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Inhibition of Programmed Cell Death in Tobacco Plants during a Pathogen-Induced Hypersensitive Response at Low Oxygen Pressure

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The hypersensitive response (HR) of plants to invading pathogens is thought to involve a coordinated activation of plant defense mechanisms and programmed cell death (pcd). To date, little is known about the mechanism underlying death of plant cells during this response. In addition, it is not known whether suppression of pcd affects the induction of other defense mechanisms during the HR. Here, we report that death of tobacco cells (genotype NN) infected with tobacco mosaic virus (TMV) is inhibited at low oxygen pressure. In contrast, virus replication and activation of defense mechanisms, as measured by synthesis of the pathogenesis-related protein PR-1a, were not inhibited at low oxygen pressure. Bacterium-induced pcd was also inhibited at low oxygen pressure. However, pcd induced by TMV or bacteria was not inhibited in transgenic tobacco plants expressing the mammalian anti-pcd protein Bcl-X_L. Our results suggest that ambient oxygen levels are required for efficient pcd induction during the HR of plants and that activation of defense responses can be uncoupled from cell death. Furthermore, pcd that occurs during the interaction of tobacco with TMV or bacteria may be distinct from some cases of pcd or apoptosis in animals that are insensitive to low oxygen or inhibited by the Bcl-X_L protein.

INTRODUCTION

The term programmed cell death (pcd) describes a cell death process that is a normal part of the life cycle of a multicellular organism (Martin et al., 1994). In animals, pcd is activated during the course of several differentiation pathways, after infection by some viral pathogens, and in response to certain cellular and environmental stimuli (Schwartzman and Cidlowski, 1993; Hacker and Vaux, 1994). Control of pcd in animals is mediated by certain gene products, including pcd activators, such as the interleukin-1β converting enzyme, and pcd inhibitors, such as the human B-cell lymphoma Bcl-2 or Bcl-X_L proteins (Korsmeyer, 1995).

In plants, pcd is thought to occur during the course of several developmental processes and in response to attack by some invading pathogens (DeLong et al., 1993; Dietrich et al., 1994; Greenberg et al., 1994; Hammond-Kosack et al., 1994; Levine et al., 1994; Mittler et al., 1995; Jones and Dangl, 1996; Mittler and Lam, 1996; Reyerson and Heath, 1996; Wang et al., 1996). Activation of cell death after recognition of invading pathogens results in the formation of a zone of dead cells localized around the site of infection. This zone of dead cells, also called a hypersensitive response (HR) lesion, is thought to inhibit the proliferation of pathogens and to prevent systemic infection (Goodman and Novacky, 1994).

Recent studies suggest that cell death occurring during the HR is a type of pcd (Dietrich et al., 1994; Greenberg et al., 1994; Levine et al., 1994). HR-associated cell death was shown to exhibit some morphological and biochemical features found in animal cells undergoing pcd. These include fragmentation of nuclear DNA, induction of specific endonucleases, and condensation and vacuolization of cytoplasm (Mittler and Lam, 1995; Mittler et al., 1995; Levine et al., 1996; Reyerson and Heath, 1996; R. Mittler, L. Simon, and E. Lam, submitted manuscript). However, it is not clear whether HR-associated pcd is mediated by a mechanism similar to that responsible for some cases of pcd in animal cells (Mittler and Lam, 1996).

