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Changes in Expression of the Cytosolic Ascorbate Peroxidase Gene, *Ca-cAPX1*, during Germination and Development of Hot Pepper Seedlings

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Ascorbate peroxidase (APX), an antioxidant enzyme, scavenges H_2O_2 that is produced by normal metabolism and cellular oxidative stresses. To investigate its role during germination and seedling growth, we isolated a cDNA encoding cytosolic APX (cAPX) in hot pepper (*Capsicum annuum* L.). The full-length clone, *Ca-cAPX1*, is 1011 bp long and has an ORF encoding 249 amino acid residues. During seedling development, cAPX activity and expression levels were higher at Days 5 and 6 post-imbibition, respectively, whereas those of catalase (CAT) increased at Days 8 and 10. The increased amount of H_2O_2 in that early developmental stage (Day 5) may have been counteracted mainly by APX, and further removed by CAT in cooperation with APX. To determine whether the accumulation of H_2O_2 via suppression of *cAPX* expression might be a factor in stimulating germination, we constructed a transformant of *Ca-cAPX1*. Compared with the wild type, the germination rate for the antisense-suppressed *Arabidopsis* increased by 26%, while its H_2O_2 content rose by 50%. Therefore, we propose that the pre-germination suppression of *cAPX* expression stimulates seed germination by promoting the accumulation of H_2O_2.

Keywords: antisense suppression, ascorbate peroxidase, *Capsicum annuum* L., hydrogen peroxide, seedling development

Peroxidases are ubiquitous enzymes found in plants, mammals, fungi, and prokaryotes. They are classified according to their physiological functioning. Group 1 comprises guaiacol peroxidase, which plays diverse roles through its oxidation products: lignin biosynthesis, degradation of indole-3-acetate, ethylene biosynthesis, cross-linking of hydroxyproline-rich glycoprotein, wound healing, and defense against pathogens. Group 2 peroxidases participate in the scavenging of hydrogen peroxide and organic hydroperoxides via the reduction to water using electron donors. Those electron donors and their prosthetic groups have diverged among organisms, with ascorbate peroxidase (APX) representing this group in plants (Everse et al., 1991; Asada, 1993). APX has been found in protozoa, eukaryotic algae, and land plants, but not in fungi. In plants, four different isoforms are present -- in the chloroplast stroma (sAPX) and thylakoid membrane (tAPX), the peroxisomes (pAPX), and the cytosol (cAPX).

Cytosolic ascorbate peroxidase (cAPX) is present in the form of a homodimer in cell compartments other than the chloroplasts and peroxisomes. Its monomer molecular size is about 27 kD. The crystal structure of cAPX has the proximal and distal sides of the heme. Amino acid residues participating in the dimerization of cAPX are assigned from its crystal structure, which is mostly inferred by electrostatic interactions (Patterson and Poulos, 1995).

Physiological and biochemical events have been studied during the initial stages of seed imbibition (Puntarulo et al., 1991; Kim et al., 2004). A number of processes, such as membrane reorganization and metabolic reactivation that occur at this stage can have profound influences on seed germination and future seedling growth. Germination requires oxygen as a final electron acceptor to yield water in the exergonic mitochondrial redox reactions coupled to ATP synthesis. Oxygen is taken up after imbibition and is reduced to H_2O by mitochondrial cytochrome oxidase. Various oxygen intermediates, e.g., superoxide radicals and hydrogen peroxide, are then produced as a result of oxidative phosphorylation and the mobilization of stored food. Cytosolic H_2O_2 production is mainly considered to be the product of mitochondrial superoxide dismutation, and is disposed of primarily by cAPX (Bewley and Black, 1994).

H_2O_2 has a harmful effect on cells at high concentrations. However, it is also regarded as a membrane-permeable molecule involved in the signaling of chilling, heat, pathogen defense, and aleurone pro-

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grammed cell death, and with its accumulation causing the induction of cAPX genes (de Tullio and Arrigoni, 1992; Fath et al., 2001). H_2O_2 can also promote seed germination by activating the oxidative pentose phosphate pathway and by oxidizing germination inhibitor(s) in the pericarp (Ogawa and Iwabuchi, 2001). It may also play an important role in protecting the emerging embryo against invasion by parasitic organisms (Fontaine et al., 1994; Schopfer et al., 2001).

