Acclimation to Diverse Environmental Stresses Caused by a Suppression of Cytosolic Ascorbate Peroxidase in Tobacco BY-2 cells

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Introduction

Many plant cells produce a range of active oxygen species (AOS), including \( \mathrm{O}_2^- \) and \( \mathrm{H}_2\mathrm{O}_2 \), during the course of normal metabolic processes. Such AOS generation is essential for various physiological processes, such as the gravitropic response in root cells (Joo et al. 2001), stomatal movements in guard cells (Pei et al. 2000) and the response to elicitors during pathological processes (Bolwell et al. 2002). On the other hand, when plants are exposed to a variety of environmental stress conditions, including low water availability, high irradiance, temperature fluctuations and nutrient deprivation, an excess amount of AOS can be generated, resulting in oxidative stress (Blokhina et al. 2003). In order to avoid cellular damage, plants have evolved highly organized mechanisms for regulating the level of cellular AOS.

AOS scavenging is carried out by a complex cross-talk of reactions including enzymes and antioxidative compounds with redox properties. The ascorbate–glutathione (AsA–GSH) cycle plays a key role in this cross-talk (Shigeoka et al. 2002). In this cycle, ascorbate peroxidase (APX) utilizes AsA as its specific electron donor to reduce \( \mathrm{H}_2\mathrm{O}_2 \) with the concomitant generation of monodehydroascorbate (MDA), a univalent oxidant of AsA. MDA is spontaneously disproportionated to AsA and dehydroascorbate (DHA). MDA is also directly reduced to AsA by the action of NAD(P)H-dependent MDA reductase. DHA reductase utilizes GSH to reduce DHA and thereby regenerate AsA. The oxidized GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD(P)H.

Components of the AsA–GSH cycle are widely distributed in the cellular compartments where AOS scavenging is needed, such as the chloroplasts, microbodies, mitochondria and cytosol. This indicates that the cycle is one of the key factors for maintaining the cellular AOS homeostasis. In view of the extensive cross-talk between the cell organelles and nuclei, the cytosol is an important buffer area for the modulation of signal molecules that are transmitted from the cellular compartments to the nuclei. In the cytosol, APX is one of the major antioxidants that regulates the reduction/oxidation (redox) states. Among the APX isoforms, the cytosolic form is the most responsive to various environmental stress conditions, supporting its physiological importance during stress exposure.

The active oxygen species (AOS) that arise from normal metabolic processes are kept under tight control by various antioxidant mechanisms. AOS are important signal molecules that regulate many physiological processes, including environmental stress responses. In this work, we have investigated the effect of lowered cytosolic ascorbate peroxidase (APX) activity in transgenic tobacco BY-2 cells, using two transformed BY-2 cell lines, cAPX-S2 and cAPX-S3, resulting from co-suppression by expression of Arabidopsis APX1 cDNA under the cauliflower mosaic virus (CaMV) 35S promoter. cAPX-S2 and cAPX-S3 possessed 50 and 75% lower cytosolic APX activity, respectively, compared with that in the untransformed cells. Chemical fluorescence analysis indicated that the AOS levels were markedly higher in the two APX-suppressed cell lines than in the wild-type cells. However, there were no substantial differences in the activity levels of the various other antioxidant enzymes. Interestingly, the APX-suppressed cells showed different responses and tolerances to environmental stresses, such as heat and salinity. Suppression subtractive hybridization revealed that several heat- and salt stress-inducible genes were up-regulated in cAPX-S3 cells. HSP70, DnaJ-like protein and purple acid phosphatase were among the constitutively induced genes. An in-gel kinase assay suggested that a mitogen-activated protein (MAP) kinase of approximately 46 kDa was predominantly active in the APX-suppressed cells, and transcript levels of both nicotiana protein kinase 1 (NPK1) and nucleoside diphosphate kinase 2 (NDPK2) were up-regulated. These data suggest the possibility that MAP kinase cascades are activated by subtle imbalances in the homeostasis of the cellular redox status caused by lowered cytosolic APX activity.

**Keywords:** Active oxygen species — Ascorbate peroxidase — Diverse stress tolerance — Redox — Tobacco BY-2

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; AsA, ascorbate; CaMV, cauliflower mosaic virus; DHA, dehydroascorbate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); H2DCF-DA, 2′,7′-dichlorofluorescein diacetate; HSP, heat shock protein; MAP, mitogen-activated protein; MBP, myelin basic protein; MDA, monodehydroascorbate; NDPK, nucleoside diphosphate kinase; NPK1, nicotiana protein kinase 1; SSH, suppression subtractive hybridization.

