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Post-Transcriptional Suppression of Cytosolic Ascorbate Peroxidase Expression during Pathogen-Induced Programmed Cell Death in Tobacco

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As a means to eliminate pathogen-infected cells and prevent diseases, programmed cell death (PCD) appears to be a defense strategy employed by most multicellular organisms. Recent studies have indicated that reactive oxygen species, such as $O_2^{\cdot -}$ and H_2O_2, play a central role in the activation and propagation of pathogen-induced PCD in plants. However, plants contain several mechanisms that detoxify $O_2^{\cdot -}$ and H_2O_2 and may inhibit PCD. We found that during viral-induced PCD in tobacco, the expression of cytosolic ascorbate peroxidase (cAPX), a key H_2O_2 detoxifying enzyme, is post-transcriptionally suppressed. Thus, although the steady state level of transcripts encoding cAPX was induced during PCD, as expected under conditions of elevated H_2O_2, the level of the cAPX protein declined. In vivo protein labeling, followed by immunoprecipitation, indicated that the synthesis of the cAPX protein was inhibited. Although transcripts encoding cAPX were found to associate with polysomes during PCD, no cAPX protein was detected after in vitro polysome run-off assays. Our findings suggest that viral-induced PCD in tobacco is accompanied by the suppression of cAPX expression, possibly at the level of translation elongation. This suppression is likely to contribute to a reduction in the capability of cells to scavenge H_2O_2, which in turn enables the accumulation of H_2O_2 and the acceleration of PCD.

INTRODUCTION

The antimicrobial response of plants and animals is often accompanied by a coordinated activation of programmed cell death (PCD) and defense mechanisms (Zychlinsky et al., 1992; Hacker and Vaux, 1994; Greenberg, 1996; Mittler and Lam, 1996). In plants, this response is termed the hypersensitive response (HR) and results in the formation of a zone of dead cells around the infection site, the synthesis of salicylic acid (SA), and the accumulation of antimicrobial agents, such as pathogenesis-related (PR) proteins and phytoalexins (Goodman and Novacky, 1994; Dangl et al., 1996; Hammond-Kosack and Jones, 1996). The layers of dead cells that surround the site of pathogen entry are thought to function as a physical barrier that inhibits further proliferation and spread of the pathogen (Goodman and Novacky, 1994).

PCD that occurs during the HR is accompanied by an increase in the production of reactive oxygen species (ROS) and lipid peroxidation (Doke and Ohashi, 1988; Mehdy, 1994; Hammond-Kosack and Jones, 1996; May et al., 1996; Yang et al., 1997). Recent studies have indicated that reactive oxygen intermediates in the form of H_2O_2 and $O_2^{\cdot -}$ are key mediators of PCD during the HR (Levine et al., 1994, 1996; Jabs et al., 1996; Mittler et al., 1996; Draper, 1997; Shirasu et al., 1997) and may function as part of a signal transduction pathway leading to the induction of PR proteins and systemic resistance in infected and noninfected parts of the plant (Chen et al., 1993; Green and Fluhr, 1995). Although the role of ROS in mediating systemic resistance was recently challenged (Bi et al., 1995; Leon et al., 1995; Neuenschwander et al., 1995), considerable evidence is accumulating in support of a role for H_2O_2 and $O_2^{\cdot -}$ in mediating PCD during the HR.

The production of $O_2^{\cdot -}$ was recently shown to activate PCD in Arabidopsis (Jabs et al., 1996), and H_2O_2 was shown to induce PCD and defense mechanisms in bean and tobacco (Levine et al., 1994, 1996; Draper, 1997; Shirasu et al., 1997). It is thought that a plasma membrane–associated NAD(P)H oxidase is activated during the response of plants to pathogens (Jabs et al., 1996; Draper, 1997). This results in the production of $O_2^{\cdot -}$, which dismutates spontaneously or via superoxide dismutase (EC 1.15.1.1) into H_2O_2. H_2O_2, which is formed extracellularly (Bestwick et al., 1997), can then diffuse into cells, leading to the activation of defense mechanisms and PCD (Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Levine et al., 1996). Additional evidence for the possible involvement of H_2O_2 and $O_2^{\cdot -}$ in mediating

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PCD is the finding that hypersensitive death, but not the activation of some defense mechanisms, is inhibited at low oxygen pressure (Mittler et al., 1996). This treatment is likely to suppress the formation of ROS (Jacobson and Raff, 1995; Shimizu et al., 1995).

Ascorbate peroxidase (APX; EC 1.11.1.11) is a key H_2O_2 scavenging enzyme found in the cytosol, chloroplast, and mitochondria of higher plants (Asada, 1992; Mittler and Zilinskas, 1992; Patterson and Poulos, 1995; Jimenez et al., 1997). Using heme as a prosthetic group, APX catalyzes the following reaction:

$$2 \text{ ascorbate} + H_2O_2 \rightarrow 2 \text{ monodehydroascorbate} + 2\ H_2O.$$

In accordance with its role in scavenging H_2O_2, APX expression is induced in response to many environmental stresses that result in the accumulation of ROS (Mittler and Zilinskas, 1992; Thomsen et al., 1992; Kubo et al., 1995; Karpinski et al., 1997). It has been suggested recently that the inhibition of APX and catalase activities by SA during the response of plants to invading pathogens results in the accumulation of H_2O_2 and the activation and acceleration of PCD (Chen et al., 1993; Durner and Klessig, 1995; Draper, 1997; Rao et al., 1997; Shirasu et al., 1997). Moreover, transgenic tobacco plants, in which the level of catalase was artificially reduced by the endogenous production of catalase antisense transcripts, developed lesions and activated some defense mechanisms (Chamnongpol et al., 1996; Takahashi et al., 1997). This finding further substantiates the hypothesis that high intracellular levels of H_2O_2 cause the activation of PCD and defense mechanisms. Unfortunately, similar studies have not been reported for APX. Suppression of cytosolic APX (cAPX) activity may have an even more dramatic effect on the induction of PCD compared with catalase, because cAPX has a higher affinity for H_2O_2 than does catalase (Asada, 1992) and because H_2O_2, which is formed at the intercellular spaces of the plant during the HR, is likely to diffuse first into the cytosol, where cAPX is localized, and only then into peroxisomes where catalase is typically found.

Because the HR is accompanied by the production of ROS, it is likely that this response is accompanied by the induction of APX expression, which is similar to its induction during environmental stress. Indeed, it was found that during the HR of tobacco plants to tobacco mosaic virus (TMV), the steady state level of transcripts encoding the cytosolic isozymes of superoxide dismutase and APX could be induced (Mittler et al., 1996). However, the presence of a high level of the cAPX protein in the cytosol of plant cells undergoing PCD may have caused a reduction in the level of H_2O_2 and thus a reduction in the rate of cell death. As indicated above, it has been suggested that SA, which accumulates during the HR, inhibits the activity of cAPX. However, it was shown recently that SA is not an effective inhibitor of cAPX (Kvaratskhelia et al., 1997; Tenhaken and Rubel, 1997). Therefore, for H_2O_2 to accumulate during the HR, cAPX activity should be suppressed via a different mechanism that is not dependent on SA. Here, we report that although the level of cAPX mRNA is induced during PCD, the translation of the mRNA encoding cAPX is inhibited. Therefore, the cAPX protein does not accumulate during the HR. This mode of post-transcriptional suppression is likely to cause a reduction in the capability of cells to scavenge H_2O_2, thereby enabling the rapid induction and progression of PCD.