The HR is also associated with induction of multiple defense mechanisms. These include the strengthening of cell walls and increased synthesis of phytoalexins, salicylic acid (SA), and pathogenesis-related (PR) proteins (Bowles, 1990; Malamy et al., 1990; Linthorst, 1991; Bradley et al., 1992; Enyedi et al., 1992; Delany et al., 1994). Previous studies suggest that activation of cell death can be uncoupled from the induction of some defense mechanisms (Gross et al., 1993; Jakobek and Lindgren, 1993; Lawton et al., 1993; Bowling et al., 1994; Century et al., 1995; Hammond-Kosack et al., 1996). However,

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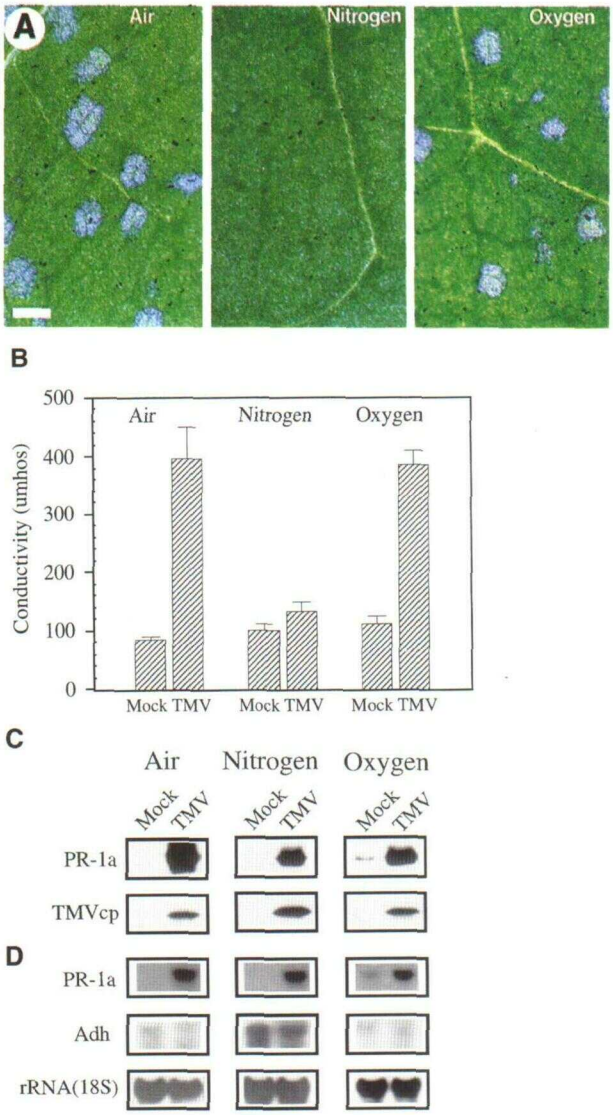

Figure 1. Effect of Low and High Oxygen Pressure on the Death of Plant Cells, Induction of the PR-1a Protein, and Virus Replication during the HR.

(A) and **(B)** Inhibition of pcd during the HR of tobacco leaves inoculated with TMV at low oxygen pressure. In **(A)**, inhibition of pcd at low oxygen pressure is shown by the suppression of visible HR lesions. In **(B)**, inhibition of pcd at low oxygen pressure was measured by the leakage of ions from leaf discs. The data presented are the mean and standard deviation of three individual measurements. umhos, micromhos. Bar in **(A)** = 2.5 mm.

(C) Protein gel blot analysis showing the induction of the PR-1a protein and accumulation of the TMV coat protein (TMVcp) under low and high oxygen pressure. The TMV coat protein was used as a measure of virus replication, indicating that this process can occur at low and high oxygen pressure. No TMV coat protein was detected when TMV-inoculated leaves were sampled immediately after infection.

(D) RNA gel blot analysis showing an increase in the steady state level of transcripts encoding PR-1a after inoculation with TMV at low and high oxygen pressure. The increase in the steady state level of transcripts encoding the anaerobic responsive protein Adh was used as a positive control for low oxygen pressure. A probe for rRNA(18S) was used as a control for equal RNA loading. Mock- and TMV-inoculated leaves were enclosed in gas-tight, temperature-controlled chambers, sampled, and analyzed 60 hr after mock or TMV infection.

it is not clear whether inhibition of pcd during the HR will affect the activation of other plant defense mechanisms, such as the synthesis of SA and induction of PR proteins.

