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Analysis of the signal transduction system involved in cytosolic ascorbate peroxidase induction

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Introduction

Active oxygen species (AOS) are produced from many cell metabolisms in aerobic organisms, even under optimal conditions. Furthermore, the imposition of biotic and abiotic stress conditions such as chilling, heat, and pathogen infection can give rise to excess concentrations of AOS, resulting in oxidative damage at the cellular level. Therefore, aerobic organisms develop a number of mechanisms to scavenge AOS rapidly. On the other hand, AOS participate in cellular signalling to regulate many gene expressions. H_2O_2, especially, seems to function as a second messenger in plant cells exposed to environmental stress. In higher plants, ascorbate peroxidase (APX; EC 1.11.1.11) isoenzymes play a key role in scavenging H_2O_2 and are distributed in at least five distinct cell compartments: the stroma and thylakoid membrane in chloroplast (chlAPX isoenzymes), cytosol (cAPX), the microbody membrane, and the mitochondrial inner membrane. Considering the specific distributions of the APX isoenzymes and the potential for AOS production in each organelle of higher plants, it seems likely that the APX isoenzymes are expressed by distinct regulatory mechanisms and play a cooperative role to protect each organelle and minimize tissue injury. Recently, we studied the expression of spinach APX isoenzymes in response to several stress conditions (Yoshimura et al. 2000). The steady-state transcript level of cAPX remarkably increased in response to high-light (HL) and paraquat treatment, while the levels of other APX isoenzymes, including chlAPX, were not changed.

It is likely that the induction of cAPX expression may be mediated by H_2O_2 because a transient accumulation of H_2O_2 is observed. In high-light-exposed *Arabidopsis,* the redox changes in electron transport through quinone B (Q_b) or plastquinone in chloroplasts have been found to be essential for cAPX induction (Karpinski et al. 1997, 1999). This result indicates that the changes in the redox status in chloroplasts may be important factors regarding the regulation of antioxidative mechanisms in whole cells even though no change of ch1APX may be observed. However, the molecular mechanisms of the gene regulation of APX isoenzymes are not fully understood. Here we studied the signal transduction system that regulates the expression of cAPX and chlAPX isoenzymes in spinach.

Materials and methods

Spinach plants were cultured for four weeks in a climate chamber under the following normal conditions: an 8-h photoperiod, low light (LL) of 300 $\mu E\ m^{-2}\ s^{-1}$, temperature of 15°C ± 2.5°C,

and a relative humidity of 75% ± 5%. For chemical treatments, detached spinach leaves were vacuum-infiltrated for 3 min in the presence of some compounds dissolved in H_2O_2 and then floated on a solution of the same compound in petri dishes under LL for 3 h and then under high light (HL) of 1600 μE m^{-2} s^{-1} for 1 h. The cDNAs encoding spinach APX isoenzymes were originally cloned into plasmid pBluescript SK(+). By Northern-blot analysis, the transcript level of each APX isoenzyme was determined using each APX cDNA probe as described previously (Yoshimura et al. 2000). The amount of H_2O_2 was measured by the homovanillic acid method (Yoshimura et al. 2000). Cloning of the promoter region of the cAPX gene (*ApxI*) from spinach was performed by the inverse PCR method. The genomic DNA was isolated from spinach leaves (Ishikawa et al. 1997).

Results and discussion

To analyze the redox changes in the electron transport of chloroplasts and the cellular H_2O_2 levels required for the expression of APX isoenzymes, LL-adapted spinach leaves were incubated with several compounds for 3 h and then exposed to HL, leading to the induction of cAPX expression. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) specifically inhibited the oxidation of the quinone A of PS II and the plastquinon pool by competitively binding to the cytochrome b_6/f complex, respectively (Jones and Whitmarsh, 1988).

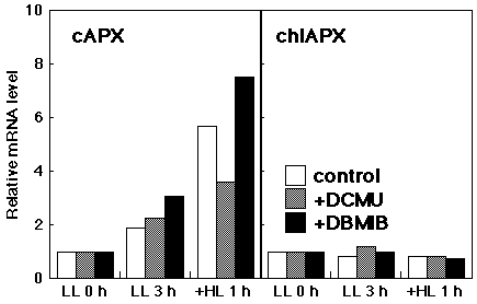

Fig. 1 Northern-blot analysis of mRNA levels of APX isoenzymes in leaf discs treated with photosynthetic electron transport inhibitors.

DCMU (10 μM) treatment suppressed the induction of cAPX expression by HL for 1 h by 37%, while DBMIB (24 μM) treatment enhanced it by 162% (Fig. 1). The two treatments failed to change the expression level of chlAPX.