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(Mittler et al. 1998, Karpinski et al. 1999, Yoshimura et al. 2000, Yabuta et al. 2004). Recently, Pnueli et al. (2003) have reported that a cytosolic APX (Apx1)-lacking Arabidopsis mutant showed late flowering, delayed development, a lower rate of photosynthetic activity, altered stomatal response and a higher level of H$_2$O$_2$ accumulation in the leaves. Microarray analysis indicated that the disruption of APX1 affected the expression of many genes under conditions of light stress. When considering the role of cytosolic APX in the stress response, it is therefore important to explore its possible functions in modulating plant redox signaling.

We have reported previously that the cytosolic APX of cultured tobacco BY-2 contributes to approximately 80% of the total cellular APX activity (Madhusudhan et al. 2003). In addition, Vacca et al. (2004) have reported that a decrease in the cytosolic APX level of BY-2 cells in the early phase of heat shock-induced programmed cell death coincided with an increase in AOS production. These observations indicated that the BY-2 cell should be a good experimental model to analyze the effect of cytosolic APX on the homeostasis of cellular AOS concentration.

In this study, to clarify the relationship between the cytosolic APX expression and stress response, we have investigated the effect of lowered cytosolic APX activity using transformed BY-2 cell lines resulting from co-suppression by the expression of Arabidopsis APX1 cDNA under the cauliflower mosaic virus (CaMV) 35S promoter. The transgenic lines showed higher levels of cellular AOS and increased tolerance to heat and salt stresses. A concomitant increase of stress-related gene mRNA and mitogen-activated protein (MAP) kinase activities was also observed. These observations suggest that the AOS accumulation in the APX-suppressed cells led to constitutive activation of stress response pathways which resulted in increased stress tolerance.

**Results**

**Production of BY-2 cells with decreased cytosolic APX activity**

To manipulate the activity of cytosolic APX in BY-2 cells, a cDNA encoding Arabidopsis cytosolic APX (APX1) was expressed in the sense orientation under the control of a CaMV 35S promoter. Although a total of >50 independently transformed BY-2 cells were obtained, none of the lines showed any substantial increase in APX activities. Fig. 1 shows the typical representative result of transformed BY-2 cells. Five lines of transgenic BY-2 cells exhibited decreased APX activity, ranging between 10 and 75% of that of non-transformed BY-2 cells (Fig. 1B). Immunoblot analysis with a monoclonal antibody raised against spinach cytosolic APX, which cross-reacts with all kind of cytosolic APXs we have tested so far, including one from tobacco (Yoshimura et al. 2001), revealed that the expression level of cytosolic APX protein in the transformed cell lines resulted in a corresponding decrease in the level of APX activity (Fig. 1C). These results suggest that the repression of APX activities in transformed cell lines is due to co-suppression. Accordingly, we selected cAPX-S2 and cAPX-S3 exhibiting different APX activities and used them for further analysis.

**Characterization of transgenic BY-2 cell lines with decreased APX activity**

The effect of decreased expression of cytosolic APX on cell growth and the antioxidative system was investigated in detail in two representative co-suppression lines, cAPX-S2 and
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cAPX-S3. In contrast to the wild-type cell cultures, the time required to reach the stationary phase was significantly delayed in cAPX-S2 and cAPX-S3 cell lines (Fig. 2), suggesting that the suppression of cytosolic APX affects cell growth in BY-2 cells. We decided to use cell lines in the stationary phase in the subsequent experiments because we speculated that this would minimize the effects of the cell growth rate difference on the results.

There were no significant differences between the wild-type and cytosolic APX-suppressed cell lines in the activities of the enzymes related to the AsA–GSH cycle (MDA reductase, DHA reductase and GSH reductase) (Table 1). Additionally, the AsA and GSH content was unaffected by the altered cytosolic APX activities. The concentration of DHA, however, was decreased in cytosolic APX-suppressed cell lines by approximately 20% compared with the wild-type cell line.

