Expression of Spinach Ascorbate Peroxidase Isoenzymes in Response to Oxidative Stresses[1]

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We studied the response of each ascorbate peroxidase (APX) isoenzyme in spinach leaves under stress conditions imposed by high light intensity, drought, salinity, and applications of methyl viologen and abscisic acid. The steady-state transcript level of cytosolic APX remarkably increased in response to high-light stress and methyl viologen treatment, but not in response to the other stress treatments. The transcript levels of the chloroplastic (stromal and thylakoid-bound) and microbody-bound APX isoenzymes were not changed in response to any of the stress treatments. To explore the responses of the APX isoenzymes to photooxidative stress, the levels of transcript and protein and activities of each isoenzyme were studied during high-light stress and following its recovery. The cytosolic APX activity increased in parallel with transcript abundance during high-light stress, while the protein level was not altered. The other isoenzymes showed no significant changes in transcript and protein levels and activities, except for the gradual decrease in chloroplastic isoenzyme activities.

Even under optimal conditions, many metabolic processes, including chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems, produce active oxygen species such as the superoxide radical, H_2O_2, and the hydroxyl radical (Foyer et al., 1994; Asada, 1997). Furthermore, the imposition of biotic and abiotic stress conditions can give rise to excess concentrations of active oxygen species, resulting in oxidative damage at the cellular level. Therefore, antioxidants and antioxidant enzymes such as ascorbate, glutathione, superoxide dismutase, ascorbate peroxidase (APX; EC 1.11.1.11), and catalase function to interrupt the cascades of uncontrolled oxidation in some organelles (Noctor and Foyer, 1998). APX isoenzymes play an important role in eliminating H_2O_2 and are distributed in at least four distinct cell compartments, the stroma (sAPX) and thylakoid membraine (tAPX) in chloroplasts, the microbody (mAPX), and the cytosol (cAPX) (Asada, 1992; Miyake and Asada, 1992; Ishikawa et al., 1998). A second family of cAPX has also reported in various plant species such as spinach, Arabidopsis, soybean, and rice (Ishikawa et al., 1995; Santos et al., 1996; Jespersen et al., 1997; Caldwell et al., 1998). More recently, Jiménez et al. (1997) reported the detection of APX activity in pea mitochondria, but the corresponding protein and cDNA have not yet been identified.

The cDNAs encoding the APX isoenzymes were isolated from various plant species and have been well characterized by many research groups (Mittler and Zilinskas, 1991; Kubo et al., 1992; Webb and Allen, 1995; Bunkelmann and Trelease, 1996; Yamaguchi et al., 1996; Mano et al., 1997). We isolated and characterized the cDNAs encoding tAPX, sAPX, mAPX, cAPX, and an unknown putative cytosol-soluble isoenzyme, SAP1, from spinach leaves (Ishikawa et al., 1995, 1996a, 1998). The two chloroplastic APX (chlAPX) isoenzymes are encoded by only one gene (*ApxII*) and their mRNAs are regulated by the alternative splicing of its two 3′-terminal exons (Ishikawa et al., 1996a, 1997; Yoshimura et al., 1999). A similar finding was also observed in pumpkin (Mano et al., 1997), *Mesembryanthemum crystallinum* (accession nos. AF069315, tAPX; AF069316, sAPX), and tobacco (accession nos. AB022273, tAPX; AB022274, sAPX).

Recent studies have focused on the changes in the cAPX expression level under environmental stresses such as ozone, UV-B radiation, low temperature, high-light stress, salinity, water stress including drought, and pathogen infection (Tanaka et al., 1985; Schöner and Krause, 1990; Mittler and Zilinskas, 1992, 1994; Mishra et al., 1993; Willekens et al., 1994; Conklin and Last, 1995; Hernández et al., 1995; Kubo et al., 1995; Rao et al., 1996; Örvar et al., 1997; Mittler et al., 1998). Considering the specific distributions and roles of the APX isoenzymes and the potential for active oxygen species production in each organelle of higher plants, it seems likely that the APX isoenzymes are expressed by distinct regulatory mechanisms. However, no studies have presented simultaneous analysis of the stress responses of all of the APX isoenzymes. In fact, the lack of specific probes to detect the APX isoenzymes at the mRNA and protein levels has limited the understanding of

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the expression of the respective isoenzymes. In this study, we report the responses of all of the APX isoenzymes in spinach leaves to high-light stress, salinity, drought, and treatment with methyl viologen (MV) and abscisic acid (ABA) by northern-blot analysis using each cDNA as a probe. The steady-state mRNA expression of each APX isoenzyme revealed that only the expression of the cAPX isoenzyme responds to high-light stress and MV treatment, while the other isoenzymes are constitutively expressed under normal and stressful conditions.

RESULTS

Expression of APX Isoenzymes in Response to Abiotic and Biotic Stresses

The transcripts of chlAPX, mAPX, cAPX, and SAP1 were detected at the respective predicted size in 4-week-old spinach leaves (Fig. 1). The steady-state transcript level of cAPX in response to either high-light stress or MV treatment for 1 h increased by approximately 2.7-fold compared with those of the control plants. Under stresses of salinity, drought, and ABA, the transcript levels of cAPX were not altered. The steady-state transcript levels of chlAPX, mAPX, and SAP1 were also not changed in response to any of the stresses or treatments. The same results were obtained for the total RNAs prepared from 2- or 8-week-old plants (data not shown).