Reactive oxygen species (ROS) have been implicated in signaling or mediating death of plant cells during the HR (Levine et al., 1994; Baker and Orlandi, 1995; Tenhaken et al., 1995). In animals, recent studies indicated that some cases of pcd, in the form of apoptosis, can occur at very low oxygen pressure (Jacobson and Raff, 1995; Shimizu et al., 1995; Graeber et al., 1996). Because treatment of cells at low oxygen pressure is likely to suppress production of reactive oxygen (Jacobson and Raff, 1995; Shimizu et al., 1995), these studies suggest that at least for some cases, reactive oxygen is not involved in signaling or mediating pcd. We tested whether death of plant cells and activation of defense mechanisms during the HR can occur at low oxygen pressure. In addition, we examined whether pathogen-induced pcd that occurs during the HR in tobacco is inhibited by a protein that inhibits pcd or apoptosis in many different types of animal cells.

RESULTS

Inhibition of Tobacco Mosaic Virus-Induced pcd at Low Oxygen Pressure

To study the effect of low oxygen pressure on the death of plant cells and activation of defense mechanisms during the HR, tobacco mosaic virus (TMV)–inoculated and mock-treated leaves of tobacco plants (genotype NN) were enclosed in gas-tight, temperature-controlled, transparent chambers, while the rest of the plant remained exposed to ambient air. The atmosphere within the chamber was replaced with ambient air, pure oxygen (high oxygen pressure), or oxygen-free nitrogen (low oxygen pressure). When the atmosphere within the chamber was replaced with nitrogen, the level of oxygen within the chamber was <0.3%. As shown in Figure 1, death of tobacco cells infected with TMV, as observed by the appearance of visible lesions (Figure 1A) and measured by an increase in ion leakage (Figure 1B), was inhibited at low oxygen pressure (nitrogen). In contrast, virus replication, as observed by the abundance of the TMV coat protein (Figure 1C), and the activation of defense mechanisms, as measured by an increase in the steady state level of the PR-1a protein (Figure 1C) and transcript (Figure 1D), were not affected at low oxygen pressure. TMV replication was also assayed by measuring the level of TMV RNA (data not shown) and was determined to cor-