To identify any differences in the intracellular AOS levels between wild-type and cytosolic APX-suppressed cell lines, we used the chemical fluorescent probe, 2′,7′-dichlorofluorescein diacetate (H$_2$DCF-DA) which has been used extensively as a non-invasive, in vivo measurement of intracellular AOS (Zhu et al. 1994). Interestingly, as shown in Fig. 3, the level of fluorescence was approximately 1.5 and 2.3 times greater in cAPX-S2 and cAPX-S3 cells, respectively, compared with wild-type cells. We carefully repeated the experiment and obtained the reproducibility. Moreover, treatment of cAPX-S2 and cAPX-S3 cell lines with exogenous H$_2$O$_2$ caused a prominent rise in intracellular fluorescence of H$_2$DCF-DA comparable with wild-type cells (data not shown). These results clearly indicated that the suppression of cytosolic APX activity in BY-2 cells results in an imbalance in the cellular redox state toward oxidation.

**Table 1** AOS scavenging enzyme activities and antioxidant levels in the wild-type and transformed cell lines

<table>
<thead>
<tr>
<th>Enzyme activities (µmol min$^{-1}$ mg$^{-1}$ protein)</th>
<th>Wild-type</th>
<th>cAPX-S2</th>
<th>cAPX-S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>APX</td>
<td>8.2 ± 0.6</td>
<td>4.1 ± 0.4*</td>
<td>2.1 ± 0.1*</td>
</tr>
<tr>
<td>Catalase</td>
<td>12.3 ± 0.8</td>
<td>9.9 ± 0.5*</td>
<td>13.6 ± 3.8</td>
</tr>
<tr>
<td>MDA reductase</td>
<td>0.32 ± 0.02</td>
<td>0.34 ± 0.01</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>DHA reductase</td>
<td>0.65 ± 0.04</td>
<td>0.50 ± 0.03</td>
<td>0.64 ± 0.01</td>
</tr>
<tr>
<td>GSH reductase</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Antioxidants (nmol g FW$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AsA</td>
<td>123.7 ± 1.2</td>
<td>130.7 ± 2.6</td>
<td>125.5 ± 1.5</td>
</tr>
<tr>
<td>DHA</td>
<td>85.3 ± 0.7</td>
<td>69.9 ± 8.7*</td>
<td>66.5 ± 0.5*</td>
</tr>
<tr>
<td>GSH</td>
<td>138.1 ± 36.5</td>
<td>157.2 ± 19.4</td>
<td>154.4 ± 25</td>
</tr>
<tr>
<td>GSSG</td>
<td>3.9 ± 2.2</td>
<td>4.1 ± 0.1</td>
<td>4.1 ± 1.6</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD of three independent measurements. An asterisk indicates statistical difference (Student’s $t$-test, $P < 0.05$) between wild-type and transformed cell lines.

**Fig. 3** Detection of cellular AOS levels. Cellular AOS was measured using H$_2$DCF-DA as described in Materials and Methods. Relative cellular AOS abundance in wild-type, cAPX-S2 and cAPX-S3 cell lines. Measurements were made using cell lines that had reached the stationary phase. The values were normalized with cell weights. Data are expressed as means ± SD of three independent measurements.

**Fig. 4** Viability of wild-type, cAPX-S2 and cAPX-S3 cell lines during the treatment of heat and salt stresses. Experiments were made using cell lines that had reached the stationary phase. The stress treatments were performed as described in Materials and Methods. The Evan’s blue assay was then performed to examine the cell viability. Data are expressed as means ± SD of three independent experiments.
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Decreased activity of cytosolic APX results in an increased tolerance against heat and salt stresses

Next, we assessed the effect of the suppression of cytosolic APX activity in BY-2 cells on their ability to tolerate environmental stress. As shown in Fig. 4, the viabilities of cAPX-S2 and cAPX-S3 cell lines remained high during a 8 h incubation at 37°C, whereas the wild-type cells exhibited a progressive decrease in cell viability. We also checked the effect of salt stress on the cell viabilities. After a 3 h treatment with 400 mM NaCl, the viability of the wild-type cells had decreased to 23% compared with the cAPX-S2 and cAPX-S3 cell lines which had decreased to only 53 and 65%, respectively (Fig. 4). These results clearly indicated that the suppression of cytosolic APX endows the cells with a considerable tolerance to heat and salt stresses.

