of 753 bp in length (Figure 7). The cDNA is initiated by an ATG, terminated by TAA, and contains an 118-bp 5' untranslated region and 21-bp 3' untranslated region. The GC content of the coding region was 48%. Figure 7 also shows the locations of

BamHI and HindII, and sequences of M13 primers and T7 promoter.

Evolutionary analysis of plant APX gene

The sequence of the APX cDNA (753 bp) showed a significant level of similarity with *Solanum tuberosum* (AB041343), *Solanum lycopersicon* (Y16773), *Nicotiana tabacum* (U15933), and *Capsicum annum* (AY078080). The deduced APX amino acid sequences from five plant species were aligned and compared. Figure 8 represents a phylogenetic tree from the conserved region using the Neighbor-Joining method. Phylogenetic analysis revealed that eggplant APX is more closely related to potato cAPX than to tomato cAPX. Eggplant and potato showed 64% homology of identity to cAPX. Specific groups of cAPX evolved and expanded independently into two groups. Moreover, these two trees inferred from the cAPX protein sequences did not show a violation of the plant taxonomy. Eggplant, potato, and tomato are members of Solanum, and were clustered together. However, pepper was clustered with tobacco with a bootstrap 99, which formed a clade with Solanum species.

DISCUSSION

Increased RNA levels were sustained up to 72 h after 6 h of flooding treatment in leaf and root of EG117 (Figure 3). Therefore, APX transcripts were up-regulated in response to flooding. Higher RNA levels were induced by the elevated flooding stress. This result is consistent with our previous report that APX activity increased markedly in roots of EG117 subjected to flooding stress (Lin et al., 2004). In addition to flooding, several other environmental factors, such as chilling, light, drought, ozone, paraquat treatment, and air pollutants, have also been known to up-regulate APX expression in higher plants (Mittler and Zilinskas, 1994; Ovar and Ellis, 1997; Morimura et al., 1999; Yoshimura et al., 2000; Kornyejev et al., 2003; Murgia et al., 2004).

The APX gene of eggplant differs greatly in its expression pattern in different plant tissues. Although the APX transcripts were detected in all tissues, this gene was most strongly active in root. In contrast, APX gene

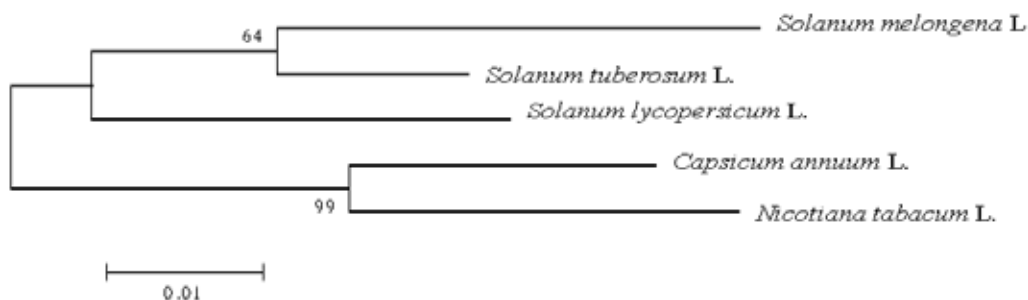


Figure 8. Phylogenetic tree inferred from the APX protein sequences by the Neighbor-Joining method. Numbers indicate bootstrap support for individual nodes. The scale on the bottom shows the number of substitutions per amino acid site.

showed a rather low activation in flowers (Figures 3 and 4). This clearly suggests the functional differentiation of plant tissues. Furthermore, the genotypic response to APX transcript under flooding was inconsistent and did not follow any pattern. Different plant genotypes were prepared for oxidative injury by up-regulating their APX transcripts during waterlogged conditions. In general, the RNA levels of EG117 tissues at 0 h, 1 h, 3 h, 24 h and 72 h of flooding were higher than those of EG203 tissues. Particularly, when flower tissues across flooding time were compared, EG117 (Figure 3) exhibited a higher APX RNA level than EG203 (Figure 4). These results indicate that APX transcripts responded differently under waterlogged conditions for different plant genotypes.