Previous studies have only examined the activity of APX during seed germination and seedling development; no reports have been made on changes in *APX* gene expression during those growth phases. Therefore, our objective was to investigate the relationship between *cAPX* and H_2O_2 at those important stages.

MATERIALS AND METHODS

Plant Materials

Seeds of hot pepper (*Capsicum annuum* cv. Nok-Kwang) and *Arabidopsis thaliana* (Columbia) were surface-sterilized for 1 min in 70% ethanol and for 1 to 2 min in a 1.3% hypochlorite solution with 0.01% Triton X-100. The seeds were rinsed three times in sterile distilled water and air-dried. They were placed on sterile Petri plates containing a Murashige and Skoog (MS) medium (Sigma, USA) with 0.2% sucrose in 0.9% bacto-agar (Sigma). Growth-room conditions included 16-h of light at 24°C (*Arabidopsis*) or 27°C (hot pepper) followed by 8 h of darkness at 22°C (*Arabidopsis*) or 25°C (hot pepper).

Isolation and Sequencing of cDNA Clone for APX

cDNA clone for APX was isolated by screening the hot pepper cDNA library (Kim et al., 1977) as described by Kwon and An (2001). The insert of the partial cDNA for hot pepper cAPX, which was kindly provided by Dr. Kim (Sogang Univ, Korea), was labeled with ^{32}P (random primer labeling kit) and used as a probe. Hybridization was performed according to the techniques of Sambrook et al. (1989). Nucleotide sequencing of a positive clone was carried out by the method of Sanger et al. (1977), using the Sequenase Version 2.0 DNA sequencing Kit and ^{35}S-dATP. Sequence analyses were then conducted by a Blast search, DNAsis, and the Clustal Program.

Enzyme Activities

One gram of pepper seedling was ground with mortar and pestle into a medium containing 30 mM MOPS (pH 7.3), 3 mM EDTA, 25 mM cysteine, 0.3 M mannitol, and 0.3% (w/v) BSA. For the cytosolic fraction, the chloroplasts were sedimented by centrifugation at 1600 *g* for 5 min. The supernatant was then recovered after centrifugation at 10,000 *g* for 10 min (Jiménez et al., 1997). Protein content was determined using Coomassie brilliant G-250, with BSA as the standard, according to the dye-binding method of Bradford (1976). The spectrophotometric assay for APX activity (Mittler and Zilinskas, 1991) evaluated a 1-mL reaction mixture containing 0.5 mM sodium ascorbate and 0.1 mM H_2O_2 in 50 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA. The decrease in absorbance at 290 nm was monitored to follow the oxidation of ascorbate over time. These raw data were corrected for the non-enzymatic oxidation of ascorbate by H_2O_2. Catalase activity was assayed at 25°C in a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and the enzyme. After the reaction was started by adding 10 mM H_2O_2, activity was determined from the decrease in absorbance at 240 nm due to peroxide consumption. All activities were calculated from their initial linear rates and corrected for non-enzymatic destruction of H_2O_2.

Detection of APX Activity in Native Gels

In-gel activity of APX was assessed according to Mittler and Zilinskas (1993). Soluble protein was extracted from 1 g of frozen pepper seedling tissue in 100 mM of sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. Cytosolic fractionation was carried out as described by Jiménez et al. (1997). This fraction was assumed to contain mostly cAPX, although possible contamination by other types of APX was not excluded. Samples were subjected to discontinuous PAGE under non-denaturing and non-reducing conditions. After electrophoresis the gels were incubated for 20 min with 50 mM PBS (pH 7.0), which contained 4 mM ascorbate and 2 mM H_2O_2. The H_2O_2 was added to this solution immediately before incubation with the gel. Afterward, the gels were washed with sodium phosphate buffer (50 mM, pH 7.0) for 1 min, then submerged with gentle agitation in a solution of 50 mM PBS (pH 7.8), 28 mM TEMED, and 2.45 mM NBT. APX activity was defined by an achromatic band on a purple-blue background.