Isolation of differentially expressed genes in response to imbalanced cellular redox conditions using SSH

The suppression subtractive hybridization (SSH) technique was utilized to identify redox-inducible genes in cAPX-S3. With sequence and public database search analysis, 17 independent cDNA clones were identified. To confirm that the cloned genes were indeed selected due to changes in their expression, we performed Northern blot analysis. Using each isolated clone as a probe, an increase of the mRNA accumulation in cAPX-S3 cells was confirmed with seven out of 17 clones (Fig. 5). The seven confirmed clones were sequenced. Two of them encode heat shock protein 70 (Hsp70) and DnaJ-like protein, which are involved in the heat stress response (Cyr et al. 1994) (Table 2). One clone displayed a close match to a purple acid phosphatase from Nicotiana tabacum, which is known to be a salt stress-inducible gene (Liao et al. 2003). The other three clones showed a marked homology with known proteins, such as PRT1 from Arabidopsis, alcohol NADP+ oxidoreductase (drd-1) from Solanum tuberosum, and phenylpropanoid:glucosyltransferase from N. tabacum. Another clone showed a high similarity with an unidentified gene from Oryza sativa that was annotated as a drought stress-inducible gene in the expressed sequence tag (EST) database.

To assess the redox responsiveness of these genes, Northern blot analysis was carried out on wild-type cells subjected to exogenous H2O2 (Fig. 5). After treatment with 10 mM H2O2, prominent induction of HSP70, DNA-J, PRT1 and drd-1 was observed in a time-dependent manner, supporting the notion that these genes are up-regulated under imbalanced cellular redox conditions.

Expression analysis of the HSP genes and redox-responsive protein kinases

Using reverse transcription (RT)–PCR analysis, we next analyzed the mRNA expression levels of other types of HSP
genes, nicotiana protein kinase 1 (NPK1) and nucleoside diphosphate kinase (NDPK), in the cells grown under standard conditions (25°C). The mRNA levels of NPK1 and NDPK are responsive to redox change (Kovtun et al. 2000, Moon et al. 2003). As a loading control, we also monitored the expression level of the actin gene. As shown in Fig. 6, most of the HSP genes tested were highly expressed in cAPX-S3 cells. Analysis of NDPK and NPK1 genes also revealed a notable variation in mRNA content between cAPX-S3 and wild-type cells, suggesting the activation of the redox signaling pathway in cAPX-S3 cells. We also determined the MAP kinase activity by an in-gel kinase assay using myelin basic protein (MBP) as a phosphorylating substrate. Fig. 7 shows that the activation of the predominant 46 kDa kinase occurs in cAPX-S3 cells, but not in wild-type cells. Although the treatment with exogenous H$_2$O$_2$ activated major MAP kinases at 48 and 46 kDa in both wild-type and cAPX-S3 cells within 5 min, the activities found in cAPX-S3 cells were higher than those of wild-type cells. These results suggest that in cAPX-S3 cells, the signaling pathway mediated by the redox-responsive protein kinase is indeed activated in response to an imbalance in cellular redox conditions.

**Discussion**

In this study, we have investigated the effect of lowered cytosolic APX activity on cellular redox homeostasis and its consequences on the stress responses of tobacco BY-2 cells. Plants have evolved multiple enzymes to control AOS that are located in the cytosol and various cell organelles (Shigeoka et al. 2002, Mittler 2002). In several studies on the manipulation of selected antioxidant enzymes, a common phenomenon of compensatory changes in the levels of other antioxidant enzymes was observed (Mittler 2002). In tobacco plants, the antisense suppression of APX1 resulted in the up-regulation of catalase, superoxide dismutase and GSH reductase (Rizhsky et al. 2002). Arabidopsis APX1 knock-out mutant plants exhibited decreased expression of chloroplastic Cu/Zn-superoxide dismutase, perhaps in an attempt to reduce the formation of H$_2$O$_2$ in chloroplasts (Pnueli et al. 2003). In contrast, even a 75% decrease in the cytosolic APX activity in cAPX-S3 cells did not alter the levels of the various antioxidant enzymes, or the levels of the reduced forms of both AsA and GSH (Table 1). We thought the residual activities of APX in cAPX-2 and cAPX-3 could be sufficient to maintain the cellular antioxidant networks, because the APX activity in tobacco BY-2 cells is an order of magnitude higher than that in the leaf tissues of tobacco or Arabidopsis plants (Madhusudhan et al. 2003, Vacca et al. 2004). However, even the 50% reduction in the cytosolic APX activity in cAPX-S2 cells resulted in an increase in the steady-state levels of AOS under the mild growth conditions (Fig. 3). No compensatory changes in the other antioxidant enzymes examined suggests that the higher steady-state levels of AOS could be attributed solely to the decrease in the cytosolic APX activity. These data demonstrate that the level of cytosolic APX plays a crucial role in maintaining the homeostasis of the cellular redox state.