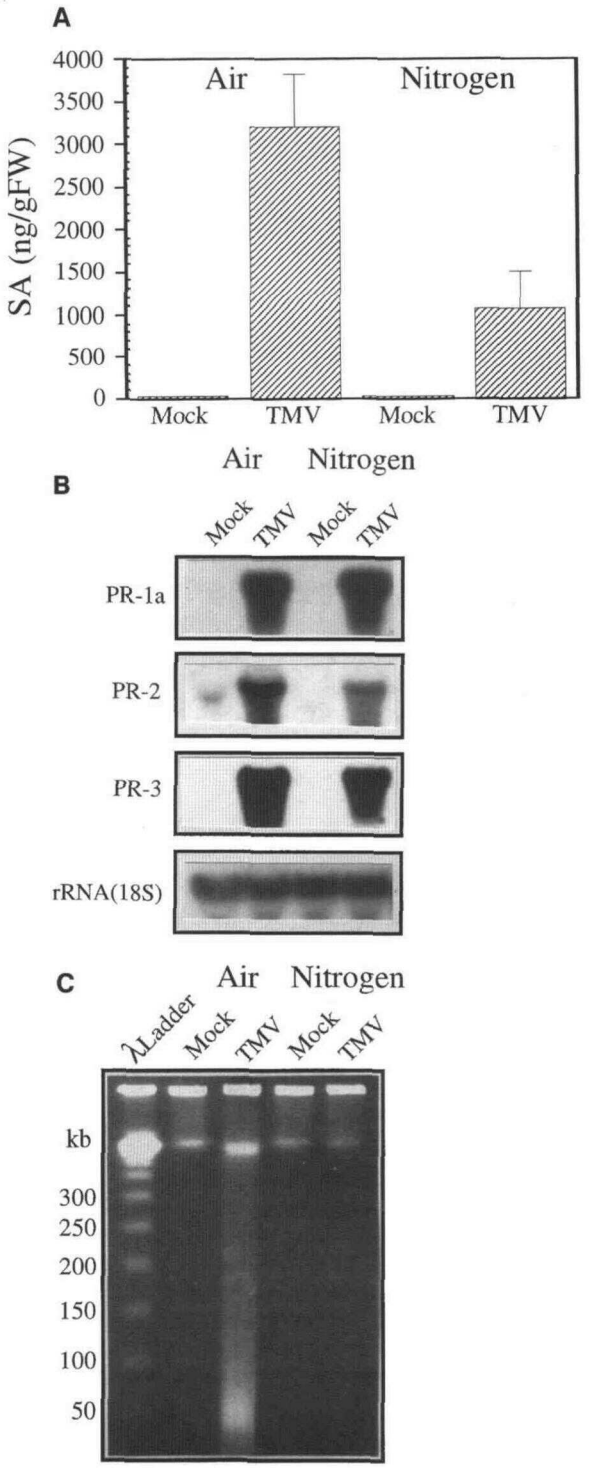

Figure 2. Suppression of Cell Death but Not Activation of the Defense Response during the HR of Tobacco to TMV at Low Oxygen Pressure.

(A) Accumulation of SA after virus infection at ambient (Air) and low (Nitrogen) oxygen pressure. The data presented are the mean and standard deviation of three individual samples. FW, fresh weight.

respond to the findings presented in Figure 1C. No TMV coat protein was detected with anti-TMV coat protein antibodies in tissues obtained from TMV-infected leaves sampled immediately after inoculation (data not shown; see also Mittler et al., 1995).

In addition to measuring ion leakage and examining leaves for visible HR lesions (Figures 1A and 1B), leaf tissue was fixed, sectioned, and stained with eosin and hematoxylin, as previously described (Mittler et al., 1995), and examined by light microscopy for the existence of small lesions that might not be visible. Examination of fixed tissues from TMV- and mock-infected leaves treated at ambient and low oxygen pressure for 60 hr confirmed the absence of lesions in tissues kept under low oxygen (data not shown). To evaluate the sensitivity of this detection method, leaf tissues infected with TMV at ambient oxygen pressure (23°C, continuous illumination of 200 μmol m^{-2} sec^{-1}) were fixed at different times after infection and examined for the appearance of small lesions, as described above. Light microscopy of these samples revealed that small lesions (approximately five to eight cells in diameter) could be detected as early as 24 hr after viral infection (data not shown).

As a positive internal control for low oxygen pressure, changes in the level of transcripts encoding alcohol dehydrogenase (Adh) were followed. *Adh* is known to be highly induced under anoxygenic conditions (Xie and Wu, 1989). The increase in the steady state level of transcripts encoding Adh (Figure 1D; nitrogen) indicated that leaves treated with nitrogen were exposed to low oxygen pressure.

Replacing the atmosphere within the chamber with a mixture of 99.7% nitrogen–0.3% carbon dioxide also inhibited cell death (a decrease of 60 to 75% in lesion size; data not shown). This inhibition was not as strong as the inhibition obtained by replacing the atmosphere within the chamber with pure nitrogen, suggesting that intracellular oxygen, which may be produced during photosynthesis, that uses the CO_2 within the chamber is sufficient to support the death of tobacco cells during the HR.

We also tested the effect of low oxygen pressure on the activation of other defense mechanisms, such as synthesis of SA and induction of the PR proteins PR-2 (β-1,3-glucanase) and PR-3 (chitinase). As shown in Figure 2, the induction of these defense mechanisms occurred at low oxygen pressure.