RESULTS

Suppression of cAPX Expression during TMV-Induced PCD in Tobacco

The recognition of TMV by tobacco plants containing the *N* gene (Xanthi NN) results in the activation of PCD. However, this response is inhibited at 30°C, thereby allowing the systemic infection of the plant. When systemically infected plants are shifted from 30 to 22°C, the inhibition of PCD is removed, and cell death occurs in all infected parts of the plants (Whitham et al., 1994; Mittler et al., 1997). As shown in Figure 1, the induction of PCD in TMV-infected plants upon a temperature shift from 30 to 22°C (Figure 1A) was accompanied by an increase in the steady state level of transcripts encoding cAPX (Figure 1B). In contrast, the level of the cAPX protein declined (45 $\pm$ 12% of control, $n = 3$; Figure 1B). This finding suggests that TMV-induced PCD is accompanied by a reduction in the capability of cells to scavenge H_2O_2 and that the level of the cAPX protein is determined post-transcriptionally.

Changes in the activity of cAPX, as measured with activity gels (Mittler and Zilinskas, 1993), corresponded to the changes in the activity of the cAPX protein (data not shown). In contrast to the suppression of cAPX expression, cells undergoing PCD were capable of accumulating transcripts encoding the PR-1 protein, suggesting that the decline in the cAPX protein did not result from the inability of cells to synthesize proteins or from their death. Fodor et al. (1997) recently reported that the activity of APX is reduced 24 hr after TMV-induced activation of the HR in tobacco plants. Although their analysis was performed with plants grown at 18 to 23°C (cell death is induced only in isolated lesions), in contrast to a temperature shift design (Figure 1A; cell death occurs in the majority of leaf cells), their findings are in agreement with our results and support the observation that during relatively early stages of the HR, the activity of APX is reduced.

In an attempt to compare the regulation of cAPX expression to that of other cellular proteins that decline during PCD, we examined the expression of the chloroplastic enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco), which is composed of two subunits (Rubisco small subunit [RbcS] and Rubisco large subunit [RbcL]). We previously observed

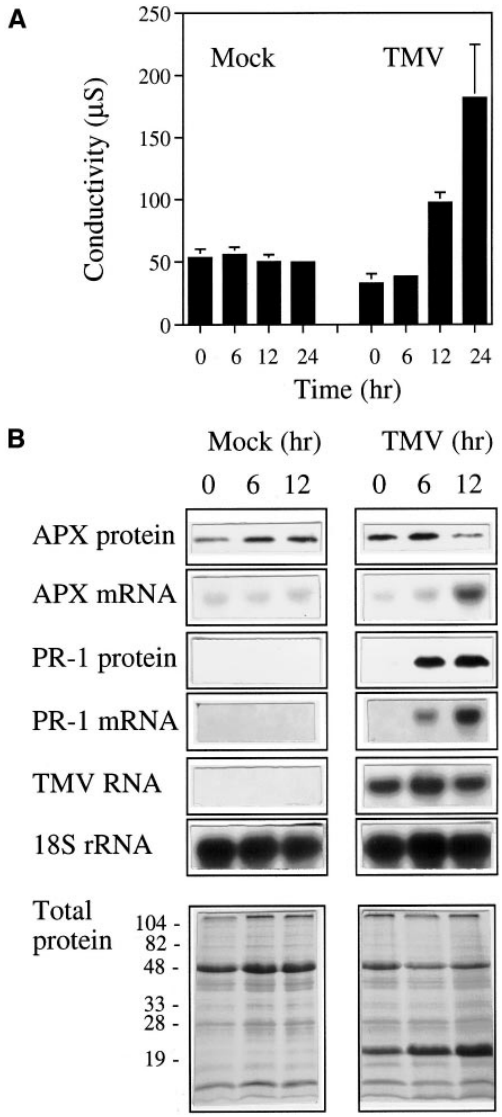

Figure 1. Suppression of cAPX Expression during TMV-Induced PCD.

(A) Ion leakage from leaf discs obtained at different time points after activation of cell death, indicating that cell death was detected 12 hr after the temperature shift. The data presented are the mean and standard deviation of three individual measurements.

(B) RNA and protein blots showing the changes in the expression of cAPX, that is, transcript induction and protein decline, and the expression of the pathogenesis-related protein PR-1, that is, transcript and protein induction, during pathogen-induced PCD. A probe for rRNA (18S) was used as a control for equal loading of RNA, and protein gels stained with Coomassie blue were used to demonstrate equal loading of protein.

Mock and systemically infected tobacco plants were subjected to a temperature shift and sampled at different time points after activation of PCD. Leaf samples were assayed for cell death by measuring ion leakage and for the expression of cAPX and PR-1 by RNA and protein blot analyses. Molecular mass markers in **(B)** are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase.

a decline in the steady state level of the RbcL and RbcS proteins at ~24 hr after cell death activation (L. Rizhsky and R. Mittler, unpublished results). We found that in contrast to the increase in the steady state level of transcripts encoding cAPX (Figure 1), the steady state level of transcripts encoding RbcS and RbcL rapidly declined during early stages of PCD (Figure 2). This finding suggests that the decline in the level of the RbcS and RbcL proteins is mediated by changes in the steady state level of their corresponding transcripts rather than by the suppression of their synthesis or an increase in their protein turnover, as may be the case with the cAPX protein. Our findings with the RbcL and RbcS proteins agree with a recent report demonstrating that in contrast to the cytosol and nuclei, the chloroplast and its genome undergo rapid degeneration during early stages of TMV-induced PCD in tobacco (Mittler et al., 1997).

The post-transcriptional suppression of cAPX gene expression during TMV-induced PCD could result from a competition between cellular mRNAs and viral RNAs for the translational apparatus of the cell. To examine this possibility, we tested the changes in cAPX gene expression in tobacco plants that do not contain the *N* gene (Xanthi nn) and in transgenic tobacco plants that express the bacterio-opsin (*bO*) gene (Mittler et al., 1995). Tobacco plants that do not contain the *N* gene do not mount an HR (Whitham et al., 1994; Figure 3A) and may therefore serve as a control for the competition of viral RNAs with cellular mRNAs in the absence of PCD. As shown in Figure 3B, the level of the cAPX protein did not decline in TMV-infected tobacco plants not containing the *N* gene after a temperature shift from 30 to 22°C. In contrast to tobacco plants that do not contain the *N*

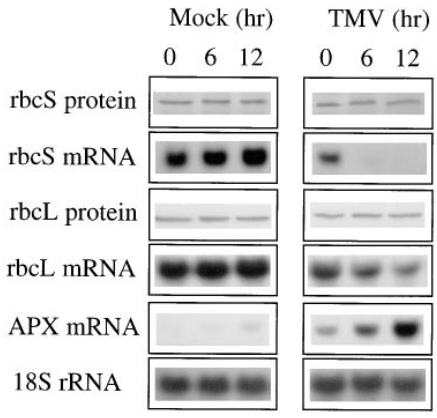

Figure 2. Suppression of rbcS and rbcL Expression during PCD.