The absence of compensatory changes in the other antioxidant enzymes in cAPX-S2 and cAPX-S3 cells suggested that the increases in AOS levels are still within tolerable limits. However, it seems likely that these marginal increases in the cellular AOS levels were sufficient to mimic a pseudo-stress situation and impart acclimation to heat and salinity (Fig. 4). It has been reported that the acquired acclimation of plants to diverse environmental stresses was induced by AOS, Funatsuki et al. (2003) found that the tolerance to chilling in certain cultivars of soybean is associated with the lack of a cytosolic APX gene. Transgenic potato and rice plants expressing glucose oxidase had increased H$_2$O$_2$ levels and enhanced resistance against attack by pathogens (Wu et al. 1995, Kachroo et al. 2003). These findings suggest that elevated levels of endogenous AOS are an early signal in activating the plant stress tolerance and defense mechanisms.

AOS play an important role as signal mediators for the activation of multiple downstream events, including gene expression, that are important for the acquisition of stress tolerance (Bowler and Fluhr 2000). By SSH analysis, we succeeded in identifying some of the up-regulated genes in cAPX-S2 cells (Table 2). The occurrence of transcripts of HSP70, DnaJ-like protein and a purple acid phosphatase among the up-

![Table 2](https://academic.oup.com/pcp/article-abstract/46/8/1264/1875161)

**Table 2** Identification of genes up-regulated in cAPX-S3 cell lines

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Clone no.</th>
<th>Gene annotation</th>
<th>Expression ratio (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB210290</td>
<td>1AI</td>
<td>PRT1 from Arabidopsis thaliana (AJ224306)</td>
<td>4.0</td>
</tr>
<tr>
<td>AB210291</td>
<td>1DI</td>
<td>Purple acid phosphatase from Nicotiana tabacum (AB017967)</td>
<td>3.0</td>
</tr>
<tr>
<td>AB210292</td>
<td>5FI</td>
<td>DNAJ protein-like from Arabidopsis thaliana (AU237345)</td>
<td>8.2</td>
</tr>
<tr>
<td>AB210293</td>
<td>11CI</td>
<td>Phenylpropanoid:glucosyltransferase from Nicotiana tabacum (AF346432)</td>
<td>3.6</td>
</tr>
<tr>
<td>AB210294</td>
<td>5DI</td>
<td>HSP70 from Petunia hybrida (X06932)</td>
<td>5.0</td>
</tr>
<tr>
<td>AB210295</td>
<td>8CI</td>
<td>Drought stress-inducible gene from Oryza sativa (BU673724)</td>
<td>2.7</td>
</tr>
<tr>
<td>AB210296</td>
<td>10EI</td>
<td>Alcohol NADP$^+$ oxidoreductase from Solanum tuberosum (AJ439993)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Expression ratio refers to the induction in cAPX-S3 cell lines relative to the wild-type cells from the results of Northern blot analysis shown in Fig. 5A.
regulated genes suggests that the higher levels of AOS in the cytosolic APX-suppressed cells are perceived by the cells as an indication of heat and salt stress. Recent transcriptome analysis focusing on the oxidative stress response also supported the fact that diverse HSP genes are up-regulated during stress (Desikan et al. 2001, Pnueli et al. 2003, Vandenabeele et al. 2003, Vandenabeele et al. 2004). In an Arabidopsis mutant with a disrupted cAPX1 gene, the transient expression level of the HSP70 gene during high-light stress was much higher than that of the wild-type plant. This suggests a close relationship between the heat shock response and \( \text{H}_2\text{O}_2 \) accumulation via the cytosolic APX during stress conditions (Pnueli et al. 2003). Successful isolation of stress-related genes by SSH justifies our decision to use cells in the stationary phase in this study.