Measurement of Hydrogen Peroxide

One gram of pepper seedling tissue was frozen in liquid nitrogen and ground in 1 mL of 3% (w/v) 0.2 M perchloric acid. This homogenate was centrifuged at 12000g for 5 min at 4°C. The supernatant was neutralized with 4 M potassium hydroxide and centrifuged at 1000g for 1 min at 4°C. Afterward, it was reacted at 25°C with 12.5 mM DMAB, 665 nM MBTH, and 0.25 units of horseradish peroxidase in 375 mM phosphate buffer. Absorbance was measured at 590 nm.

Isolation of Total RNAs and Northern Analysis

Total RNA (15 μg) was denatured, separated electrophoretically, and transferred to a nitrocellulose membrane according to the method of Sambrook et al. (1989). When loading of equivalent amounts of RNA (15 μg) for each time point was checked on gels stained with ethidium bromide, the bands for ribosomal RNA (rRNA) were at equal intensities. Northern blots were hybridized with ^{32}P-labeled DNA probes, the full-length inserts of *Ca-cAPX1* and *Ca-CAT* (Kwon and An, 2001). Afterward, they were washed twice in 2× SSC and 0.1% (w/v) SDS at 63°C for 60 min, and visualized by autoradiography.

Reverse Transcription Polymerase Chain Reaction

Total RNA was prepared from pepper seedlings with an RNeasy kit (Qiagen, USA) and was subjected to reverse transcription with an RT-PCR kit (Qiagen). PCR was performed in 50 μL of reaction mixture that comprised the cDNA and a master mix [i.e., final concentrations were 1 unit of Taq polymerase (Promega, USA) 3 mM $MgCl_2$, 20 mM Tris (pH 8.4), 300 μM of each dNTP, and 0.5 μM of gene-specific primers]. The following program was applied: initial polymerase activation at 94°C for 10 min; then 35 cycles at 94°C, 10 s; 55°C, 10 s; and 72°C, 30 s. Primer pairs for PCR included: Ca-cAPX1F; 5′-tactcaaggaactattgggt-3′, Ca-cAPX1R; 5′-catccaacaactccagtgagg-3′, AtAPX1F; 5′-gtccattcggaacaatgaggtttgac-3′, AtAPX1R; 5′-gtggcaccagataaagcgacaat-3′, AtActin2F; 5′-accttgctggacgtgaaccttactgat-3, AtActin2R; 5′-gtgtctcgtggattccagcagctt-3 (GenBank accession no. X59600 and U41998 for *APX1* and *Actin2*, respectively).

Transgenic Plants

The sequence of full-length *Ca-cAPX1* in the antisense orientation (relative to the CaMV 35S promoter) was constructed in a binary vector, pPZP221, by ligating the cDNA into plasmid pRT101 cut with *Eco*RI/*Xba*I. The plasmid was then introduced into *Agrobacterium tumefaciens* by electroporation. Antisense constructs for *Ca-cAPX1* were introduced into *A. thaliana* ecotype Columbia by floral dip transformation with *Agrobacterium* (Clough and Bent, 1998). After the seeds were harvested, they were plated on an MS medium containing hygromycin to select for transgenic lines. Transformed seedlings that arose from T3 seeds were reared on MS-bacto-agar plates in a growth chamber under a light period of 16 h at 23°C and a dark period of 8 h at 22°C. Germination frequency was recorded after 24 h of incubation by counting the number of seeds with protruding radicles.

RESULTS

cDNA Sequence of *Ca-cAPX1*

By screening the cDNA library for *C. annuum*, using a partial hot pepper cAPX cDNA as a probe, we have obtained a full-length clone, *Ca-cAPX1* (GenBank accession number, DQ002888). This clone contains an 1011-bp-long insert that consists of a 74 b leader sequence, an open reading frame of 750 b coding for 249 amino acids, and a 184 b untranslated tail. Its amino acid sequence was compared with that from other species (Fig. 1). The proximal and distal active sites (R38, W41, and H42) and the catalytic triad (H163, W179, and D208) are conserved among plant APX proteins. The lack of a transit peptide, plus high homology values (84 to 95%), suggests that this clone encodes a cytosolic APX in hot pepper. DNA blot analysis verified that *Ca-cAPX1* is a single-copy gene in the hot pepper genome (data not shown).