No strong visible effects on the leaves, flowers and roots in response to early flooding were observed. After 24 h exposure, EG203 exhibited clear stress symptoms, such as epinasty and senescence (i.e. chlorosis) in most of the leaves and flowers; however, most leaves and flowers of EG117 looked green and healthy in the pots (photos not shown). The roots of EG203 appeared brownish in color following 48 h of flooding while those of EG117 appeared brownish only after 72 h of flooding. When significant flooding-injury became apparent, the APX level of oxy-radical production might have increased. The APX gene was affected by flooding stress over different hours of the treatment. The degree and speed of flooding-injury were the result of an accumulation of APX transcripts. Plant development plays a role in the regulation of APX gene expression. Flooding stress imposed disturbances in this expression, causing alterations in transcript profiles of different tissues. APX gene was differently expressed in plant tissues and spatially regulated in eggplants. APX gene expression and regulation interacted with both plant development and stress response. Flooding stress in eggplant triggered a defense mechanism against oxidative stress. Hence, APX defenses were concretely regulated to ensure proper protection against ROS generated after exposure to flooding.

Southern analysis of *EcoRI*-, *HindIII*-, and *BamHI*-digested DNA was performed to estimate the copy number of the APX gene. The result showed that two restriction fragments from eggplant genomic DNA hybridized to the APX cDNA clone (Figure 2). Among these two bands, bands of high molecular size appeared less intense than the faster migrating bands. The intensity of the hybridization bands reflected the extent of hybridization due to fragment similarity to the APX cDNA probe. The brighter band represents a fragment bearing a greater similarity to the probe than the faint band. Southern blot was repeated to exclude these fragments with different intensities resulting from partial digestion of genomic DNA. In short, two highly homologous genes to APX genes may exist in eggplants.

A cDNA of APX was cloned from eggplant, and the full sequence was subsequently subjected to NCBI database comparison using BLASTN algorithms. The sequence

showed very high homology to other known cytosolic APXs, with the closest match to *S. tuberosum* (96%) (Kawakami et al., 2002), *S. lycopersicum* (95%) (Gadea et al., 1999), *C. annuum* (93%) (Schantz et al., 1995) and *N. tabacum* (91%) (Ovar and Ellis, 1997). Therefore, the isolated clone proved to be the most similar to cAPX of the other plants in nucleotides sequence. The cloned APX cDNA contains an open reading frame of 753 bp, which was a cytosolic type in the eggplant (Figure 7). Most full-length APX cDNAs cloned to date have been about 1,000 bp long, such as 1042 bp of maize (Bresegem et al., 1995), 1044 bp of strawberry (Kim and Chung, 1998), 1102 bp of spinach (Webb and Allen, 1995), 1040 bp of tobacco (Ovar and Ellis, 1997), 1040 bp of pea (Mittler and Zilinskas, 1992), 1046 bp of sweet potato (Park et al., 2004), and 1039 bp of potato (Kawakami et al., 2002).

cAPX is a dimer consisting of identical subunits with a molecular mass of 28 kDa. It is localized in the cytosol of both photosynthetic and non-photosynthetic tissues. The function is still obscure although it has been reported that RNA for cAPX was induced by environmental stimuli such as heat stress. The cytosolic APX gene transcript from pea strongly increased following treatment with methyl viologen, drought stress, and heat stress (Mittler and Zilinskas, 1992; Mittler and Zilinskas, 1994). Storozhenko et al. (1998) indicated that the heat shock response was very fast in the case of cAPX1 in *A. thaliana*, and this quick response to heat shock was due to the existence of a heat shock element in the promoter region of cAPX1. The expression of the cAPX gene in rice was mediated by high temperature; furthermore, rice seedlings previously subjected to high temperature showed increased tolerance to chilling stress (Sato et al., 2001). Yoshimura et al. (2000) reported that among the four APX isozymes tested, the steady-state transcript level of cAPX type markedly increased in response to high light stress and paraquat treatment, but not in response to drought and salt treatments. In the present report, we have measured the level of transcription of the APX gene in different plant tissues exposed to various flooding treatments, and concluded that the transcript level of cAPX gene increased under flooding stress. Under stress conditions, more H₂O₂ is generated in the microbody matrix and readily diffuses into the cytosol. The gene expression for cAPX is a response to environmental changes, resulting in the protection of important cellular compartments from oxidative stress and in strict control of the level of H₂O₂ in intercellular signaling. APX has a high affinity to H₂O₂ and therefore is able to scavenge it. In plant cells, an alternative and effective detoxification mechanism against H₂O₂ exists, operating both in chloroplasts and the cytosol. In this detoxification mechanism, H₂O₂ is reduced to H₂O with the ASA-GSH-NAPDH system catalyzed by APX (Asada, 1992) in addition to catalase. The regulation of APX gene expression by H₂O₂ has been reported by Lee et al. (1999). They concluded that treatment of cultured soybean cells with exogenous H₂O₂ resulted in the alteration of cytosolic APX transcription levels.