An important aspect of the redox-responsive signaling pathway has been recognized in the plant response against diverse environmental stresses (Mittler 2002, Apel and Hirt 2004). A pivotal role for redox-responsive kinases, such as NPK1, MAP kinase kinase kinase, and NDPK2, has recently begun to emerge (Kovtun et al. 2000, Moon et al. 2003). An in gel kinase assay showed that a MAP kinase of approximately 46 kDa was predominantly active in the cAPX-S3 cells. A MAP kinase with a similar molecular mass was also activated under hypoxosmotic stress in tobacco cells (Cazale et al. 1999). The molecular identity of this 46 kDa MAP kinase in tobacco cells is not yet known, but, based on its molecular size, it may be the ortholog of Arabidopsis AtMAPK6, which is responsive to a variety of abiotic stresses (Ichimura et al. 2000). Furthermore, gene expression analysis revealed that NPK1 and NDPK2, which are upstream regulators of MAP kinases (Kovtun et al. 2000, Moon et al. 2003), are also significantly up-regulated in the cAPX-S3 cells (Fig. 6). Moon et al. (2003) have reported that the transcript level of NDPK2 in Arabidopsis increases rapidly after treatment with exogenous \( \text{H}_2\text{O}_2 \). It seems likely that the stress tolerance of cytosolic APX activity-suppressed cells is due to the activation of MAP kinase cascades. It is worth noting that transgenic Arabidopsis plants with overexpressing constitutively active mutants of NPK1 and NDPK2 displayed enhanced tolerance to diverse environmental stresses, such as heat, salt, freezing and methyl viologen treatment (Kovtun et al. 2000, Moon et al. 2003).

In conclusion, the present study demonstrates that the cytosolic APX plays an important role in maintaining the cellular redox homeostasis in tobacco cells and offers a possibility for the potential enhancement of stress tolerance through the activation of stress-related genes via redox signaling.

Materials and Methods

Cell culture

Tobacco BY-2 cells (\textit{N. tabacum} L. cv. Bright Yellow 2) were grown in a modified Linsmaier and Skoog’s medium as described by Nagata et al. (1992).

Binary vector construction and transformation of BY-2 cells

The cytosolic APX1 cDNA from Arabidopsis (accession number X59600) was obtained by RT–PCR and ligated, in a sense orientation, into plant binary vector pBI121 under the control of the CaMV 35S promoter. The orientation of the final construct was confirmed by DNA sequencing using a pBI121 sequencing primer. \textit{Escherichia coli DH5}\( \alpha \) was transformed with the construct and co-cultured with \textit{E. coli} HB101 harboring pRK2013 and Agrobacterium tumefaciens LBA4404 in a minimal medium containing kanamycin (50 \( \mu \text{g ml}^{-1} \)). Agrobacterium tumefaciens were used to infect the BY-2 cells using the co-cultivation method, and transformed BY-2 cells were selected on the modified Linsmaier and Skoog’s medium containing kanamycin (100 \( \mu \text{g ml}^{-1} \)) and carbenicillin (250 \( \mu \text{g ml}^{-1} \)).

Enzyme assays

Cells were ground in liquid nitrogen and homogenized at 4°C in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM AsA and 1 mM EDTA. The resulting cell lysate was centrifuged at 15,000g at 4°C for 10 min and the supernatant was used for the APX activity assay and for immunoblot analysis. APX activity was measured as described previously (Ishikawa et al. 1996). Briefly, the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.4 mM AsA and 0.1 mM H\(_2\text{O}_2\). The oxidation of AsA was followed by a decrease in absorbance at 290 nm (\( \varepsilon = 2.80 \text{mM}^{-1} \text{cm}^{-1} \)). MDA reductase activity was assayed using MDA generated by AsA and an AsA oxidase system (Shigeoka et al. 1987a). The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.0), 1 mM AsA, 1 U of AsA oxidase (Wako, Osaka, Japan), 0.1 mM NADH and the extract. Activity was determined by the AsA oxidase-induced oxidation of NADH monitored at 340 nm (\( \varepsilon = 6.2 \text{mM}^{-1} \text{cm}^{-1} \)). DHA reductase was assayed according to Ishikawa et al. (1998) by measuring the reduction of DHA to AsA in a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 10.5 mM AsA and 2.5 mM GSH. The formation of AsA was followed by an increase in absorbance at 265 nm (\( \varepsilon = 14 \text{mM}^{-1} \text{cm}^{-1} \)). Catalase activity was assayed according to Madhusudhan et al. (2003). The reaction mixture contained 50 mM potassium phosphate (pH 7.0) and 10.5 mM H\(_2\text{O}_2\). After addition of the extract, the reaction was monitored by following the decomposition of H\(_2\text{O}_2\) at 240 nm (\( \varepsilon = 0.041 \text{mM}^{-1} \text{cm}^{-1} \)). GSH reductase was measured according to Shigeoka et al. (1987b). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 2 mM GSGG and 0.2 mM NADPH. The oxidation of NADPH was monitored at 340 nm (\( \varepsilon = 6.2 \text{mM}^{-1} \text{cm}^{-1} \)). The protein concentration was measured with the Coomassie Protein Assay Reagent (Bio-Rad).