Changes in *Ca-cAPX1* Expression during Seedling Development

Ca-cAPX1 was uniformly expressed throughout the cotyledons, hypocotyls, and roots of hot pepper; activity staining for cAPX also showed a similar pattern (Fig. 2A). Likewise, the induction of *Ca-cAPX1* did not differ when plants were grown under either light or dark conditions (Fig. 2B). Based on these observations, therefore, we chose to continue our analysis using whole seedlings grown in the light.

H_2O_2 contents reached their maxima at Day 5

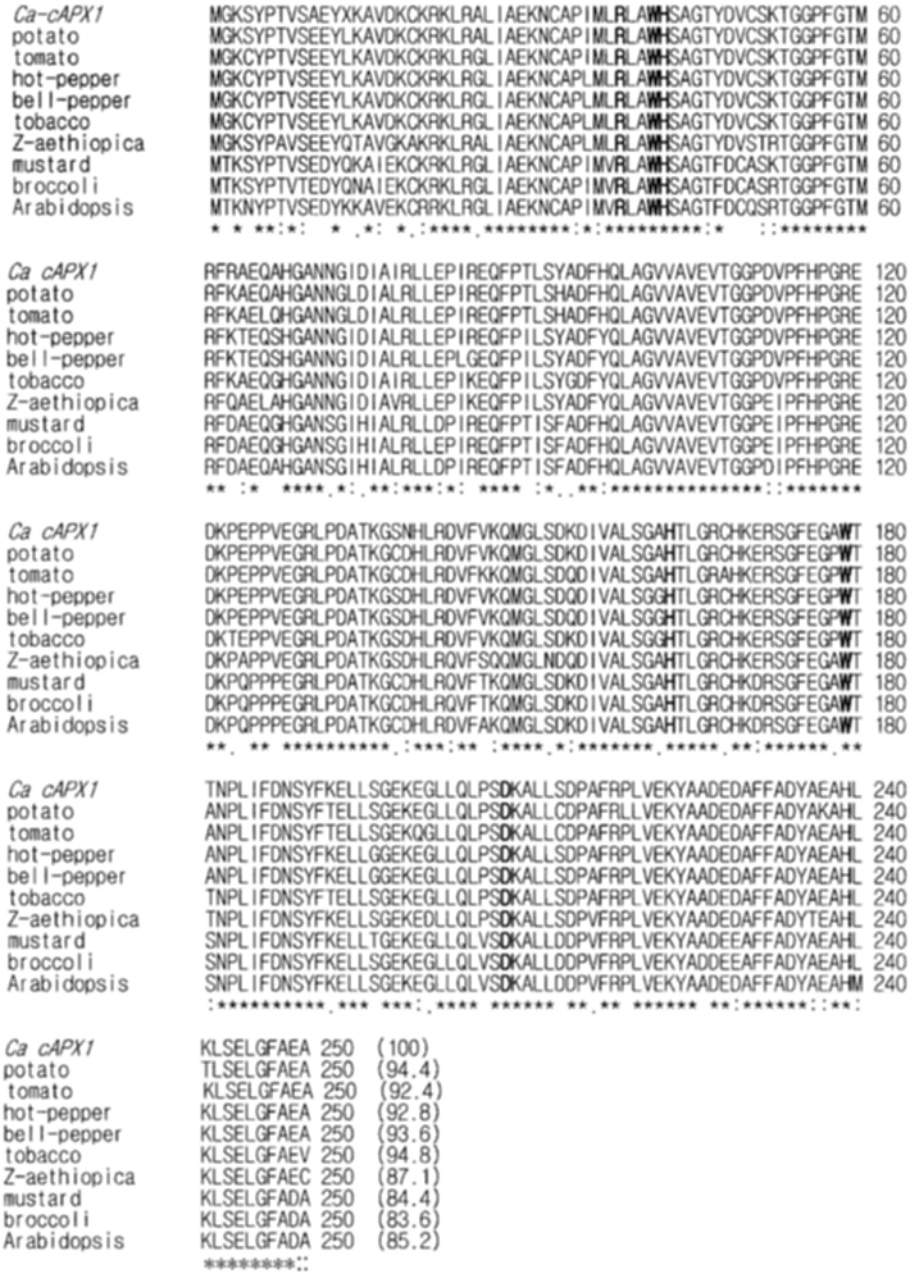

Figure 1. Comparison of cytoplasmic APX amino acid sequences. Conserved amino acids are marked with asterisks. Active sites and catalytic triads are indicated in bold, and percentages of homology are at ends of sequences.