Expression of APX Isoenzymes in Response to High-Light Stress and Following Its Recovery

Changes in Photosynthetic Parameters

Four-week-old plants were subjected to high light intensity (1,600 μE m^{-2} s^{-1}) for 1, 3, and 5 h and compared with the control plants of the same developmental stage. Recovery from high-light stress was analyzed by reexposure to the illumination at 300 μE m^{-2} s^{-1} and sampling 2 and 48 h later. CO_2 fixation decreased by approximately 30% (Fig. 2A), and PS II activity (F_v/F_m) dropped after 1 h, reaching a minimum after 5 h of high light (Fig. 2B). After 48 h of recovery, PS II activity and CO_2 fixation reached almost the same values as those in the control plants. The restoration of the CO_2 fixation and the PS II activity during the recovery period from stress indicated that high light intensity for 5 h caused mild stress to the spinach leaves.

Changes in Transcript and Protein Levels and Activity of APX Isoenzymes

As shown in Figure 3, A and B, the transcript abundance of cAPX increased remarkably after 1 h, which is in agreement with the results shown in Figure 1, and reached a peak after 3 h of the high-light period. In addition, during the recovery period from stress, the transcript abundance gradually decreased and returned to nearly the control level after 48 h in the recovered plants. However, in the high-light-stressed and recovered plants, no significant changes in the transcript levels were detected in chlAPX, mAPX, and SAP1, confirming the data shown in Figure 1.

The protein level of each APX isoenzyme was detected by immunoblotting using mAb-I raised against spinach sAPX (the preparation and characterization of this monoclonal antibody will be described else-

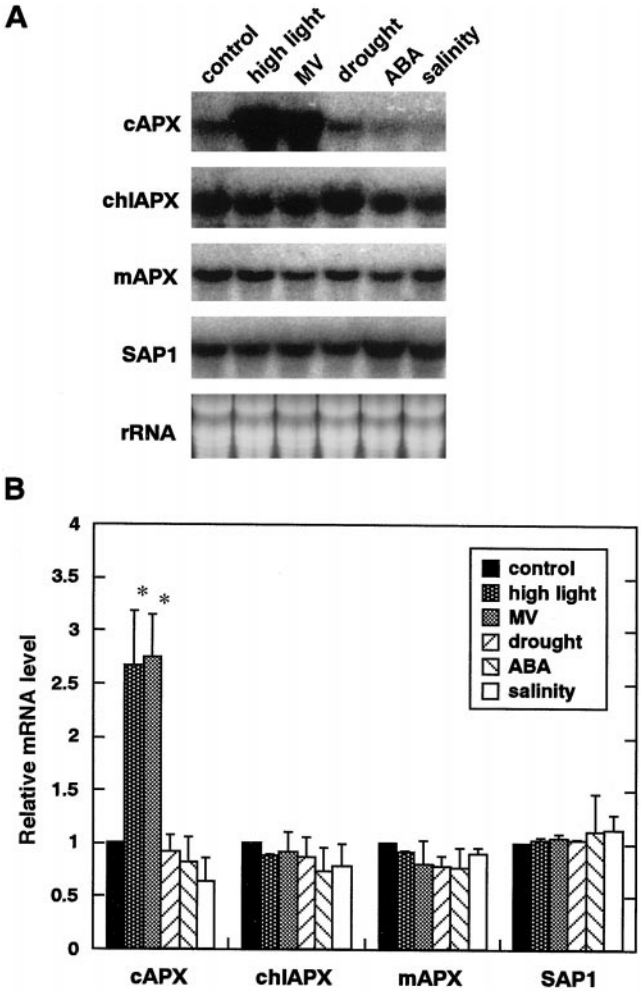

Figure 1. Changes in the steady-state transcript levels of the APX isoenzymes in response to environmental and chemical stresses. Four-week-old plants were exposed to stresses of high light, salinity, drought, and treatments with ABA and MV. A, Northern-blot analysis of APX isoenzyme transcripts. Total RNA was isolated from spinach leaves, separated by electrophoresis (30 μg each), blotted on a membrane, and hybridized with cAPX, tAPX, mAPX, and SAP1 cDNA probes as described in "Materials and Methods." B, Relative mRNA levels. The mRNA level of each sample was quantified with a phosphor imager and normalized to the respective 18S ribosomal RNA, and the values shown represent the mean value ± SD of three individual experiments. The value of the control plants was set equal to 1. Asterisks indicate that the mean values are significantly different compared with those of the control plants ($P < 0.05$).