Mock and systemically infected tobacco plants subjected to a temperature shift were sampled at different time points after the activation of PCD, as described in Figure 1. RNA and protein gel blot analyses showed the reduction in the steady state level of transcripts encoding the large and small subunits of Rubisco during TMV-induced PCD in tobacco. APX, cytosolic ascorbate peroxidase.

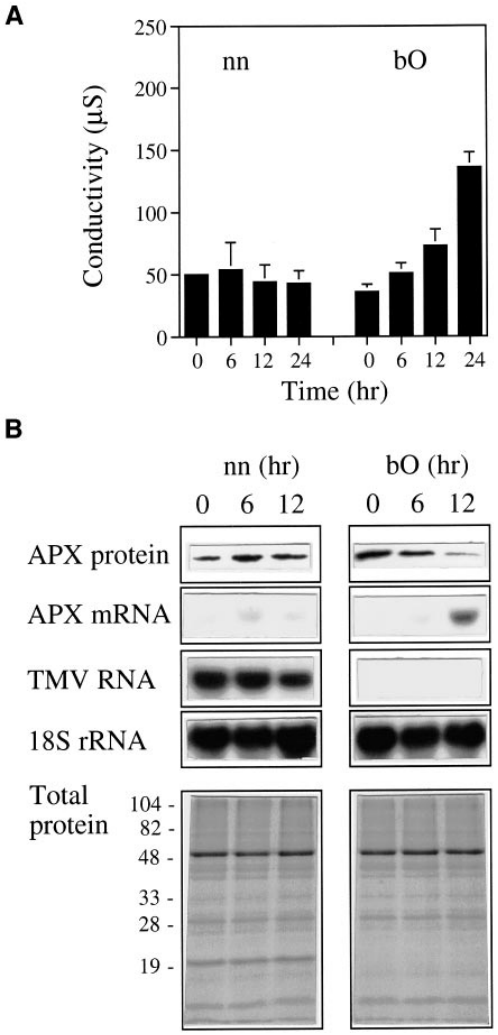

Figure 3. Suppression of cAPX Expression during Transgenically Induced PCD.

(A) Ion leakage from leaf discs obtained at different time points after activation of cell death, indicating that PCD was detected 12 hr after the temperature shift. The data presented are the mean and standard deviation of three individual measurements.

(B) RNA and protein gel blots showing the suppression of cAPX expression during transgenically induced PCD. A probe for rRNA (18S) was used as a control for equal loading of RNA, and protein gels stained with Coomassie blue were used to demonstrate equal loading of protein.

Transgenic plants expressing the *bO* gene and systemically infected tobacco plants that do not contain the *N* gene were subjected to a temperature shift and sampled at different time points after activation of PCD. Sizes of molecular mass markers in **(B)** are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase; nn, Xanthi-nc nn.

gene, tobacco plants that express the *bO* gene spontaneously activated the HR after a temperature shift from 30 to 22°C in the absence of the TMV pathogen (Mittler et al., 1995; Figure 3A). As shown in Figure 3B, PCD that occurred in transgenic tobacco plants that express the *bO* gene was accompanied by a suppression of cAPX expression (a reduction of 60%). This suppression was similar to that which occurred in TMV-infected tobacco plants that mounted an HR (Figure 1B). These findings suggest that the suppression of cAPX expression occurs as an integral part of the cell death program and that it is not effected by the presence of the TMV pathogen.

Suppression of cAPX Synthesis

To examine whether the decline in cAPX protein resulted from the inhibition of its synthesis, we labeled newly synthesized proteins with ^{35}S-methionine and subjected proteins to immunoprecipitation with the anti-cAPX serum. As shown in Figure 4, total ^{35}S-methionine uptake and incorporation were reduced in cells undergoing PCD (12 hr after a temperature shift; compare Figures 4A and 4B, at left, in which gels were loaded based on equal amount of fresh tissue weight). When protein extracts with equal amounts of ^{35}S-methionine–incorporated material were assayed for the content of ^{35}S-methionine–labeled cAPX protein via immunoprecipitation, no newly synthesized cAPX protein was detected in cells undergoing TMV-induced PCD (Figure 4B, middle). In contrast, newly synthesized PR-1 protein was precipitated (Figure 4B, right). This finding suggests that the synthesis of the cAPX protein is inhibited during PCD. Alternatively, newly synthesized cAPX protein may undergo very rapid degradation during the HR.

Association of cAPX Transcripts with Polysomes

Inhibition of protein translation is known to occur during many stress conditions in plants (Crosby and Vayda, 1991; Cohen and Mayfield, 1996; Gallie, 1996). During recovery from drought stress in pea, the expression of cAPX was found to be controlled by the association of cAPX transcripts with polysomes (Mittler and Zilinskas, 1994), suggesting that the expression of cAPX was regulated post-transcriptionally at the level of translation initiation. To examine whether the suppression of cAPX expression is regulated via a similar mechanism during pathogen-induced PCD, we examined the association of cAPX transcripts and protein with polysomes 12 hr after a temperature shift. As shown in Figure 5B, cAPX transcripts were found to associate with polysomes during PCD, suggesting that the suppression of cAPX expression is not mediated by the inhibition of its binding to polysomes, that is, a possible inhibition of translation initiation.

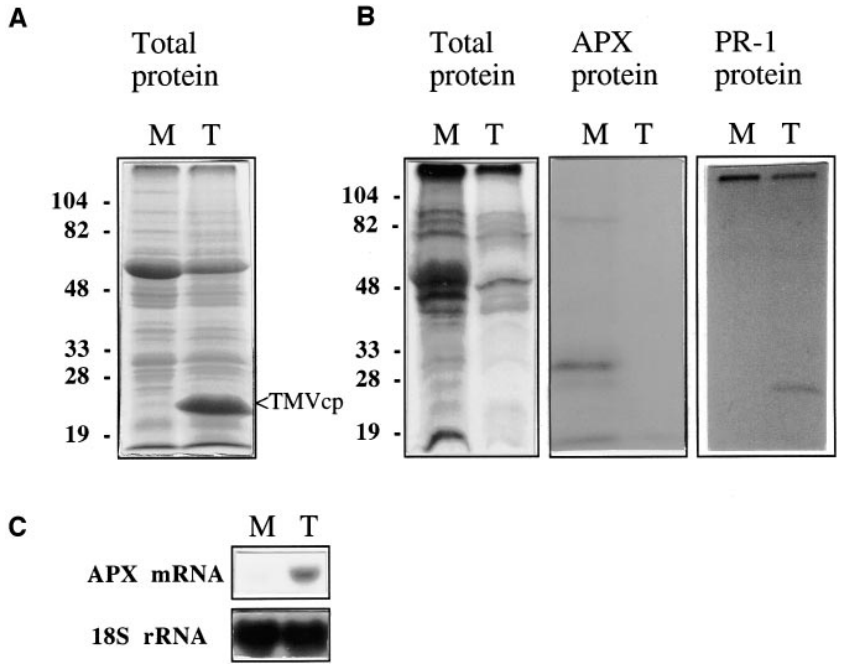

Figure 4. In Vivo Labeling of cAPX during Virus-Induced PCD.