post-imbibition before decreasing (Fig. 3B). *Ca-cAPX1* expression was most intense at Day 6, but then sharply declined from Day 10 to Day 12, i.e., when seedlings were considered full-grown (Fig. 4B). However, expression of the catalase gene, *Ca-CAT*, increased after 10 d of imbibition (Fig. 4B). APX activity also rose during this early stage of development, compared with the delay seen in heightened CAT activity (Fig. 5A and B).

Ca-cAPX1 Induction and Seed Germination

Because of their high polysaccharide contents, it was difficult to obtain pure extracts of total RNA from seeds. Therefore, we were unable to detect expression by northern analysis in the germination phase (data not shown), and, instead, performed RT-PCR. Even with that latter technique, expression was not detected before germination ensued (at Day 3) but

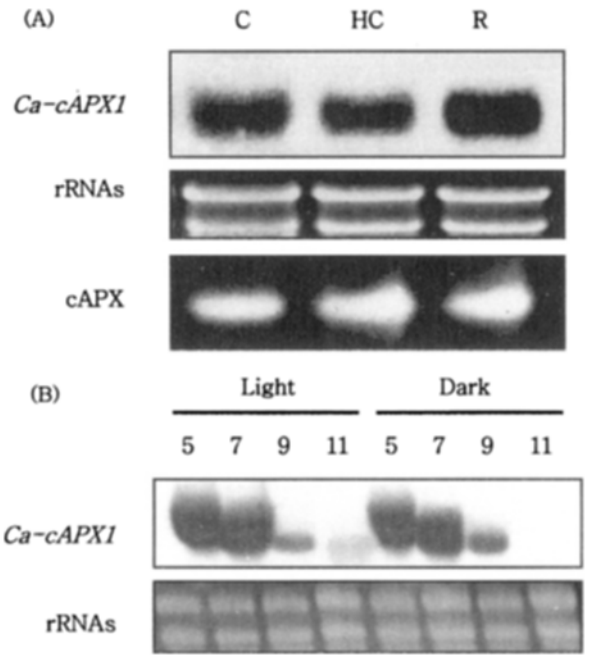

Figure 2. Expression of *Ca-cAPX1* gene in different organs and under light or dark conditions. **(A)** Northern blot analysis and in-gel activity of cAPX from cotyledons (C), hypocotyls (HC), and roots (R). **(B)** Northern blot analysis from whole seedlings grown under light or dark for days indicated. Blots were hybridized with full-length *Ca-cAPX1* gene as probe. For detection of cAPX activities on native PAGE, 100 μg protein per lane was loaded on gel.

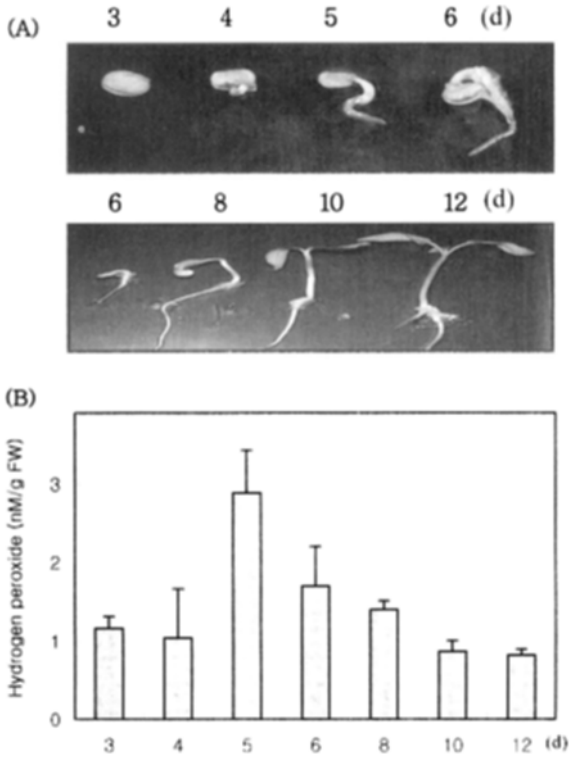

Figure 3. Morphology and hydrogen peroxide content during seedling development. Photograph of hot pepper seedlings **(A)** and H_2O_2 contents **(B)** when grown in light for days after imbibition indicated. Data are mean values with half SD from three replicates.