(B) RNA gel blot analysis showing an increase in the steady state level of transcripts encoding PR-1a, PR-2, and PR-3 after virus inoculation at ambient and low oxygen pressure. A probe for rRNA(18S) was used as a control for equal RNA loading.

(C) Field inversion gel electrophoresis showing inhibition of cellular DNA degradation during the HR of tobacco to TMV at low oxygen pressure. Cellular DNA degradation is shown by the formation of 50-kb fragments in TMV-infected leaves, in air, at 60 hr after inoculation. Monomeric chloroplast DNA (150-kb fragment; R. Mittler, L. Simon, and E. Lam, submitted manuscript) was not detected at 60 hr after infection because at this time point, most of the chloroplast DNA is degraded. The lengths of the molecular markers are given at left in kilobases. Leaves were sampled and analyzed 60 hr after mock or TMV infection.

However, in contrast to the PR-1a protein (Figures 1C, 1D, and 2B), the level of SA and transcripts encoding PR-2 and PR-3, at low oxygen pressure, was lower than that observed when tobacco leaves were inoculated in ambient air (Figures 2A and 2B). Inhibition of SA synthesis may be expected under conditions of low oxygen pressure because oxygen is one of the substrates of benzoic acid 2-hydroxylase, an enzyme involved in SA biosynthesis (Leon et al., 1995b).

Low oxygen pressure not only inhibited visible symptoms (Figure 1A) and ion leakage (Figure 1B) but also suppressed the fragmentation of cellular DNA during TMV-induced pcd (Figure 2C). The findings presented in Figures 1 and 2 suggest that the oxygen level present in TMV-infected tobacco leaves kept at low oxygen pressure is not sufficient to support cell death but is high enough to allow the induction of SA synthesis and PR proteins. Interestingly, treatment of TMV-inoculated tobacco leaves with pure oxygen did not cause a significant change in the HR (Figure 1). This may indicate that the ambient atmospheric level of oxygen (~20%) is saturating for the induction of cell death.

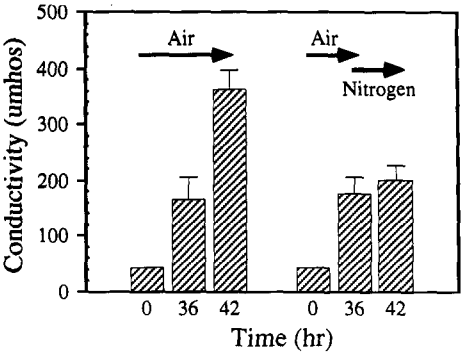

Figure 3. Suppression of Cell Death by Low Oxygen Pressure at Late Stages of the HR.

Leaves were inoculated and kept in ambient air for 36 hr. Inoculated leaves were then enclosed in sealed chambers, and the atmosphere within the chamber was replaced with nitrogen or air. Cell death was measured as the leakage of ions from leaf discs obtained at 42 hr post-viral infection. The data shown are the mean and standard deviation of three individual measurements. umhos, micromhos.

Inhibition of TMV-Induced pcd at a Late Stage of the HR

Because tobacco leaves infected with TMV were treated with low oxygen pressure immediately after virus infection, it was not clear whether the inhibition of cell death is the result of suppression at an early step in the signaling process or the disruption of a late event that may be directly associated with killing of cells. Therefore, we tested whether treatment with low oxygen pressure applied at a later stage of the HR can inhibit cell death. Tobacco leaves were inoculated with TMV and kept at ambient atmospheric conditions for 36 hr. Leaves were then enclosed in chambers, and the atmosphere within the chambers was replaced with nitrogen or air. At 36 hr after viral infection, cell death, as measured by leakage of ions from leaf discs, was detected (Figure 3). However, as shown in Figure 3, treatment of leaves with low oxygen pressure at 36 hr after viral infection resulted in suppression of further cell death. This finding may suggest that treatment with low oxygen pressure can inhibit cell death even after it has been initiated. However, TMV-induced cell death may not occur simultaneously in all infected cells. Therefore, the inhibition of cell death at a late stage of the HR (36 hr) may have resulted from inhibiting the initiation of new cell death that occurred at this stage (cells that initiated cell death at 36 hr) or the propagation of cell death that was initiated at an earlier stage of the HR (cells that initiated cell death before 36 hr).