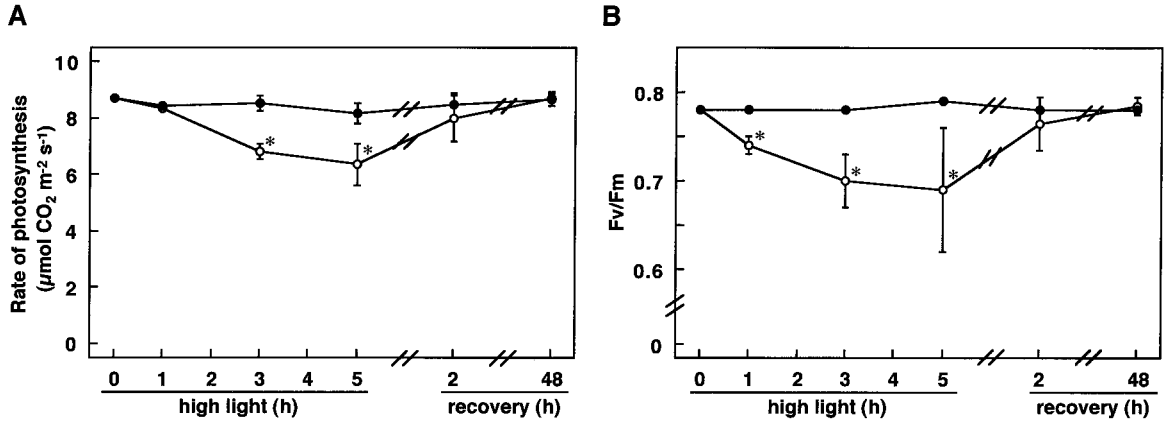

Figure 2. Photosynthetic parameters measured in spinach leaves exposed to high-light stress. Four-week-old plants were exposed to high light intensity (1,600 μE m^{-2} s^{-1}). After 5 h, stressed plants were re-exposed to moderate light at 300 μE m^{-2} s^{-1}. The CO_2 fixation (A) and chlorophyll fluorescence (F_v/F_m) (B) were measured as described in "Materials and Methods." The data are the mean value ± SD of three individual experiments. ●, Control plants; ○, stressed plants. Asterisks indicate that the mean values are significantly different compared with those of the control plants ($P < 0.05$).

where) and EAP1 raised against *Euglena* cAPX (Ishikawa et al., 1996b). The mAb-I reacted with both the 33- and 38-kD bands of sAPX and tAPX, respectively, which were present in the crude homogenate from the spinach leaves (Fig. 3, C and D). EAP1 reacted with the 28- and 31-kD bands of cAPX and mAPX, respectively. With either antibody, no other cross-reactive bands were detected. An increase in cAPX protein was not detected during high-light stress or recovery. The protein levels of the other isoenzymes were also not changed.

cAPX activity increased approximately 1.7-fold during the high-light stress and returned to nearly the control level during the recovery period (Fig. 3E). mAPX activity was not changed. Decreases in both tAPX and sAPX activities were found during the high-light stress. After 5 h of high light intensity, plants contained 46% lower activities of both the tAPX and sAPX compared with the control plants. During the recovery from the high-light stress, the activities of tAPX and sAPX returned to almost the same values as those in the control plants.

Changes in H_2O_2 and Ascorbate Levels

In the control plants, the H_2O_2 level did not change significantly during the experimental period (Fig. 4). In contrast, a transient increase (119%) in the H_2O_2 level was observed at 1 h in the high-light-stressed plants. Subsequently, the H_2O_2 level rapidly decreased and after 5 h, reached almost the same value as that in the control plants. To investigate the redox status of ascorbate in whole cells during high-light stress, the ascorbate and dehydroascorbate levels were assayed. The ascorbate level in the high-light-stressed plants was not changed, whereas the dehydroascorbate level increased approximately 3-fold after 5 h of high light intensity (Fig. 5). After 48 h in recovering plants, the dehydroascorbate level reached almost the same value as in the control plants. As a result, the decrease in the redox status of ascorbate (ascorbate/[ascorbate + dehydroascorbate]) was observed.

DISCUSSION

Only the cAPX transcript levels increased in the high-light-stressed and MV-treated plants; that is known to cause photooxidative stress (Fig. 1). Similar observations of cAPX induction by MV or high-light stress were reported in pea, maize, rice, and Arabidopsis (Mittler and Zilinskas, 1992; Pastori and Trippi, 1992; Donahue et al., 1997; Karpinski et al., 1997; Storozhenko et al., 1998; Morita et al., 1999). It seems likely that the induction in cAPX expression during an early stage of oxidative stress plays an important role in removing H_2O_2 and minimizing photooxidative damage. Transgenic tobacco plants expressing antisense RNA for cAPX showed increased susceptibility to ozone (Örvar and Ellis, 1997). Furthermore, overexpression of cAPX in tobacco plants provides increased resistance against MV treatment (Allen et al., 1997).

To confirm the early response of APX isoenzymes under high-light stress, we analyzed the changes in the transcript and protein levels and activities of each isoenzyme during the progression of high-light stress and following its recovery (Fig. 3, A–E). The cAPX transcript level reached a maximum in 3 h, the level of cAPX protein was only slightly changed, and the cAPX activity increased in response to the high light intensity. Mittler and Zilinskas (1994) reported that, during recovery from drought stress, cAPX expression in pea leaves is regulated at the post-transcriptional level, which is at least in part at the level of protein synthesis, protein stability, and/or enzyme activation. Furthermore, during pathogen-induced programmed cell death in tobacco leaves, cAPX expression was