Immunoblot analysis

Proteins were separated on a 12.5% polyacrylamide gel using SDS–PAGE and blotted onto a PVDF membrane, Immunoblot (Millipore, Bedford, MA, USA), using the transfer buffer of Towbin et al. (1979) and a semi-dry electroblot apparatus (Taitec, Shizuoka, Japan). The blot was incubated with a monoclonal antibody EAP1 al. (1979) and a semi-dry electroblot apparatus (Taitec, Shizuoka, Japan). The blot was incubated with a monoclonal antibody EAP1 al. (1979) and a semi-dry electroblot apparatus (Taitec, Shizuoka, Japan).

AsA and GSH assays

AsA contents were determined colorimetrically by the 2,4-dinitrophenylhydrazine method as described previously (Shigeoka et al. 1987c). Cells (1.0 g FW\(^{-1} \)) were homogenized with the same volume of cold 3% (w/v) metaphosphoric acid using a mortar and pestle. The
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homogenate was centrifuged at 15,000g for 15 min at 4°C, and the supernatant assayed for AsA content.

GSH content was measured with an enzymatic recycling assay based on GSH reductase according to Brehe and Burch (1976). Cells (1.0 g FW–1) were homogenized in an equal volume of 100 mM potassium phosphate buffer, pH 7.0, and 1.5 vols of 10% trichloroacetic acid. The homogenate was centrifuged at 15,000×g for 10 min at 4°C, and the supernatant used for the GSH assay. The assay mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.2 mg ml–1 NADPH, 75 µg ml–1 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and cell extract. After a 5 min incubation, the reaction was started by the addition of 4 U of GSH reductase (Wako), and the reduction rate of DTNB was monitored at 412 nm for 3 min. GSSG was selectively measured by assaying the sample in which GSH was masked by treatment with N-ethylmaleimide.

Detection of AOS

The intercellular production of AOS was measured using 2,7′-dichlorofluorescin diacetate (H2DCF-DA; Molecular Probes, CA, USA). H2DCF-DA was added to the cells at a final concentration of 15 µM. After a 15 min incubation, the cells were collected by microcentrifugation and the supernatant removed. Fluorescence was measured with a spectrophotometer (Model RF-1500 Shimadzu, Kyoto, Japan). Hybridization was carried out in Church-phosphate buffer using Gene-specific RT–PCR

Stress experiments

Stationary cultures (7 d old for the wild-type and 9 d old for cAPX-S2 and cAPX-S3) were exposed to increased heat and salinity. Heat stress was imposed by incubating cultures for 8 h at 37°C. Salt stress was imposed by the addition of NaCl at a final concentration of 400 mM to the culture medium and incubating the cells for 3 h at 25°C. Cell viability was measured as previously described using a Trypan blue dye exclusion test (de Pinto et al. 1999).

Suppression subtractive hybridization

SSH was performed using the Clontech PCR-Select cDNA Subtraction kit (Clontech Laboratories, Palo Alto, CA, USA) following the manufacturer’s recommendations. Starting material consisted of 2 µg of mRNAs from cAPX-suppressed (tester) and non-transformed BY-2 cells (driver), respectively. Products from the secondary PCR were inserted into pT7Blue T-vector (Novagen, Madison, WI, USA).

Northern analysis

A 20 µg aliquot of total RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde, blotted onto Hybond N membrane (Amersham Pharmacia, Buckinghamshire, UK) and hybridized with a 32P-labeled cDNA obtained from SSH analysis. The radiolabeled probe was prepared by priming with each gene-specific primer using EcoRI-BEST polymerase (TAKARA SHUZO CO., LTD, Kyoto, Japan). Hybridization was carried out in Church-phosphate buffer (Church and Gilbert 1984) at 60°C for 16 h. The hybridized membrane was washed with 0.1× SSC containing 0.1% SDS at 60°C for 1 h. Autoradiography was carried out with a BAS 1500 imager (Fuji Film, Tokyo, Japan).