Inhibition of Bacterium-Induced pcd at Low Oxygen Pressure

We next examined whether the HR of tobacco plants infected with a bacterial pathogen is also inhibited at low oxygen pressure. *Pseudomonas syringae* pv *phaseolicola* is a bean pathogen that induces an HR in tobacco (Lindgren et al., 1986). As shown in Figure 4A, infiltration of leaves with a *P. s. phaseolicola* strain that induces the HR (NPS3121) resulted in activation of cell death. In contrast, infiltration of leaves with a *P. s. phaseolicola* strain that is *Hrp*$^-$ (NPS4000), and therefore does not induce the HR, did not trigger cell death. Bacterium-induced pcd of tobacco cells was inhibited at low oxygen pressure (Figure 4A).

Suppression of bacterium-induced cell death at low oxygen pressure is not likely the result of inhibiting or killing bacteria at low oxygen because the numbers of viable bacteria obtained from leaves treated at low oxygen were similar to those obtained from leaves treated with air (Figure 4B). We also tested whether bacterium-induced pcd is inhibited by treatment with low oxygen pressure at later stages of the HR. As shown in Figures 5A and 5B, the application of low oxygen pressure at 3 hr after infection did not result in the inhibition of pcd. Thus, in contrast to the inhibition of TMV-induced cell death at low oxygen pressure (Figure 3), the inhibition of bacterium-induced cell death at low oxygen pressure occurred only if this treatment was applied at an early stage of the HR.

Because bacteria grown in rich broth (King's B) do not produce the HR-inducing protein harpin before they are infiltrated into leaves (He et al., 1993), it was possible that the inhibition of bacterium-induced pcd resulted from the inhibition of harpin synthesis in bacteria after it was infiltrated into leaves treated with low oxygen pressure. To exclude this possibility, we tested whether low oxygen pressure can inhibit bacterium-induced pcd that is induced by bacteria grown in a minimal medium, which was shown previously to induce harpin synthesis in culture (Huynh et el., 1989; He et al., 1993). Although bacteria grown in minimal medium induced pcd earlier than bacteria grown in rich medium, we found no difference in the inhibition of pcd at low oxygen pressure between bacteria grown in rich or minimal medium (data not shown).

Increase in the Steady State Level of Transcripts Encoding Ascorbate Peroxidase and Superoxide Dismutase during TMV-Induced pcd in Tobacco

Previous studies indicated that the HR is accompanied by an increase in the production of ROS and lipid peroxidation (Goodman and Novacky, 1994; May et al., 1996; Rusterucci et al., 1996). Our findings that pcd induced by TMV or bacteria was inhibited by low oxygen pressure may also indicate that ROS are involved in this process (Jacobson and Raff, 1995; Shimizu et al., 1995). If the HR is accompanied by an increase in the production of ROS, then it is possible that it is also accompanied by the activation of several defense mechanisms that are responsible for the removal of ROS. Therefore, we examined whether the HR is accompanied by the induction of enzymes involved in the removal of ROS, such as ascorbate peroxidase (APX) and superoxide dismutase (CuZnSOD; Bowler et al., 1989; Asada, 1992; Mittler and Zilinskas, 1994). As shown in Figures 6A and 6B, the HR of tobacco plants infected with TMV is accompanied by an increase in the steady state level of transcripts encoding the cytosolic antioxidant enzymes APX and CuZnSOD. We also detected an increase in