(A) A gel of total protein from mock- and TMV-infected tobacco plants, 12 hr after a temperature shift, stained with Coomassie blue.
(B) A gel of total ^{35}S-methionine–labeled protein (left), a gel of immunoprecipitated in vivo–labeled cAPX protein (middle), and a gel of immunoprecipitated in vivo–labeled PR-1 protein (right) showing the suppression of cAPX expression 12 hr after activation of PCD. ^{35}S-methionine–labeled protein was detected by SDS-PAGE followed by fluorography.
(C) An RNA gel blot showing the steady state level of transcripts encoding cAPX in the samples used for the analysis shown in **(A)** and **(B)**.
The gel shown in **(A)** and the gel shown at left in **(B)** were loaded based on an equal amount of fresh tissue weight. The gels shown at middle and right in **(B)** are the result of immunoprecipitating cAPX and PR-1 protein from protein extracts with an equal amount of ^{35}S-methionine–incorporated material. Molecular mass markers in **(A)** and **(B)** are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase; M, mock infected; T, TMV infected; TMVcp, TMV coat protein.

Interestingly, partly synthesized cAPX polypeptides were found to associate with polysomes isolated from both control cells and cells undergoing PCD (Figure 5A). The size of these putative steady state intermediates of cAPX (25,500 D) suggests that the synthesis of the cAPX protein is coupled to its interaction with heme, because all three amino acids involved in heme binding should be present in these intermediate polypeptides. It has been suggested recently that the binding of heme to globin occurs during its translation (Komar et al., 1997).

In Vitro Translation of cAPX

Because cAPX expression is suppressed post-transcriptionally after the binding of its transcripts to polysomes, we examined whether this suppression will occur in an in vitro translation system as well. As shown in Figure 6A, the translational products of total mRNA isolated from control cells and of total mRNA isolated from cells undergoing PCD (12 hr after a temperature shift) differed in the pattern of proteins synthesized in vitro. When in vitro–synthesized proteins were subjected to immunoprecipitation with the cAPX antiserum, we found that although mRNA from cells undergoing PCD contained transcripts encoding cAPX (Figure 6B), no cAPX protein was found among their translational products, suggesting that the suppression of cAPX expression also occurred in a heterologous translation system from rabbit reticulocytes.

Because cAPX transcripts were found to associate with polysomes, we examined whether polysomes loaded with cAPX transcripts (12 hr after activation of cell death; Figure 5) were capable of synthesizing cAPX protein in vitro (a polysome run-off assay; Vayda, 1995). As shown in Figure 6C, no cAPX protein was found in the in vitro translation assays that contained polysomes from cells undergoing PCD.

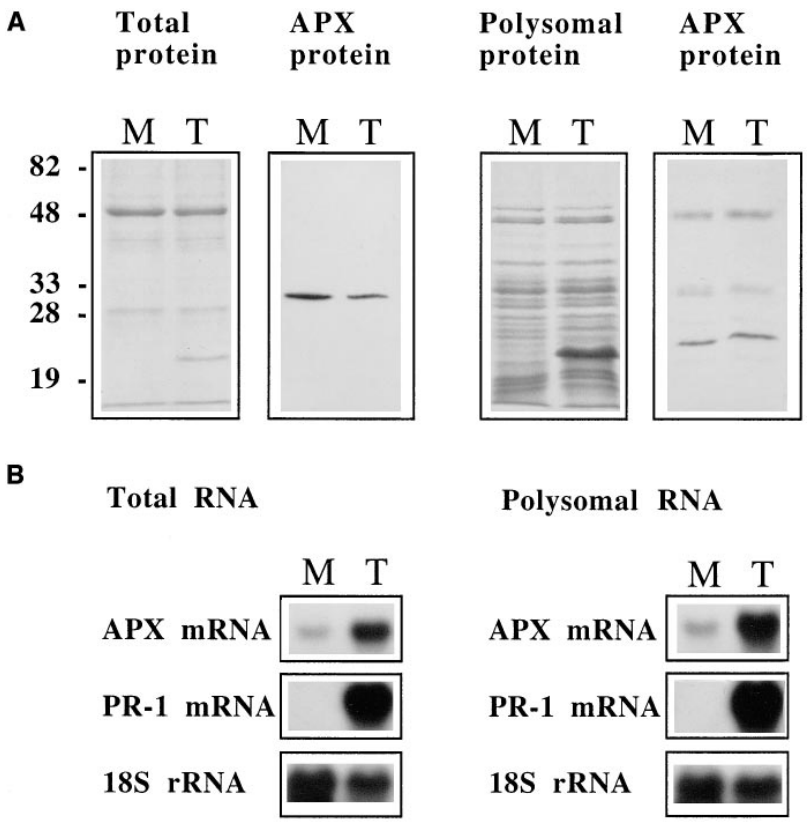

Figure 5. Association of cAPX Transcripts and Protein with Polysomes during TMV-Induced PCD.

(A) Protein gels stained with Coomassie blue (Total protein and Polysomal protein) and blots (APX protein) of total protein extracts (two left-hand gels) and of polysomal protein extracts (two right-hand gels) showing that a partially synthesized form of cAPX protein is bound to polysomes during normal metabolism and PCD.

(B) RNA gel blots of total RNA (left) and polysomal RNA (right) showing that transcripts encoding cAPX and PR-1 are bound to polysomes during PCD.

Polysomes were isolated from mock- and systemically infected plants 12 hr after a temperature shift and assayed for the content of cAPX transcript and protein by using RNA and protein gel blot analyses. Molecular mass markers in **(A)** are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase; M, mock infected; T, TMV infected.

Effect of Methyl Jasmonate and SA on cAPX Synthesis

The plant hormones SA and methyl jasmonate (MJ) accumulate during the HR (Hammond-Kosack and Jones, 1996; Wasternack and Parthier, 1997). MJ was previously shown to suppress the translation of several plant genes (Reinbothe et al., 1993b). In addition, MJ was found to affect the production of functional *rbcL* transcripts (Reinbothe et al., 1993a). In contrast, SA suppresses the effect of MJ on protein expression (Wasternack and Parthier, 1997). Because it was found that cAPX translation is inhibited during the HR (Figures 1 and 3), and because transcripts encoding cAPX isolated from cells undergoing PCD could not be translated in an in vitro translation system (Figure 6), it is possible that the expression of cAPX is affected by MJ. Therefore, we examined the effect of MJ on cAPX expression.

The steady state level of cAPX transcripts was previously shown to be induced by MJ (Orvar et al., 1997). However, as shown in Figure 7A, the expression of the cAPX protein appears to be suppressed by MJ, and the steady state level of the cAPX protein rapidly declined at 12 and 24 hr after MJ application and recovered, perhaps due to depletion of MJ, at 48 hr. Further, as shown in Figure 7B, the in vitro translation of mRNA encoding cAPX isolated from plant cells treated with MJ for 24 hr was also inhibited. These results may suggest that cAPX expression is controlled post-transcriptionally by MJ in a manner that may be similar to the control of cAPX expression during PCD. As shown in Figure 7C, SA was found to protect the expression of cAPX from the inhibitory effect of MJ. These findings may suggest that the steady state level of the cAPX protein is determined by an interplay between these two plant hormones during PCD.

However, additional studies are required to elucidate the effect of these hormones on the expression of cAPX during PCD.