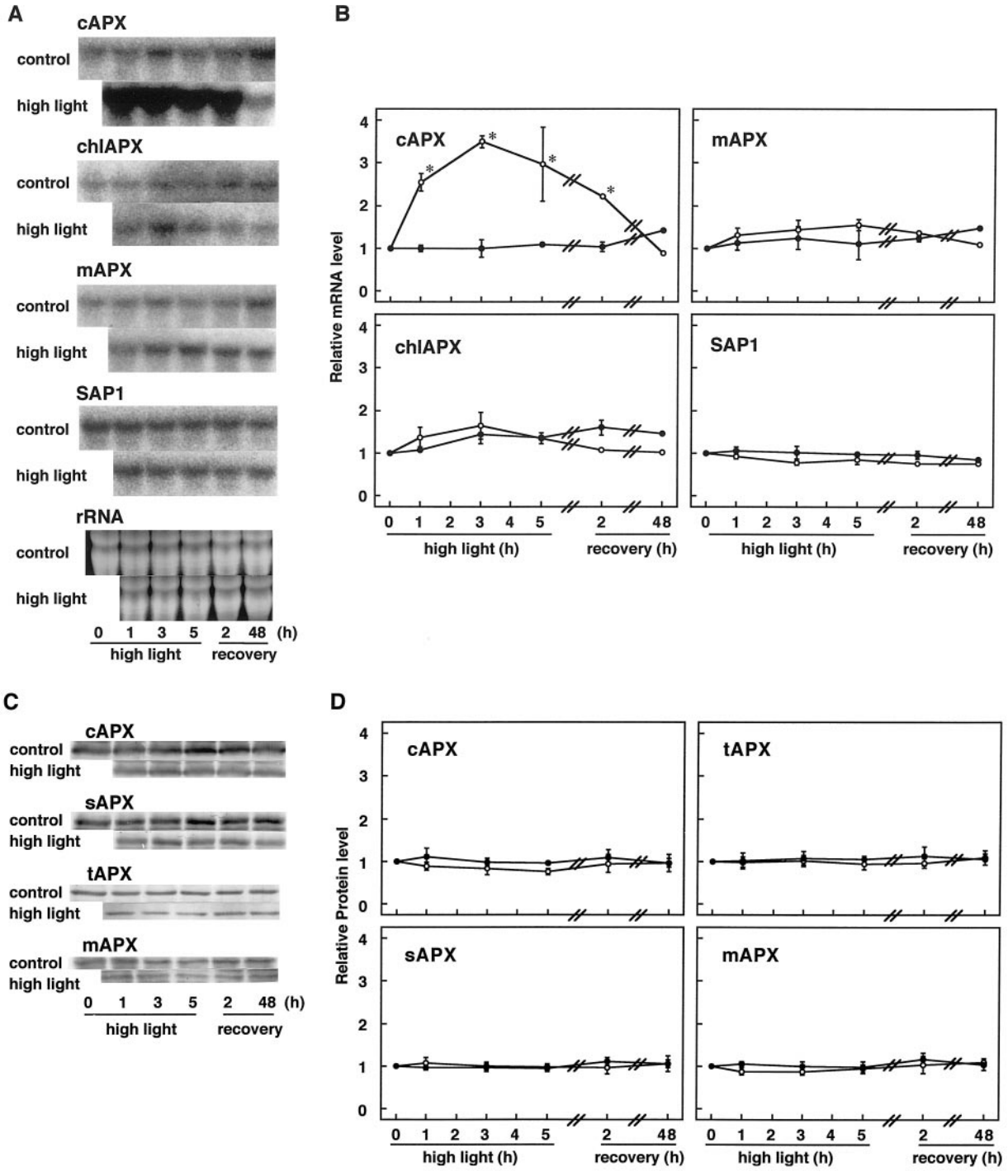

Figure 3. (Continues on facing page.)

suppressed by inhibition of protein synthesis in the polysome (Mittler et al., 1998). Based on our present data and on the data reported so far, it is clear that the protein level of cAPX does not directly correlate with the increase in the transcript level and activity. Therefore, it is likely that the activation state of cAPX pool increases under high-light stress. The inconsistencies between the unchanged level of protein and the increase in activity for cAPX will be clarified by further analysis, for example, by testing the effect of a mRNA or protein synthesis inhibitor.

The level of H_2O_2 reached a peak at 1 h during the progression of high-light intensity, exhibited a rapid decrease, and then returned to the initial level of the control plants at 5 h (Fig. 4). It has been suggested that H_2O_2 functions as a second messenger in plant