became visible at from Day 4 (Fig. 4A). To exclude the possibility of RT-PCR error, we also performed activity-staining for cAPX, and were able to clearly observe it during germination (Fig. 4A). This increased expression and activity of cAPX was similar to the trend seen with H_2O_2 content (Fig. 4B, 5A), which suggests that cAPX is important in H_2O_2-scavenging during the early stage of seedling development.

Stimulation of Germination in Antisense-Suppressed Transformants of *Ca-cAPX1*

To test whether the suppression of *Ca-cAPX1* expression affected H_2O_2 accumulation and stimulated seed germination through the accumulation of H_2O_2, we examined the germination rate and H_2O_2 content in antisense-suppressed *Arabidopsis* transformants. Four homozygotic lines (AS1-1, AS1-2, AS2-1, and AS2-2) were chosen from two independent F2 plants -- AS1 and AS2. APX activity in AS1-2 was 51.3% of the level measured from the Col wild type, while levels in AS1-1, AS2-1, and AS2-2 were 83%, 79%, and 61%, respectively (Fig. 6B). We also used RT-PCR to compare this with the expression of intrinsic *cAPX* of *Arabidopsis*, or *APX1*. Because *APX1* expression in the AS1-2 line was fully suppressed (Fig. 6A), we selected that line as the antisense-suppressed transformant, and found that its germination rate and H_2O_2 content were higher (by 26% and 50%, respectively) than those of the wild type (Fig. 7).

DISCUSSION

Changes in APX and CAT Activities from Seed to Seedling Stage

H_2O_2-producing and -consuming processes are active early in the germination of soybean embryonic axes (Puntarulo et al., 1988). Mitochondrial electron transfer appears to be the most important source for its production. If H_2O_2 is generated at an unexpected site or reaches a level that exceeds the scavenging

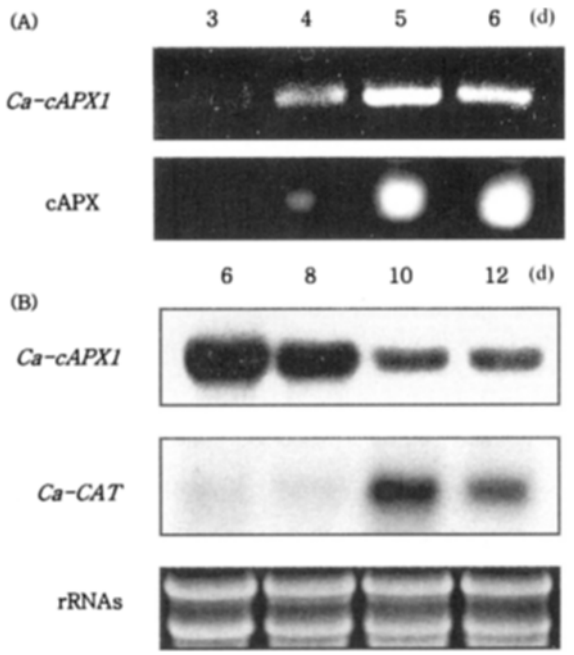

Figure 4. Expression of *Ca-cAPX1* gene by RT-PCR **(A)** and-northern blot analysis along with *Ca-CAT* gene **(B)** from whole seedlings grown for days after imbibition indicated. Blots were hybridized with full-length *Ca-cAPX1* and *Ca-CAT* genes as probes. Total RNA (15 μg) was used for analysis.