It was previously shown that transgenic plants with reduced levels of cAPX are more sensitive to ozone, which is a known oxidant (Orvar and Ellis, 1997). Therefore, it is possible that a reduction in the level of cAPX causes cells to become more susceptible to the presence of ROS. We examined whether the reduction in the steady state level of the cAPX protein during MJ treatment resulted in greater sensitivity of plants to ROS. As shown in Figure 7D, MJ-treated tobacco plants were found to be more sensitive than were control plants to the application of the superoxide-generating agent paraquat. This finding supported the hypothesis that a reduction in the level of cAPX during the HR results in greater accumulation of ROS. In support of this hypothesis, treatment of parsley cells with MJ before the

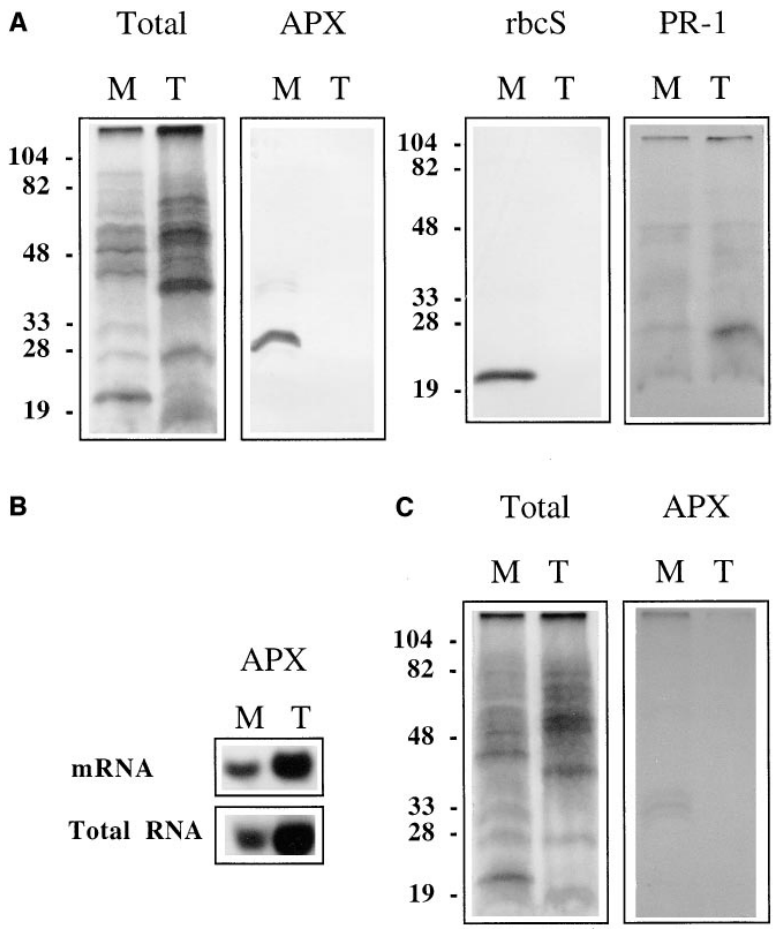

Figure 6. In Vitro Synthesis of the cAPX Protein.

(A) In vitro–translated products of mRNA (0.1 μg) from control cells and cells undergoing PCD (Total, left) were subjected to immunoprecipitation with antibodies raised against cAPX (APX, second gel from the left), rbcS (third gel from the left), and PR-1 (right-hand gel). Results show that the expression of cAPX is suppressed in an in vitro translation system. Total and immunoprecipitated ^{35}S-methionine–labeled proteins were assayed by SDS-PAGE followed by fluorography.

(B) RNA gel blot shows the level of transcripts encoding cAPX in total RNA and mRNA used for the analysis in **(A)**.

(C) In vitro–translated products of polysomes (12 μg) from control cells and from cells undergoing PCD (Total, left) and the level of in vitro–synthesized cAPX protein, as measured by immunoprecipitation (APX, right), demonstrate that the expression of cAPX by polysomes from cells undergoing cell death is suppressed in vitro.

Polysomes and mRNA were isolated from mock- and systemically infected plants 12 hr after a temperature shift and subjected to in vitro translation and immunoprecipitation. Molecular mass markers in **(A)** and **(C)** are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase; M, mock infected; T, TMV infected.

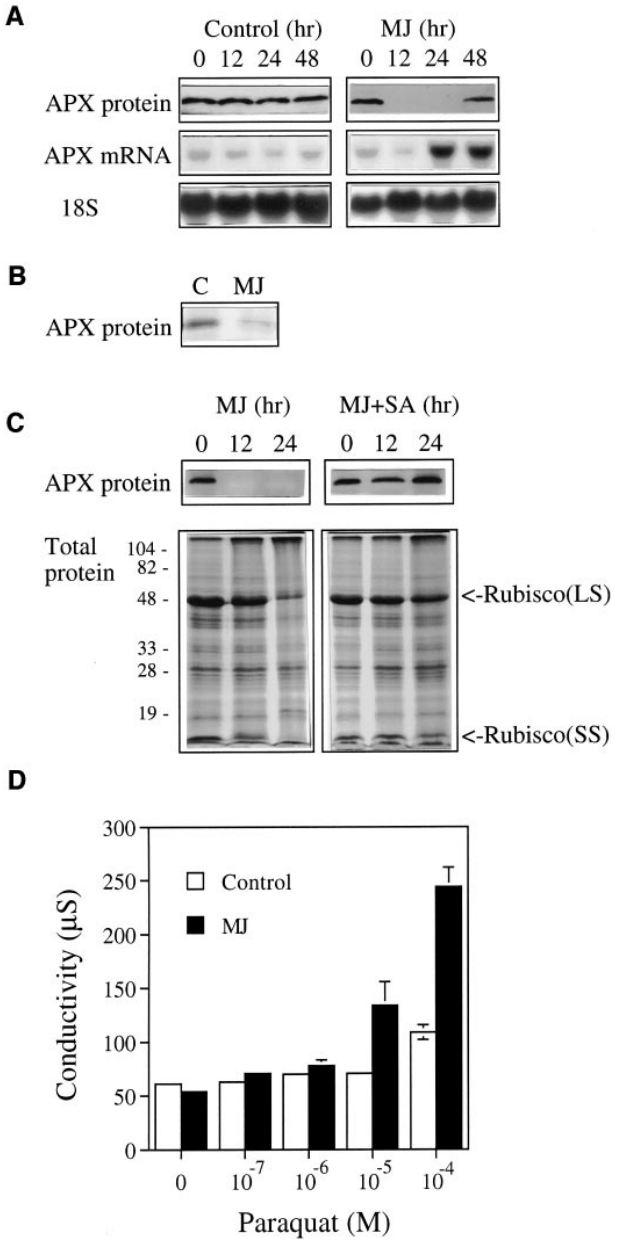

Figure 7. Effects of MJ and SA on cAPX Expression.

(A) Protein and RNA gel blots showing that MJ treatment causes the induction of cAPX steady state transcript level and the suppression of cAPX protein.

(B) Immunoprecipitated ^{35}S-methionine–labeled cAPX translated in vitro from total mRNA isolated from control and MJ-treated cells 24 hr after MJ application.