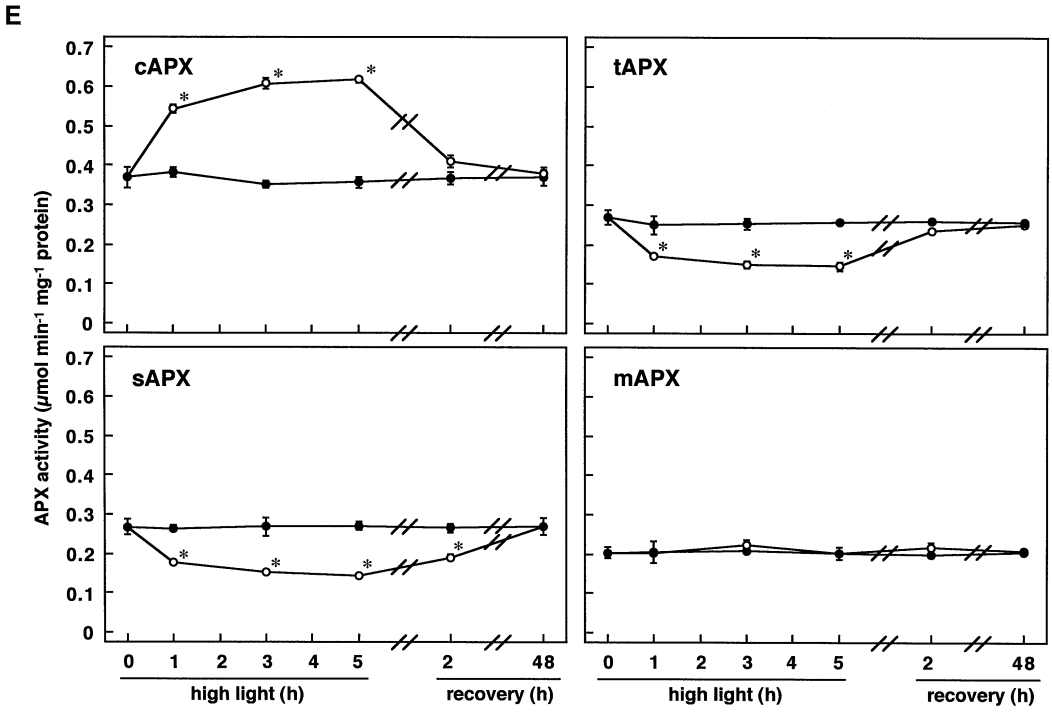

Figure 3. (Continued from facing page.)
The effect of high-light stress and its recovery on transcript and protein levels and activities of APX isoenzymes. A, Northern-blot analysis. Each APX isoenzyme transcript was detected as described in the legend of Figure 1. B, Relative mRNA levels. The mRNA levels of each sample were quantified with a phosphor imager and normalized to the respective 18S ribosomal RNA, and the values shown represent the mean value ± SD of three individual experiments. The value at time 0 was set to 1. C, Immunoblot analysis. The crude homogenates (50 μg each) of spinach leaves were subjected to SDS-PAGE and immunoblotting using mAb-I for sAPX and tAPX or EAP1 for cAPX and mAPX, as described in "Materials and Methods." D, Relative protein levels. The protein levels of each sample were densitometrically quantified and represent the mean value ± SD of three individual experiments. The value at time 0 was set to 1. E, Activities. Detailed procedures are described in "Materials and Methods." The data represented are the mean value ± SD of three individual experiments. ●, Control plants; ○, stressed plants. Asterisks indicate that the mean values are significantly different compared with those of the control plants ($P < 0.05$).

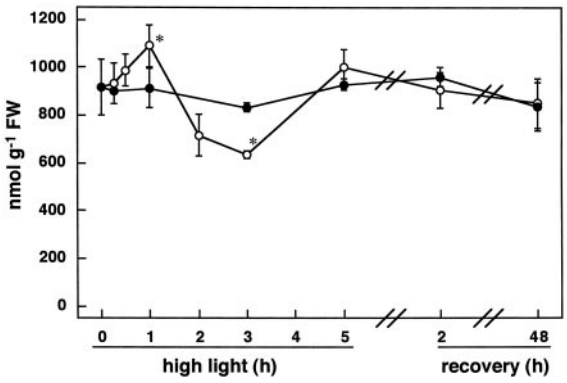

Figure 4. The effect of high-light stress and its recovery on the H_2O_2 level, which was determined as described in "Materials and Methods." The data represented are the mean value ± SD of three individual experiments. ●, Control plants; ○, stressed plants. Asterisks indicate that the mean values are significantly different compared with those of the control plants ($P < 0.05$).

cells exposed to environmental stresses such as chilling (Prasad et al., 1994), heat (Dat et al., 1998), and pathogens (Levine et al., 1994). The response of the spinach cAPX to oxidative stress caused by high light and MV, therefore, may be mediated by the transient accumulation of H_2O_2. The subsequent rapid decrease in H_2O_2 level may be caused by the increase in the cAPX activity (Figs. 3E and 4). The transient accumulation of H_2O_2 following cAPX expression has been observed in high-light-exposed Arabidopsis (Karpinski et al., 1997). In catalase (Cat-1)-deficient tobacco, cAPX protein and APX activity were increased by accumulated H_2O_2 under high-light conditions (Willekens et al., 1997). Photorespiration was identified as the principal source of H_2O_2 in tobacco exposed to high light intensity. It could be that catalase is light sensitive and undergoes photoinactivation with subsequent degradation, allowing H_2O_2 to escape destruction and move to the cytosol (Feierabend et al., 1992; Hertwig et al., 1992). In addition to H_2O_2 accumulation, Karpinski et al. (1999) suggested that redox changes in electron transport through qui-