Gene-specific RT–PCR

First-strand cDNA was synthesized in total volume of 20 µl using SuperScript III reverse transcriptase (Invitrogen) and 5 µg of total RNA. A 2 µl aliquot of the products obtained from this reaction was used in a 50 µl PCR with Ex Taq polymerase (TAKARA SHUZO CO., LTD, Kyoto, Japan) which contained 0.2 µM each of the gene-specific primers. Thermocycle conditions consisted of 25 cycles of 94°C, 30 s; 52°C, 30 s; 72°C, 90 s without a final extension. To monitor whether an equal amount of cDNA was synthesized, a cDNA fragment of the constitutively expressed actin gene (accession number AB158612) was amplified simultaneously in 25 or 30 cycles. The primer sequences and predicted amplicon sizes were as follows: forward 5′-GTTAATGGTGTTACAGCCTCTTACG-3′ and reverse 5′-ACGCTTACCTCCTCTCCTCATACTG-3′ for NtHSP 26 (665 bp, D88584); forward 5′-GATGGCTATGTCCTACAGTTCTCTTTG-3′ and reverse 5′-TAAACGAGATGCTCAATAGCTTCTTTCAACCC-3′ for NtHSP 82 (264 bp, X63195); forward 5′-CCGTGCGTGATGAAAGGTATG-3′ and reverse 5′-AGATTTGTTCACTGATCCTC-3′ for NtHSP 101 (874 bp, AF083343); forward 5′-ATGGGTTGTCTGTTGGTTAAAGGC-3′ and reverse 5′-TTAATCGTACACACAGGTCTTTGGAACG-3′ for NtNPK1 (2,092 bp, D26601); and forward 5′-ATGGCGGATGGGGAGGACATCATTACCC-3′ and reverse 5′-TATAGGACTTTTCGGTGGCATAAGG-3′ for actin (1,134 bp, X16432).

In-gel kinase assay

The in-gel kinase assay was performed as described previously (Zhang and Klessig 1999) with some modifications. Cells (1.0 g FW–1) were ground in liquid nitrogen and homogenized at 4°C in 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM dithiothreitol, 50 mM EDTA, 2.5 mM NaF. The resulting cell lysate was centrifuged at 15,000g at 4°C for 10 min and the supernatant was used for the assay. A 20 µg aliquot of the protein extracts was separated on a 12.5% SDS–polyacrylamide gel embedded with 0.25 mg ml–1 MBP (from bovine brain, Sigma) as a substrate for kinase activity. After electrophoresis at 250 V for 1 h, SDS was removed from the gel by washing with three washes with 100 ml of buffer A (50 mM Tris–HCl, pH 8.0, 20% 2-propanol) for 1 h each at room temperature and followed by three washes with washing buffer B (50 mM Tris–HCl, pH 8.0, 5 mM 2-mercaptoethanol) for 1 h each. The proteins were renatured by soaking the gel in 100 ml of renaturation buffer A (50 mM Tris–HCl, pH 8.0, 5 mM 2-mercaptoethanol, 6 µM guanidine hydrochloride) at room temperature for 1 h followed by an overnight incubation in 100 ml of renaturation buffer B (50 mM Tris–HCl, pH 8.0, 5 mM 2-mercaptoethanol, 0.05% Tween-20) at 4°C, with at least three changes of the buffer. The gel was then incubated three times for 1 h each in 100 ml of reaction buffer (40 mM Tris–HCl, pH 8.0, 0.1 mM EGTA, 10 mM MgCl2, 2 mM dithiothreitol, 50 mM NaCl, 20 mM KCl) at room temperature. Phosphorylation was performed for 1 h at room temperature in 100 ml of the same reaction buffer supplemented with 50 µM ATP and 50 µCi of [γ-32P]ATP (3,000 Ci mmol–1). The reaction was stopped and any incorporated radioactivity removed by washing the gel (with three changes of solution) for 2 h at room temperature in 5% trichloroacetic acid (w/v) and 1% sodium pyrophosphate (w/v). The gel was dried on 3MM paper and subjected to autoradiography.

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Acclimation to stresses with APX suppression

References