(C) Protein gel blots demonstrating that the application of SA protects cAPX protein expression from the inhibitory effect of MJ. Protein gels stained with Coomassie blue were used to demonstrate equal loading of protein.

(D) Ion leakage from leaf discs obtained from MJ-treated and control plants that were infiltrated with different concentrations of the superoxide-generating agent paraquat. The data presented are the mean and standard deviation of three individual measurements.

Tobacco plants were enclosed in glass chambers, treated with MJ, and sampled at different time points. To examine the effect of SA, SA-treated and untreated plants were enclosed together in the same glass chambers and treated with MJ. To examine the ROS-scavenging capability of MJ-treated plants, treated and control plants (24 hr after MJ application) were infiltrated with paraquat and sampled and analyzed for ion leakage 18 hr after paraquat application. In **(C)**, molecular mass markers are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase; C, control for MJ treatment; LS, large subunit; SS, small subunit; 18S, rRNA.

addition of elicitors was found to result in greater accumulation of H_2O_2 during the oxidative burst, perhaps due to the suppression of antioxidative mechanisms (Kauss et al., 1994).

Suppression of cAPX Expression during Bacteria-Induced PCD

We extended our study of cAPX expression during PCD to the interaction between tobacco plants and bacterial pathogens that induce the HR. As shown in Figure 8A, infiltration of tobacco leaves with a *Pseudomonas syringae* pv *phaseolicola* strain that induces the HR (NPS3121) triggered PCD. In contrast, infiltration of leaves with a *P. s. phaseolicola* strain that is *Hrp*$^-$ (NPS4000) did not induce the HR. As shown in Figure 8B, PCD induced by the *Hrp* cluster was accompanied by the suppression of cAPX expression. Thus, although the steady state level of cAPX mRNA was induced, the level of the cAPX protein declined. This mode of cAPX suppression appears to be similar to the suppression of cAPX expression during TMV-induced PCD (Figure 1).

DISCUSSION

Pathogen-induced PCD in tobacco resulted in an increase in the steady state level of transcripts encoding the cytosolic antiperoxidative enzyme APX, suggesting that this process is accompanied by the production of ROS (Figure 1; Mittler et al., 1996). However, in contrast to the increase in cAPX transcript, the level of the cAPX protein declined (Figure 1). A similar response occurred in cells undergoing transgenically induced PCD in the absence of a pathogen (Figure 3). In contrast, the presence of TMV had no effect on cAPX synthesis in the absence of PCD (Figure 3). Therefore, a mechanism acting to suppress the expression of cAPX may be an integral component of the cell death program of tobacco plants. Because pathogen-induced PCD is accompanied by an increase in the production of ROS (Doke and Ohashi, 1988; Mehdy, 1994; Baker and Orlandi, 1995; Hammond-Kosack

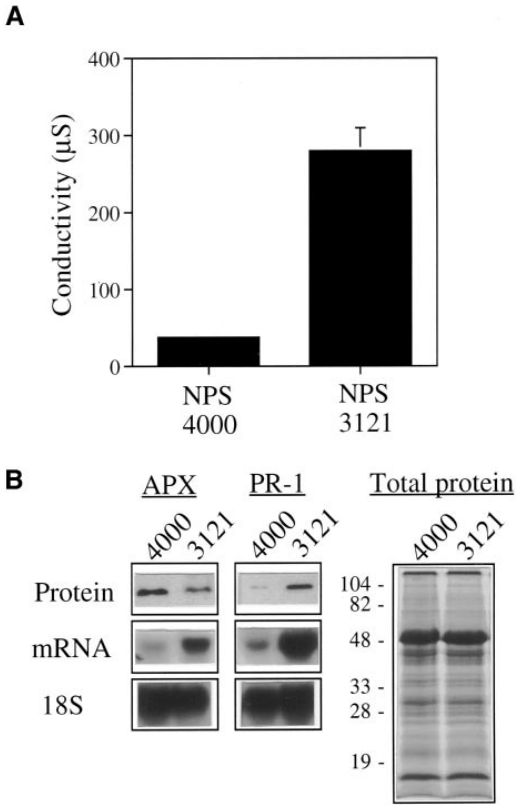

Figure 8. Suppression of cAPX Expression during Bacteria-Induced PCD.

(A) Ion leakage from leaf discs obtained 12 hr after infection with bacteria showing that the *P. s. phaseolicola* NPS3121 induced PCD. The data presented are the mean and standard deviation of three individual measurements.

(B) Protein and RNA gel blots showing that the increase in cAPX transcript is accompanied by a reduction in cAPX protein during bacteria-induced PCD. A probe for rRNA (18S) was used as a control for equal loading of RNA, and protein gels stained with Coomassie blue were used to demonstrate equal loading of protein.

Leaves were infiltrated with *P. s. phaseolicola* NPS3121 (3121; induced the HR) and NPS4000 (4000; no PCD induction) and sampled 12 hr after infection. Cell death was assayed by measuring ion leakage, and cAPX expression was examined by using RNA and protein gel blot analyses. Molecular mass markers in **(B)** are indicated at left in kilodaltons. APX, cytosolic ascorbate peroxidase; 18S, rRNA.

and Jones, 1996; Bestwick et al., 1997; Fodor et al., 1997), which is thought to mediate cell death (Levine et al., 1994, 1996; Chamnongpol et al., 1996; Jabs et al., 1996; Mittler et al., 1996; Draper, 1997; Shirasu et al., 1997; Takahashi et al., 1997), the suppression of cAPX expression may play an important role in preventing the scavenging of H_2O_2, which would have resulted in the inhibition of PCD.

It was suggested previously that SA, which accumulates during pathogen-induced PCD in tobacco, inhibits the activity of APX and catalase, thereby reducing the capability of cells to scavenge H_2O_2 (Chen et al., 1993; Durner and Klessig, 1995; Rao et al., 1997). However, recent reports have indicated that SA may not inhibit these activities during the HR (Kvaratskhelia et al., 1997; Tenhaken and Rubel, 1997). Our findings suggest that in addition to the possible inhibition of cAPX activity by SA, the expression of the cAPX protein is suppressed. Thus, no newly synthesized cAPX protein may replace the SA-inactivated cAPX molecules during pathogen-induced PCD. On the other hand, because SA may not inhibit the activity of APX (Kvaratskhelia et al., 1997), because it was found that H_2O_2 application stimulated the production of SA (Leon et al., 1995), and because SA application in the absence of pathogen infection was found to act as a rather weak signal for the induction of defense mechanisms (Shirasu et al., 1997), it is possible that the suppression of cAPX expression acts before or independent of the accumulation of SA. It is also possible that the reduced capability of cells to remove H_2O_2 together with the extracellular production of $O_2^{\cdot -}$ and H_2O_2 induce the synthesis of SA and enable the rapid and efficient activation of defense mechanisms and PCD.

The inhibition of cAPX expression was detected 12 hr after activation of cell death. Therefore, it is possible that this response plays a role during relatively late stages of PCD. Thus, suppression of cAPX expression may play a role in the H_2O_2-derived propagation of cell death that occurs during late stages of PCD. This suggestion is supported by the findings that late stages of pathogen-induced PCD are accompanied by the production of ROS (Goodman and Novacky, 1994; Mehdy, 1994; Baker and Orlandi, 1995; Hammond-Kosack and Jones, 1996) and that low oxygen pressure suppresses TMV-induced cell death when applied during late stages of the HR (Mittler et al., 1996).