A phylogenetic tree constructed using the deduced amino acid sequences of APX from five species showed the division of the sequences into two classes. Some lineages appear to be conserved across plant species with large evolutionary differences between them. Eggplant and potato contain smaller-subunit APX sequences (64%) than pepper and tobacco (99%). The APX gene from eggplant and potato is highly conserved, suggesting that the domestication of eggplant to potato substantially affects the evolution of APX in these plants. The divergence of APX genes is in the context of the phylogenetic separation between Solanaceae. The diversity of APX genes is probably an adaptive function to counteract the rapid evolution of plants.

Shigeoka et al. (2002) demonstrated that APX isozyme evolution in higher plants and algae can be divided into four groups: cAPXI, cAPXII, chlAPX and mAPX. The cAPXI, chlAPX and mAPX share common features conserved among plant species while the cAPXII group may have evolved from cAPX in species-species manner. Genes encoding cAPX respond to stress-related signals of a biotic or abiotic nature, and are believed to play key roles in these processes. Phylogenetic analysis indicates that in eggplant and potato the expression degree of cAPX gene may correlate with flooding stress.

In conclusion, we cloned the full-length cDNA of APX in eggplant. The transcripts regulated by flooding stress encode cytosolic APX isoform, and the genome of eggplant contained two related cAPX genes. Phylogenetic relationships among the plant APX sequences were separated into two classes. The results suggest that the induction of APX during the flooding stress is affected through a transcriptional mechanism. The differential and coordinated tissue and genotype specific expressions of APX gene occurred in response to flooding stress conditions. This is consistent with our previous report that plant roots under waterlogged conditions generate H₂O₂ that may then be removed by APX. The elevated APX activity may be one factor adding to the increased waterlogging tolerance of eggplant roots. The assessment of transcript kinetic accumulation of APX may help to propose new strategies to analyze APX gene knockout or to engineer transgenic plant (ie. tomato) tolerance to flooding stress. To our knowledge, this is the first suggestion that APX-encoding gene expression in eggplant is regulated by flooding stress.

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茄子淹水逆境下抗壞血酸過氧化酶基因之選殖與表現分析

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我們先前發現淹水造成茄子根部抗壞血酸過氧化酶的活性增加，本研究目的為選殖抗壞血酸過氧化酶基因，並測定淹水逆境下茄子不同組織抗壞血酸過氧化酶基因表現之調節情形。即時聚合鏈反應結果發現，茄子不同組織的表現圖譜顯示其變異性廣，其中以 EG117 淹水 72 小時之抗壞血酸過氧化酶轉錄子最多，每種組織與遺傳型之差異性表現直接與淹水逆境反應有關。在 NCBI 資料庫篩選並比較抗壞血酸過氧化酶的基因序列，根據番茄與馬鈴薯設計消退引子組 (degenerate primer sets)，利用逆轉錄即時聚合鏈反應方法擴增茄子抗壞血酸過氧化酶的 cDNA，並以 5' 與 3' 快速擴增 cDNA 端技術完成全長之抗壞血酸過氧化酶 cDNA，cDNA 系的開放編閱架構 (open reading frame) 長度為 753 鹼基對，譯碼出細胞液之抗壞血酸過氧化酶，茄子抗壞血酸過氧化酶的序列與馬鈴薯、番茄、番椒與菸草的抗壞血酸過氧化酶序列分別有 96%、95%、93% 與 91% 的相似度；以 Neighbor-Joining 法作導譯胺基酸序列 (deduced amino acid sequence) 之演化分析，植物的 cAPXs 分為兩主群，茄子 cDNA 與馬鈴薯的親緣關係比番茄更近，南方墨點分析顯示茄子譯碼抗壞血酸過氧化酶的基因有兩套。這些結果顯示茄子的 cAPX 可能與過氧化氫解毒有關，因此幫助克服淹水誘導之逆境。

關鍵詞：抗壞血酸過氧化酶；即時聚合鏈反應；淹水；茄子；演化。