The treatment of 3-aminotriazole (3AT: 10 mM), an irreversible inhibitor of catalase, or paraquat (PQ: 10 μM), an enhancer of the production of O_2^-, for 4 h under LL increased the expression of cAPX but not that of chlAPX isoenzymes (Fig. 2A, B).

H_2O_2 levels in 3AT- and PQ-treated leaves increased to 244% and 132%, respectively, compared with those in non-treated leaves (Table1).

The increase in the transcript level of cAPX by the paraquat treatment was partially suppressed by *N*, *N*-diethyldithiocarbamate (DDC: 1 mM), which inhibited Cu/Zn-SOD (Fig. 2B).

The endogenous H_2O_2 (50 mM) induced an increase in the transcript level of cAPX but not in that of chlAPX isoenzymes (Fig. 3). These results indicate that the expression of cAPX is mediated by both the redox changes in electron transport through Q_b and/or plastoquinone and the intracellular H_2O_2 level.

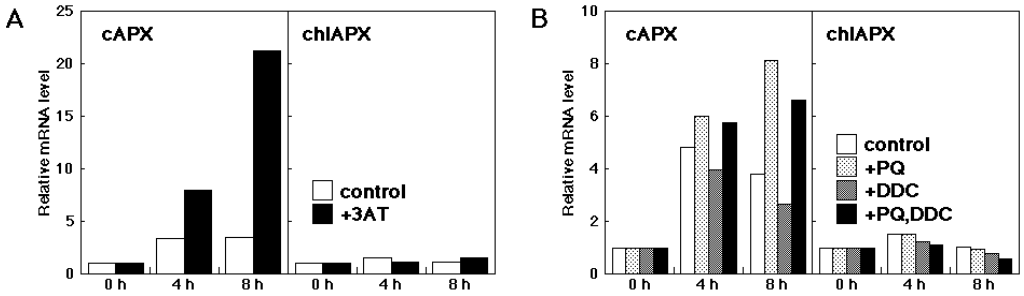

Fig. 2 Northern-blot analysis of mRNA levels of APX isoenzymes in leaf discs treated with 3AT, PQ, and DDC.

To investigate the *cis*-acting elements involved in the induction of cAPX, we isolated the promoter region of the cAPX gene (*ApxI*) by the inverse PCR method. A 3.5-kbp fragment obtained contained the partial sequence of cAPX cDNA and the 1.7 kbp-promoter region. The TATA box was found in psitions –49 to –52 and two putative CAAT boxes were found in 94 and 122 upstream from the TATA box. The first intron is located upstream of the ATG initiation codon (Fig. 4).

A similar structure was observed in the cAPX gene from several sources. In tomato, this first intron is required to confer constitutive gene expression in leaves but not in other organs of the plant (Gadea et al., 1999). Now, we are constructing genes with the *ApxI* promoter, including the first intron fused to the β-glucronidase reporter gene, and transforming *Arabidopsis* with these constructs.

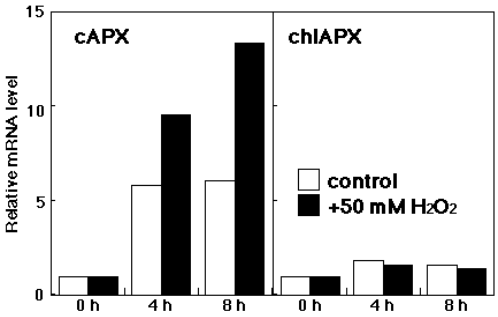

Fig. 3 Northern-blot analysis of mRNA levels of APX isoenzymes in leaf discs treated with H_2O_2.

Table 1 cellular H_2O_2 level within spinach leaves treated with 3AT and PQ.

Treatment	H_2O_2 µmol g^{-1} FW
None	1.08 ± 0.02
+ 10 mM 3AT	2.64 ± 0.01
+ 10 µM PQ	1.43 ± 0.06

Data are represented as mean ± deviation (n=3).

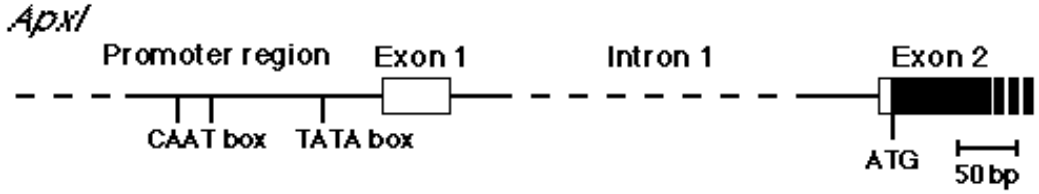

Fig. 4 Structure of the *ApxI* promoter region. Exons are represented by open (untranslated) and closed (translated) boxes.

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