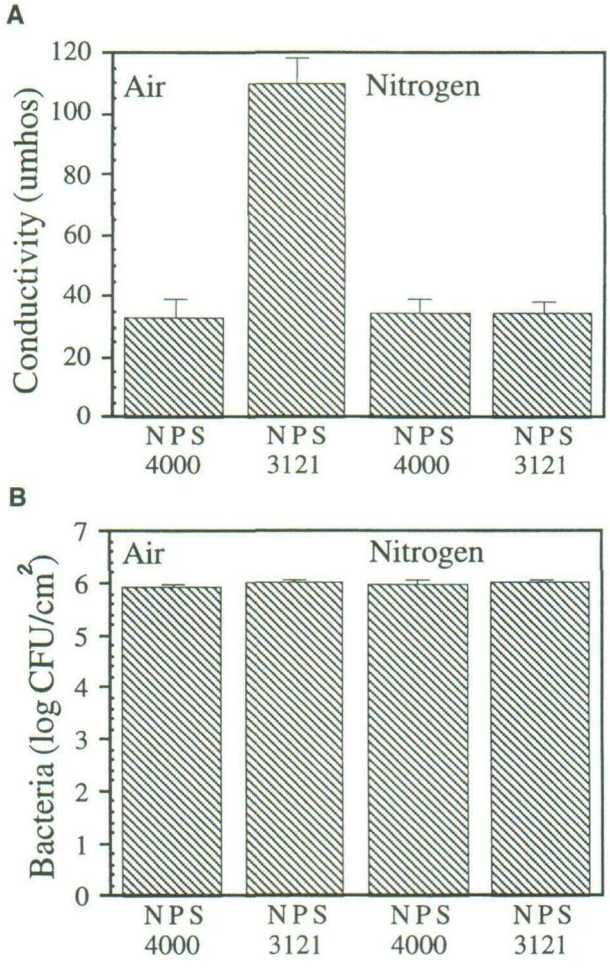

Figure 4. Inhibition of Bacterium-Induced pcd at Low Oxygen Pressure.

(A) Inhibition of bacterium-induced pcd at low oxygen pressure (Nitrogen), as measured by the leakage of ions from leaf discs. The data presented are the mean and standard deviation of three individual measurements. umhos, micromhos.

(B) Treatment with low oxygen pressure does not inhibit the growth of bacteria. Bacteria (*P. s. phaseolicola*) were extracted from leaves treated at low (Nitrogen) or ambient (Air) oxygen levels and analyzed for viability by growth on agar plates. The data presented are the mean and standard deviation of three individual measurements. Leaves were infiltrated with a *P. s. phaseolicola* strain that induces the HR (NPS3121) or a *P. s. phaseolicola* strain that is *Hrp*⁻ (NPS4000) and therefore does not induce the HR. Leaves were sampled and analyzed 24 hr after infection. CFU, colony-forming units.

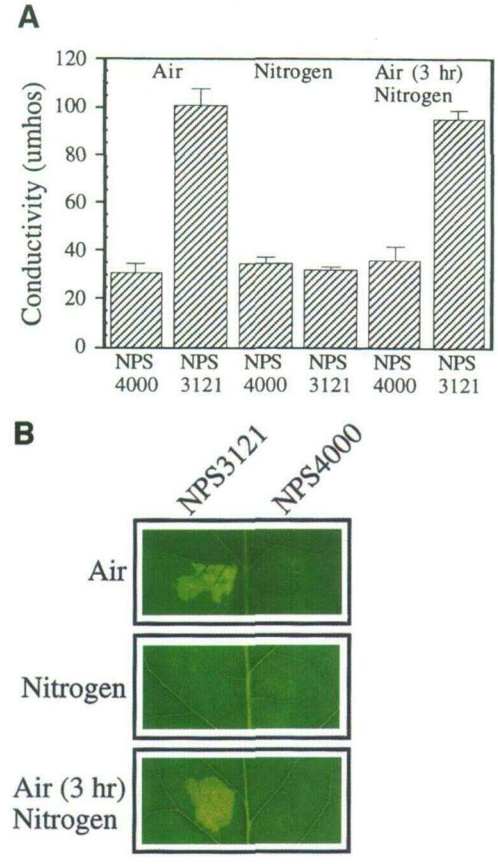

Figure 5. Application of Low Oxygen at Late Stages of Bacterium-Induced HR Does Not Inhibit the Death of Plant Cells.