Several lines of evidence suggest that the suppression of cAPX expression during PCD results from the inhibition of its translation: the level of the cAPX protein declined, although the steady state level of its transcript was induced (Figures 1 and 3); cAPX transcripts were found to associate with polysomes during PCD (Figure 5); however, no newly synthesized cAPX protein was found in cells undergoing PCD (Figure 4) and no cAPX protein was produced by polysomes isolated from cells undergoing cell death in an in vitro polysome run-off assay (Figure 6). These findings point to the inhibition of polypeptide elongation as being the rate-limiting step in cAPX synthesis during PCD. The response of plants to abiotic stresses, such as hypoxia, wounding, heat shock, or drought, involves a reduction in global protein translation, followed by the selective translation of specific stress-induced or preexisting mRNAs. This response involves the alteration of preexisting components of the translational machinery and often requires specific sequences present within the stress-translated mRNAs (Gallie, 1996).

During the wound response of plants, the translation of a specific set of cellular proteins is inhibited at the level of polypeptide elongation. In contrast, the expression of several

wound-associated proteins is elevated (Crosby and Vayda, 1991). Our findings suggest that during pathogen-induced PCD in tobacco, the expression of cAPX is inhibited via a similar mechanism. Thus, in contrast to PR proteins that are efficiently transcribed and translated during the HR, the expression of cAPX is post-transcriptionally suppressed. Because the wound response and PCD may result in the activation of similar mechanisms (Mittler and Lam, 1997), it is possible that the suppression of cAPX expression is mediated by the same mechanism during these two processes.

An alternative explanation to the inhibition of cAPX synthesis is that cAPX-encoding transcripts that are found in cells undergoing PCD may be untranslatable due to specific or nonspecific modifications. This possibility may account for the inability to synthesize cAPX protein from mRNA isolated from cells undergoing PCD in an in vitro translation system (Figure 6). Interestingly, it was found that MJ treatment causes the production of untranslatable *rbcL* transcripts (Reinbothe et al., 1993a). This finding is in accordance with our inability to synthesize the cAPX protein in vitro from mRNA isolated from TMV-infected or MJ-treated cells (Figures 6 and 7). Another possible explanation to the post-transcriptional suppression of cAPX expression is that a protease found in cells undergoing PCD rapidly degrades the newly synthesized cAPX protein. This protease may be encoded by mRNA, or mRNA-loaded polysomes, from cells undergoing PCD, thus accounting for our inability to detect the cAPX protein after in vivo and in vitro protein synthesis assays. The protease that may degrade the cAPX protein may function in a manner similar to proteases that are activated during apoptosis in animals (Fraser and Evans, 1996). The possibility that a protease activity may be involved in the activation of pathogen-induced PCD in plants was recently strengthened by the finding that protease inhibitors can suppress pathogen-induced PCD (Levine et al., 1996).

The possibility that heme availability is the cause of cAPX suppression, whether it be by inhibition of protein synthesis or by rapid protein degradation, was examined by δ-aminolevulinic acid feeding experiments. However, it was found that during PCD, the steady state level of the cAPX protein was not affected by manipulating the intracellular level of heme via δ-aminolevulinic acid feeding (data not shown).

The plant hormone MJ is thought to act as a master switch that controls the expression of many genes during stress or pathogen infection (Wasternack and Parthier, 1997). Interestingly, MJ was found to control the expression of cAPX in a manner that may be similar to the control of cAPX expression during PCD (Figure 7). Thus, it is possible that MJ causes the suppression of cAPX expression during TMV-induced PCD. However, the application of MJ, which resulted in the complete suppression of cAPX expression, did not induce PCD (Figure 7D). Therefore, it is possible that the suppression of cAPX expression will result only in the activation of cell death when the production of ROS is elevated, for example, during the HR. This hypothesis is supported by the finding that MJ-treated plants are more susceptible to the application of paraquat (Figure 7D). A similar observation was made when transgenic plants with reduced cAPX activity were studied. These plants did not show any significant cell death under normal growth conditions; however, cell death was induced upon ozone stress, a treatment that causes the formation of ROS (Orvar and Ellis, 1997).

The finding that SA acts to prevent the inhibitory effect of MJ (Figure 7C) may explain why cAPX activity is not completely eliminated during the HR (in contrast to the inhibition of cAPX expression by MJ). During the HR, both MJ and SA accumulate (Hammond-Kosack and Jones, 1996). Therefore, SA may act to prevent some of the inhibitory effect of MJ on the steady state level of the cAPX protein.

Taken together, our findings suggest that the HR of plants to invading pathogens involves the activation of a mechanism that acts post-transcriptionally to suppress the expression of cAPX. This mechanism is likely to reduce the capability of cells to detoxify H_2O_2 and therefore facilitate H_2O_2-derived PCD. Although other isozymes of APX may function to remove H_2O_2, which diffuses from the extracellular spaces, their localization within the chloroplast and mitochondria makes it unlikely that they will be efficient in protecting the cytosol. The activity of at least three other antioxidative enzymes—glutathione reductase, superoxide dismutase, and glutathione *S*-transferase—was found to be suppressed during relatively early stages of the HR in tobacco (Fodor et al., 1997; R. Mittler and E. Lam, unpublished data). Combined with our findings on cAPX expression, these observations support the possibility that the expression of antioxidative enzymes is suppressed during early stages of the HR.

However, it should be noted that although the proposed role of H_2O_2 in mediating cell death has gained considerable experimental support, the production of H_2O_2 after the recognition of a bacterial pathogen was insufficient to trigger PCD (Glazener et al., 1996), at least in one instance. This example may suggest that additional mechanisms, such as a mechanism suppressing the antiperoxidative activity of cells, may be required for elevated H_2O_2 levels to trigger PCD. Thus, the signal transduction cascade that is activated upon pathogen recognition may result not only in the enhanced production of $O_2^{\cdot -}$ and H_2O_2 but also in the suppression of antiperoxidative enzymes such as cAPX.

METHODS

Conditions for Plant Growth, Programmed Cell Death Activation, and Hormone Treatment

Nicotiana tabacum cv Xanthi-nc NN, Xanthi-nc nn, and Samsun NN (R1 of El-301A; Mittler et al., 1995) plants were grown at 30°C under continuous illumination provided by cool-white fluorescent lamps (100 mmol m^{-2} sec^{-1}). Tobacco plants were inoculated with tobacco mosaic virus (TMV) strain U1 in 5 mM potassium phosphate buffer, pH 7, or mock infected with 5 mM phosphate buffer by gently rub-

bing the leaves with carborundum, as previously described (Mittler et al., 1995) and kept at 30°C. Programmed cell death (PCD) was induced by shifting plants to 22°C, as previously described (Mittler and Lam, 1995). Although this method of inducing PCD resulted in the complete death of all TMV-infected cells present within the area of the leaves sampled, not all cells undergo a precisely synchronized cell death process (Mittler et al., 1997). Therefore, samples obtained 24 hr after the temperature shift contained a mixture of cells at different stages of death, including cells in which cell death initiation was delayed. Adequate biochemical analysis of cytosolic ascorbate peroxidase (cAPX) expression was therefore possible only during the relatively early stages of cell death, that is, 0, 6, and 12 hr after the temperature shift.