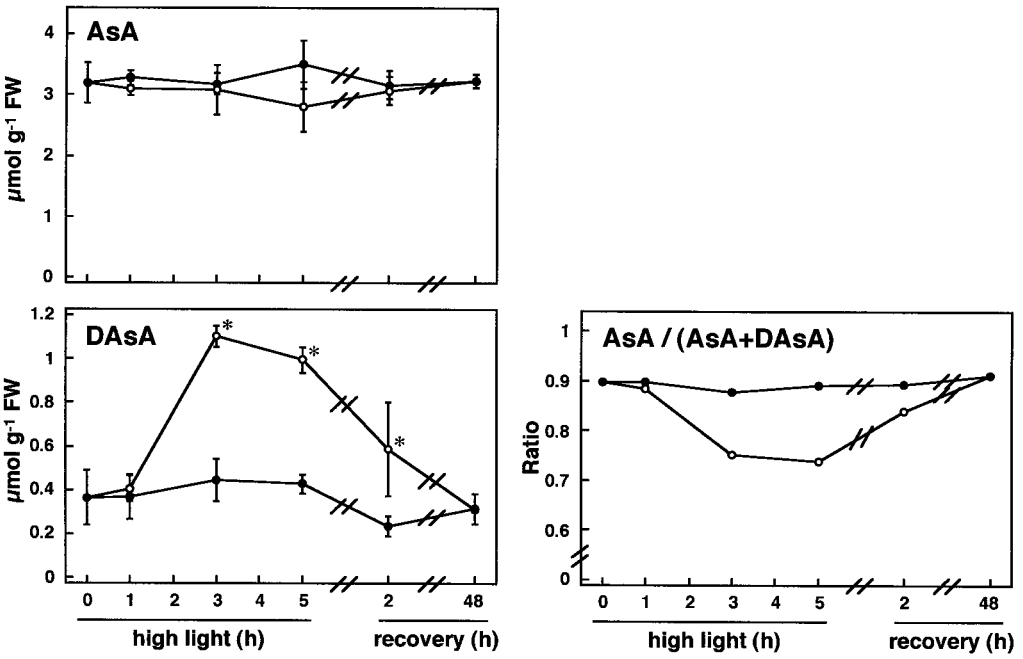

Figure 5. The effect of high light intensity and its recovery on the levels of ascorbate and dehydroascorbate and the redox status of ascorbate. Experimental conditions are described under "Materials and Methods." The redox status of ascorbate (ascorbate/[ascorbate + dehydroascorbate]) was calculated. The data represented are the mean value ± SD of three individual experiments. ●, Control plants; ○, stressed plants. Asterisks indicate that the mean values are significantly different compared with those of the control plants ($P < 0.05$).

none B or plastoquinone in chloroplasts could be essential for cAPX induction under high-light intensity.

Various plants contained two or more putative cAPXs, indicating that cAPX is encoded by a multigene family (Santos et al., 1996; Jespersen et al., 1997; Karpinski et al., 1997; Caldwell et al., 1998). In spinach, SAP1, which appears to be a member of a second family of cAPXs, has been cloned and characterized (Ishikawa et al., 1995), but its subcellular localization has not yet been clarified. We could not detect SAP1 at the protein level in the crude homogenate of spinach leaves, whereas its mRNA was found to be constitutively expressed under several conditions (Fig. 1). When the soluble extract prepared from spinach leaves was loaded onto a DEAE-Sephacel column and eluted, the APX activities were separated into only two fractions derived from sAPX and cAPX (S. Shigeoka and K. Yoshimura, unpublished data). Thus, the expression of SAP1 seems to be suppressed by translational regulation.

The spinach mAPX and chlAPX isoenzymes showed no significant changes in the steady-state transcript level in response to several stresses (Figs. 1 and 3, A and B). The constitutive expression of the transcript levels of chlAPX and mAPX supported the hypothesis that these isoenzymes function to immediately detoxify H_2O_2 generated in each organelle under normal and stress conditions. Wang et al. (1999) indicated that the protective action provided by the expression of mAPX seems to be specific against oxidative stress originating from microbodies. The protein level and activity of mAPX were not changed during high-light intensity and following its recovery (Fig. 3, C–E). Zhang et al. (1997) indicated that the transcript level of peroxisomal APX in Arabidopsis slightly increased in response to cold, UV light, and treatment with H_2O_2 and MV. While further study of the response of mAPX under oxidative stress is required, the present data suggest that the regulation of mAPX may be different among the species due to microbody biogenesis and metabolism.

The activities of the chlAPX isoforms decreased during the progression of high light intensity despite the fact that their transcript and protein levels were not altered (Fig. 3, A–E). We have previously demonstrated that the chlAPX isoforms in tobacco leaves are completely inactivated under stress conditions, while phosphoribulokinase remains active; this indicates that APX is much more strongly inactivated by oxidative stress than phosphoribulokinase, which is believed to be one of the thiol-modulated enzymes most sensitive to H_2O_2 (Shikanai et al., 1998). One of the characteristic properties of the chlAPX isoforms is rapid inactivation when the level of ascorbate is too low for the operation of the catalytic cycle of the APX isoenzymes (Miyake and Asada, 1996). It has been suggested that the level of ascorbate in chloroplasts affects the stability of the chlAPX isoforms under oxidative stress conditions. However, in spite of a decrease in the activities of the chlAPX isoforms, the

level of ascorbate was not changed during the high light intensity (Fig. 5).

The increase in the dehydroascorbate level in spinach leaves caused a decrease in the redox status of ascorbate (ascorbate/[ascorbate + dehydroascorbate]), and after 48 h in recovering plants the dehydroascorbate level reached almost the same value as in the control plants (Fig. 5). It is worth noting that during the recovery from stress, the activities of the chlAPX isoforms still had the capacity to return to the initial levels, in parallel with the recovery of the redox status of ascorbate. Thus, the reversible inactivation of chlAPX isoforms under oxidative stress conditions seems to be involved in the redox status of ascorbate in spinach chloroplasts. This would be true under mild environmental conditions. In conclusion, it is likely that the expression patterns of the APX isoenzymes are individually regulated at each cellular compartment under several stress conditions, and that each expressed APX isoenzyme plays a cooperative role to protect each organelle and minimize tissue injury.