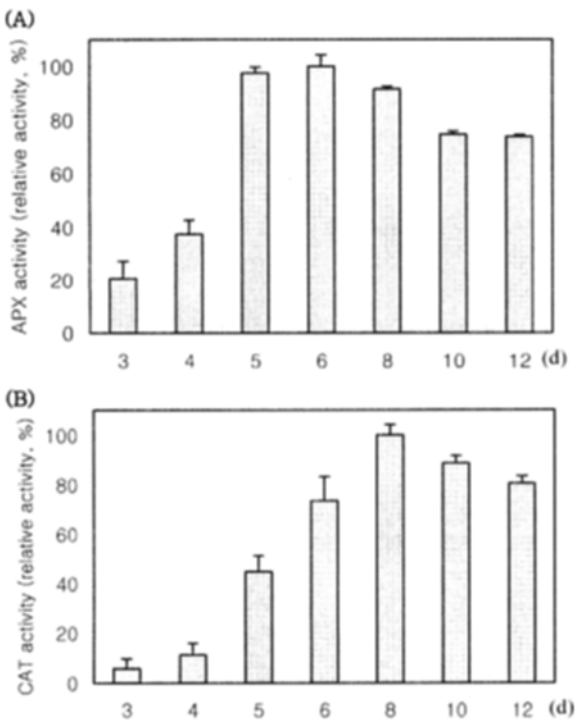

Figure 5. Relative activities of APX **(A)** and CAT **(B)** at days after imbibition indicated. Data are mean values with half SD from three replicates.

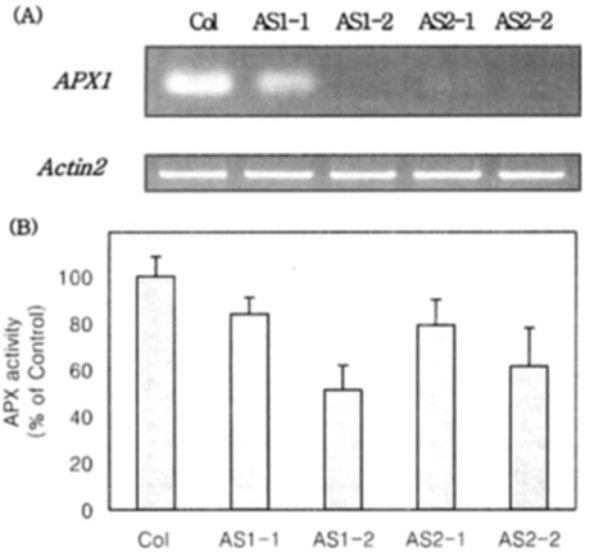

Figure 6. Expression of *Arabidopsis* cAPX gene, *APX1*, in wild-type (Col) and antisense-suppressed transformants (AS1-2) per RT-PCR **(A)** and enzyme activity **(B)**. Data are mean values with half SD from three replicates.

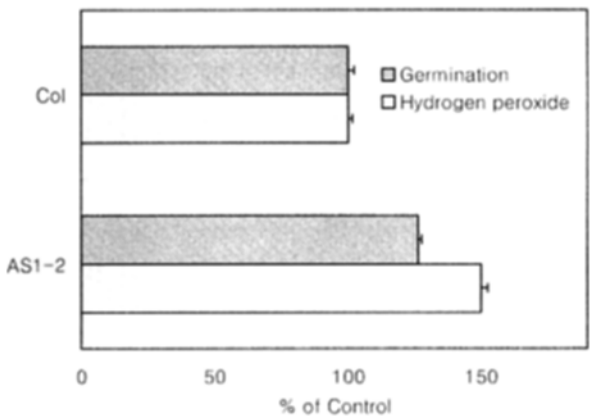

Figure 7. Hydrogen peroxide content and germination frequency in wild-type (Col) and antisense-suppressed transformant (AS1-2) after 24 h of incubation. Values are shown as percentage of control. Data are mean values with half SD from three replicates.

capacity, it causes oxidative damage to plants. Therefore, it should be scavenged where its generation or utilization occurs.

Catalase appears to be the predominant H_2O_2-consuming enzyme in soybean axes at the onset of germination (Puntarulo et al., 1991). However, our hot pepper study showed that *CAT* expression began later than did *cAPX* expression during seedling development (Fig. 4B). In fact, CAT has a very low affinity for H_2O_2, so it seems to be ineffective in destroying per-

oxide at low concentrations (i.e., under normal metabolisms). Moreover, H_2O_2 can easily permeate membranes and diffuse from the glyoxysomes/peroxisomes to other cellular compartments that lack CAT (Chance et al., 1979; Klapheck et al., 1990; Tommasi et al., 1995).