Bacteria-induced PCD was activated by infecting leaves of tobacco plants grown at 25°C with *Pseudomonas syringae* pv *phaseolicola* (NPS3121), as described by Lindgren et al. (1986). As a control for PCD induced by bacteria, leaves were infiltrated with a *Hrp*$^-$ derivative of NPS3121 (NPS4000; Lindgren et al., 1986).

Treatment of plants with salicylic acid (SA) was performed as previously described (Mittler and Lam, 1995), and treatment of plants with methyl jasmonate (MJ) was performed essentially as described by Constable et al. (1995). Briefly, tobacco plants were enclosed in gas-tight glass chambers and kept under continuous illumination (60 mmol m^{-2} sec^{-1}). Control plants were placed in similar chambers and treated with the same amount of ethanol used to apply MJ. For studying the effect of SA on MJ application, SA-treated and untreated plants were enclosed together in the same glass chambers and treated with MJ. For examining the scavenging capability of MJ-treated tobacco plants, MJ-treated and control plants (24 hr after MJ application) were infiltrated with paraquat (0 to 10^{-4} M prepared in water) or water, kept under continuous illumination (60 mmol m^{-2} sec^{-1}), and sampled and analyzed for ion leakage (an indication of damage to membranes and death) 18 hr after paraquat application.

For all treatments, plant material was collected at various time points and flash-frozen in liquid nitrogen.

Measurements of Ion Leakage

Cell death was assayed by measuring ion leakage from leaf discs. For each measurement, five leaf discs (9 mm in diameter) were floated abaxial side up on 5 mL of distilled water for 3 hr at room temperature. After incubation, the conductivity of the bathing solution was measured with a conductivity meter (model K511; Consort, Turnhout, Belgium).

Isolation of RNA and RNA Gel Blot Analysis

Leaves were frozen in liquid nitrogen and ground to a fine powder. RNA was extracted as previously described and subjected to RNA gel blot analysis (Mittler et al., 1995). RNA gel blots were first hybridized with either cAPX or the PR-1 probes and then with a probe for 18S rRNA (Mittler and Zilinskas, 1994). RNA gel blot hybridization and membrane washing were performed as previously described (Mittler et al., 1995). cAPX transcripts were detected with a tobacco cAPX cDNA (gift of B. Zilinskas and D. Klessig, Rutgers University, New Brunswick, NJ), the level of PR-1 transcripts was determined with a tobacco cDNA probe (gift of D. Klessig), the level of ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (*rbcS*) transcripts was determined with a pea cDNA probe (gift of G. Coruzzi, New York University, New York, NY), the level of TMV RNA was determined with a cDNA encoding the TMV coat protein (gift of N. Tumer, Rutgers University), and the level of Rubisco large subunit (*rbcL*) transcripts was determined with a tobacco cDNA probe (gift of P. Maliga, Rutgers University).

Isolation of Protein and Protein Gel Blot Analysis

Immunodetection of cAPX (Mittler and Zilinskas, 1991, 1993), the pathogenesis-related protein PR-1, and the subunits of rbcS and rbcL was performed by protein blot analysis of total leaf protein with a chemiluminescence detection system (SuperSignal; Pierce, Rockford, IL). To determine the steady state level of cAPX protein, total protein extracts (5 to 10 μg; the linear range for quantification of cAPX via protein gel blots was determined to be between 1 and 40 μg) were subjected to protein blot analysis as described by Mittler et al. (1995). Protein blots were quantified using a laser scanner (model SL-TRFF; Zeineh, Fullerton, CA). Although several cAPX isoforms may exist in tobacco, the polyclonal antibody used in this study was found to react with the majority of cAPX activity detected in tobacco total protein extracts by APX activity gels (Mittler and Zilinskas, 1993). The cAPX antiserum did not react with protein extracts prepared from isolated intact tobacco or pea chloroplasts (data not shown; Mittler and Zilinskas, 1991). To demonstrate equal loading of protein for protein blot analysis, protein extracts were subjected to protein gels and stained with Coomassie Brilliant Blue R 250. Anti–PR-1 serum was a gift of R. Fluhr (Weizmann Institute, Rehovot, Israel), and anti-RbcS serum and anti-RbcL serum were a gift of R. Nechushtai (Hebrew University, Jerusalem, Israel).

In Vivo Protein Labeling and Immunoprecipitation

In vivo protein labeling was performed by floating three leaf discs (9 mm in diameter) abaxial side up on 3 mL of distilled water containing 0.2 mCi of ^{35}S-methionine (ICN, Costa Mesa, CA) for 2 hr at room temperature. After incubation, leaf discs were rinsed briefly in distilled water and extracted in 100 μL of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 μg/mL phenylmethylsulfonyl fluoride, and 1% Triton X-100 (ice cold). Three volumes of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, and 0.25% gelatin (ice cold) were added to the sample. After centrifugation at 12,000*g* (4°C) for 5 min, the supernatant was used for immunoprecipitation. Samples with an equal amount of ^{35}S-methionine–incorporated material (final volume of 0.35 mL) were mixed with 2.5 mg of protein A–Sepharose (Sigma) and incubated on a rotary shaker for 20 min at 4°C. Samples were then centrifuged for 1 min at 12,000*g* (4°C), and the anti-cAPX serum (5 μL; Mittler and Zilinskas, 1991) was added to the supernatant. After addition of the anti-cAPX serum, samples were incubated for 3 hr on a rotary shaker at 4°C. After incubation, 2.5 mg of protein A–Sepharose was added, and samples were incubated for an additional 30 min. After centrifugation for 1 min at 12,000*g* (4°C), the pellet was washed twice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, and 0.25% gelatin (ice cold), resuspended in Laemmli sample buffer, heated, and subjected to SDS-PAGE and fluorography, as described below. In control incubations, preimmune serum was used instead of the anti-cAPX serum to confirm that the immunoprecipitated polypeptide is cAPX. To assess the rate of ^{35}S-methionine incorporation and protein synthesis, protein extracts from an equal amount of tissue (0.2 g fresh weight) were subjected to SDS-PAGE followed by fluorography, as described below.

In Vitro Protein Labeling and Immunoprecipitation

For preparation of mRNA, total RNA was extracted with the Trizol reagent, as suggested by the manufacturer (Gibco BRL, Grand Island, NY). mRNA was isolated using the Oligotex mRNA isolation kit (Qiagen, Chatsworth, CA). Polysomes were isolated according to Vayda (1995). RNA was extracted from polysomes with the Trizol reagent and subjected to RNA blot analysis. mRNA (0.05 to 0.1 μg) was translated in a rabbit reticulocyte lysate (Boehringer Mannheim) supplemented with ^{35}S-methionine (ICN), as suggested by the manufacturer. Polysomes (12 μg) were translated in the same in vitro translation mix; however, they were modified according to Vayda (1995). Total in vitro–translated protein was subjected to SDS-PAGE. After electrophoretic separation, gels were equilibrated with Amplify (Amersham, Amersham, UK), dried, and exposed to x-ray films. For immunoprecipitation, the volume of the in vitro translation reaction was adjusted to 0.4 mL with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, and 0.25% gelatin (ice cold), and immunoprecipitation was performed as described above.

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