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea*) seedlings, individually planted in pots, were grown in a climate chamber under the following normal conditions: an 8-h photoperiod, illumination of 300 μE m^{-2} s^{-1}, temperature of 15°C ± 2.5°C, and a relative humidity of 75% ± 5%. The cDNAs encoding the spinach APX isoenzymes were originally cloned into plasmid pBluescript SK(+) (Ishikawa et al., 1995, 1996a, 1998). All other chemicals were of the highest purity grade commercially available.

Plant Stress Conditions

Four-week-old plants were exposed to stresses of high light intensity, salinity, drought, and treatments of ABA and MV. High-light stress was accomplished with exposure to illumination at 1,600 μE m^{-2} s^{-1}. Recovery from the high-light stress was assayed by the following: plants exposed to high light for 5 h were transferred to normal conditions under 300 μE m^{-2} s^{-1} illumination and sampled 2 and 48 h later. Salinity stress was imposed by transferring the plants to Hoagland solution containing 300 mM NaCl and growing for 3 d under normal conditions. Chemical treatments were imposed by spraying with 1 mM ABA or 10 μM MV, each prepared in 0.05% (v/v) Tween 20 as described previously (Mittler and Zilinskas, 1992). ABA-treated plants were sampled following a 24-h incubation under normal conditions. MV-treated plants were sampled following a 1-h incubation under illumination at 1,600 μE m^{-2} s^{-1}. Drought stress was imposed by harvesting the whole plants, washing gently, and then subjecting them to dehydration on a paper towel for 1 h. Control plants were maintained under normal conditions and sampled at the same time as the stressed plants. Visible injury, such as the degradation of chlorophyll, was not observed in any of the stressed plants. The leaves were collected from approximately 30 plants per treatment, divided into four portions, flash-frozen in liquid N_2, and stored at −80°C for northern blotting, immunoblotting, and the activity assay for each APX isoenzyme and for the measurement of their biochemical parameters.

RNA Extraction and Northern-Blot Analysis

The total RNA (30 μg each) was isolated from spinach leaves (1 g fresh weight) as previously described (Yoshimura et al., 1999), subjected to electrophoresis on 1.2% (v/v) agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond N membrane (Amersham, Buckinghamshire, UK). Prehybridization took place at 55°C for 6 h in buffer containing 5× SSC, 5× Denhard's solution, 1% (w/v) SDS, and 100 μg mL^{-1} denatured salmon sperm DNA. The membrane was hybridized at 55°C for 12 h in the presence of the individual ^{32}P-random primed spinach cDNA of the APX isoenzymes (Table I). We have previously shown that the tAPX and sAPX isoenzymes arise from a common pre-mRNA, which was generated from an identical gene (*ApxII*) by alternative splicing of the 3′-terminal exons (Ishikawa et al., 1997). As a result, four

Table I. *Probes used in northern-blot and immunoblot analysis*

Isoenzyme	Localization	Probe		Reference
		cDNA	Antibody[b]	
cAPX	Cytosol	cAPX	EAP1	Ishikawa et al. (1995)
SAP1	Putative cytosol	SAP1	EAP1	Ishikawa et al. (1995)
mAPX	Microbody membrane	mAPX	EAP1	Ishikawa et al. (1998)
chlAPX/tAPX	Thylakoid membrane	tAPX[a]	mAb-I	Ishikawa et al. (1996a)
/sAPX	Stroma	tAPX[a]	mAb-I	Ishikawa et al. (1997); Yoshimura et al. (1999)

[a] The tAPX cDNA probe was used for detection of the transcript level of chlAPX added together with those of tAPX and sAPX. The cDNA sequences are available in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession nos.: D85864 (cAPX), D49697 (SAP1), D83104 (mAPX), D77997 (tAPX), and D83669 (sAPX). [b] EAP1 and mAb-I were raised against cAPX and tAPX proteins from spinach, respectively. Detailed procedures are described in "Materials and Methods."

mRNA variants, one form of thylakoid-bound APX (tAPX-I), and three forms of stromal APX (sAPX-I, sAPX-II, and sAPX-III) are generated (Yoshimura et al., 1999). sAPX-II and sAPX-III mRNAs contain a sequence derived from exon 13 including the coding sequence of the membrane anchoring segment of tAPX as a untranslated region. Accordingly, the respective transcript levels of the sAPX and tAPX isoenzymes are difficult to determine by northern-blot analysis. Therefore, in this experiment, we measured the transcript level of chlAPX added together with those of tAPX and sAPX using a tAPX cDNA as the probe. The membrane was washed twice in 2× SSC and 0.1% (w/v) SDS for 10 min each at room temperature, and in 0.1× SSC and 0.1% (w/v) SDS at 60°C for 60 min. The membrane was then exposed to an imaging plate and the relative expression ratio of each APX transcript was calculated using a phospor imager (Mac BAS 2000, Fuji, Tokyo).