Although APX activity peaked at Days 5 and 6, that of CAT was maximized at Day 8 of imbibition (Fig. 5A and B). The amount of H_2O_2 was highest at Day 5, but then decreased (Fig. 3B). Therefore, this early, increased level of H_2O_2 may have been scavenged mainly by APX until Day 8, when CAT perhaps began to cooperate with APX.

Ca-cAPX1 Induction and Seed Germination

Previous reports have shown that, when oxidative metabolism is high in the first stage of seed development, H_2O_2 production is also remarkable, and consequently, APX is utilized to efficiently remove it (de Gara et al., 1997). In the embryos and endosperms of *Pinus pinea* seeds, Tommasi et al. (2001) have detected a single band with APX activity after 24 h of germination. This band becomes more intense during germination, thus confirming the increase in APX activity, but no extra bands appear in the following hours.

In contrast, for barley aleurone cells, decreased APX activity likely contributes to an accumulation of H_2O_2 and eventual cell death during seed germination (Fath et al., 2001). According to this explanation, reactive oxygen species (ROS) may scavange peroxide membrane lipids, causing the plasma membrane to rupture, an event characteristic of gibberellin-induced programmed cell death in those protoplasts. GA treatment causes that down-regulation of antioxidants, resulting in the accumulation of ROS. Other evidence for this relationship is that phenolic compounds, which act as antioxidants, inhibit germination in a dose-dependent manner in *Zinnia elegans* (Ogawa and Iwabuchi, 2001). Therefore, the suppressed expression of *Ca-cAPX1* until germination that we observed here might be an important factor in the accumulation of H_2O_2 (Fig. 4A). The increased levels of *Ca-cAPX1* and H_2O_2 after germination may then be interpreted as a mechanism for protecting cells against the by-product of greater oxidative metabolism (Fig. 3B, 4B).

Stimulation of Germination in Antisense-Suppressed Mutant of *Ca-cAPX1* from *Arabidopsis*

Imbibition of dry seeds is associated with a rapid increase in oxygen uptake and mitochondrial respiration, thereby supporting ATP synthesis (Hendry, 1993). Up to 2% of the mitochondrial O_2 consumption in seeds is estimated to be involved in the generation of H_2O_2. Most previous investigations with seeds or seedlings have been concerned with the destructive effects of ROS, such as lipid peroxidation (Bewley and Black, 1994). Nevertheless, interest has grown in understanding the functional significance of ROS in germination and early seedling development (Schopfer et al., 2001). For example, in radish seeds, the release of superoxide radicals, H_2O_2 and $OH^{\cdot -}$, can be detected before the onset of visible germination in the seed coat. A rise in ROS release is initiated shortly before the radicle protrudes. Therefore, ROS production by germinating seeds indicates an active, developmentally controlled physiological function that is connected with high germination capacity and vigorous development, presumably for protecting the emerging seedling against pathogen attack.

Another explanation for the role of ROS is that exogenously applied H_2O_2 ameliorates seed germination in many species (Chien and Lin, 1994; Fontaine et al., 1994; Naredo et al., 1998), even in a dose-dependent manner (Ogawa and Iwabuchi, 2001). However, it is not known whether all ROS species or only H_2O_2 are involved in germination or how they may enhance that process. Fontaine et al. (1994) have suggested that H_2O_2 activates the oxidative pentose phosphate pathway, resulting in the promotion of germination. Another hypothesis is that H_2O_2 is helpful in cracking hard seed coats, allowing them to imbibe water. The temporally oxidized state of the embryo that is induced by H_2O_2 might even initiate germination (Chien and Lin, 1994; Ogawa and Iwabuchi, 2001).

In this study, the germination rate for transformants of *Ca-cAPX1* antisense-suppressed *Arabidopsis* increased by 26%, while their H_2O_2 contents increased by 50%, compared with those values for the wild type (Fig. 7). This suggests that pre-germination suppression of *cAPX* expression is an important factor in the accumulation of H_2O_2 for seed germination. However, further research is needed concerning the suppression mechanism of *cAPX* before germination as well as the stimulatory effect provided by H_2O_2 and/or ROS.

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