Enzyme Assays

One of the specific properties of APX isoenzymes is rapid inactivation in an ascorbate-depleted medium. This is especially true for chloroplastic APX isoforms, whose half-inactivation time is only 15 s (Miyake and Asada, 1992; Yoshimura et al., 1998). Both the cAPX and the mAPX isoenzymes are less sensitive to depletion of ascorbate than the chloroplastic APX isoforms, so the half-inactivation times of cAPX and mAPX were approximately 60 min and over 24 h, respectively (Chen and Asada, 1989; Miyake and Asada, 1992; Ishikawa et al., 1998). On the basis of this characterization, the activities of the APX isoenzymes were separately assayed by a modified method reported by Amako et al. (1994). Spinach leaves were ground to a fine powder in liquid N_2 and then homogenated in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate, 20% (w/v) sorbitol, 1 mM EDTA, and 0.1% (w/v) phenylmethanesulfonyl fluoride using a mortar and pestle. The homogenate was squeezed through four layers of cheesecloth and then centrifuged at 100,000*g* for 30 min. The obtained soluble fraction contained activities of sAPX and cAPX isozymes. The supernatant (5 μL) was added to 2.0 mL of N_2-bubbling 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM H_2O_2. At 1, 2, 3, and 5 min after the start of the incubation, the incubated mixture (1.98 mL) was sampled and mixed with 10 μL of 100 mM ascorbate to terminate the inactivation. The residual oxidizing activity of ascorbate was then assayed by adding 10 μL of 20 mM H_2O_2. The oxidation of ascorbate was followed by a decrease in the A_{290} (ϵ = 2.8 mM^{-1} cm^{-1}), and the results are plotted on the graph. The ratio of cAPX and sAPX activities was calculated from the inactivation curve of each isoenzyme. The 100,000*g*-membrane fraction was washed and suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate. The suspended-membrane fraction contained activities of tAPX and mAPX isoenzyme, which were assayed separately by the same method using each half-inactivation time as measurements of activities of sAPX and cAPX isoenzymes. The protein was quantified according to the method of Bradford (1976).

SDS-PAGE and Immunoblotting

To measure the protein levels of the APX isoenzymes, the spinach leaves were homogenized with SDS-loading buffer (150 mM Tris-HCl, pH 6.8, 4% [w/v] SDS, and 10% [v/v] 2-mercaptoethanol). The homogenates were boiled for 5 min and centrifuged at 10,000*g* for 10 min. The supernatants were quantified with respect to protein content and subjected to SDS-PAGE and immunoblotting. SDS-PAGE was performed in 12.5% (v/v) slab gels according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting, the gels were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) using an electroblot apparatus (model 200/2.0, Bio-Rad Laboratories) at 15 V for 1 h. The membranes were treated with the *Euglena* cAPX monoclonal antibody (EAP1; Ishikawa et al., 1996b) to detect cAPX and mAPX or the spinach sAPX monoclonal antibody (mAb-I; S. Shigeoka and K. Yoshimura, unpublished data) to detect sAPX and tAPX (Table I). The membranes were visualized with alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories).

Assay of Photosynthesis

The CO_2 fixation was measured with the portable photosynthesis system (model LI-6400, LI-COR, Lincoln, NE). Net CO_2 assimilation rates were measured using fully expanded leaves under the following conditions: 300 μE m^{-2} s^{-1}, 1,000 ppm CO_2, 15°C, and 75% relative humidity. The change in chlorophyll fluorescence was measured at 15°C with a chlorophyll fluorometer (Mini PAM, Waltz, Effeltrich, Germany). The minimum fluorescence yield (F_0) was determined after a 30-min dark adaptation followed by illuminating the sample with a low-irradiance measuring light (approximately 0.12 μE m^{-2} s^{-1}). A saturating pulse of white light (0.8 s, 10,000 μE m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (F_m). The maximal quantum yield of PS II (F_v/F_m) was determined from the following equation: $F_v/F_m = F_m - F_o/F_m$.

Determination of H_2O_2

Leaves (1 g fresh weight) frozen in liquid N_2 were ground using a pestle and mortar with 1 mL of 3% (v/v) $HClO_4$ containing 2.5 mM EDTA. The homogenate was centrifuged at 12,000*g* for 5 min at 4°C. The supernatant was neutralized with 2.5 M KOH to pH 7.5, and then centrifuged at 12,000*g* for 5 min at 4°C. The supernatant obtained was filtered (DISMIC-25HP filter, Advantec Toyo, Tokyo) and then passed through an anion-exchange column (Okuda et al., 1991). The elute was used for the determination of H_2O_2 by the homovanillic acid method with some modifications (Guilbault et al., 1968).

Determination of Ascorbate and Dehydroascorbate

Ascorbate and dehydroascorbate were measured as described by Wise and Naylor (1987). Leaves (0.5 g wet weight) frozen in liquid N_2 were ground using a pestle and

mortar with 5 mL of 6% (v/v) $HClO_4$ and centrifuged at 10,000*g* for 10 min at 4°C. A 100-μL aliquot of the obtained leaf extract was added directly to 900 μL of a 200 mM succinate buffer (pH 12.7, adjusted with NaOH) in the spectrophotometer. The final pH was very near 6.0. The A_{265} was immediately recorded and again 5 min after the addition of 5 units of ascorbate oxidase from *Cucurbita* sp. (Wako, Osaka). For determination of total ascorbate, the leaf extract was adjusted to pH 6.0 with 1.25 M K_2CO_3 and centrifuged at 10,000*g* for 5 min. The supernatant was incubated with 10 mM dithiothreitol in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH buffer (pH 7.5) for 10 min at 25°C. A 100-μL aliquot of the solution was directly added to 900 μL of 200 mM succinate buffer (pH 6.0) in the spectrophotometer. The resultant solution was assayed as described above. The difference between the total ascorbate and ascorbate contents was taken to be the content of dehydroascorbate.

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