(A) The application of low oxygen at 3 hr postinfection (Air [3 hr] Nitrogen) does not inhibit bacterium-induced pcd. Cell death was measured by the leakage of ions from leaf discs. The data presented are the mean and standard deviation of three individual measurements. umhos, micromhos.

(B) Visible HR lesions induced by bacteria are inhibited by the application of low oxygen pressure immediately after infection (Nitrogen), but not by the application of low oxygen at 3 hr after infection. Leaves were infiltrated with a *P. s. phaseolicola* strain that induces the HR (NPS3121) or a *P. s. phaseolicola* strain that is *Hrp*⁻ (NPS4000), as described for Figure 4.

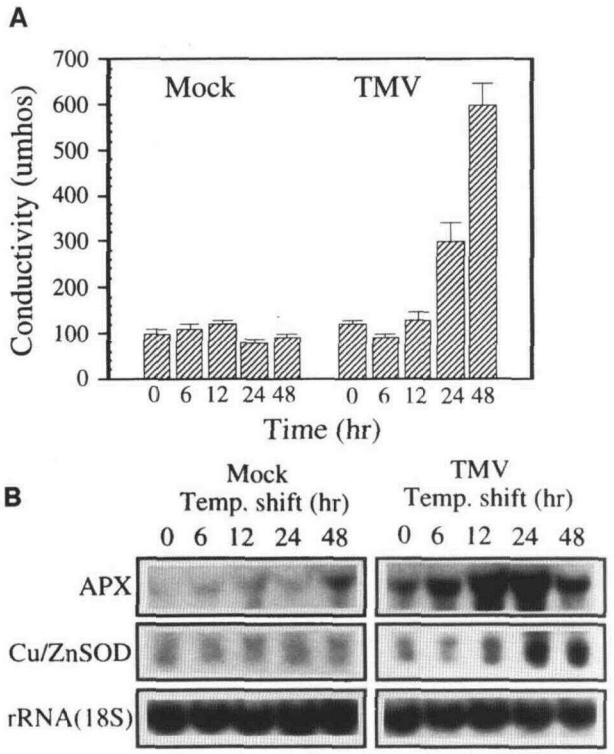

Figure 6. Increase in the Steady State Level of Transcripts Encoding APX and CuZnSOD during TMV-Induced HR.

(A) Ion leakage from leaf discs obtained from TMV- and mock-infected plants after a temperature shift from 30 to 23°C. The activation of pcd is shown by an increase in ion leakage in TMV-infected plants at 24 and 48 hr after the temperature shift. The data presented are the mean and standard deviation of three individual measurements. umhos, micromhos.

(B) Increase in the steady state level of transcripts encoding the cytosolic antioxidant enzymes APX and CuZnSOD during TMV-induced pcd. A probe for rRNA(18S) was used as a control for equal RNA loading. Treatment of plants and RNA gel blots was performed as described in Methods.

lipid peroxidation, as measured by elevated levels of malonyldialdehyde (data not shown). These findings support the hypothesis that ROS are produced during the HR, and they may be involved in signaling or mediating pathogen-induced pcd.

Expression of a Mammalian Anti-pcd Gene Does Not Inhibit Death of Plant Cells during the HR

In animals, a variety of proteins was shown to inhibit or activate pcd (Ellis and Horvitz, 1986; Lakshmi et al., 1992; Boise et al., 1993; Korsmeyer, 1995). The Bcl-X_L protein is a member of the Bcl-2 family of anti-pcd proteins. Bcl-2 was initially identified as an oncoprotein that is highly expressed in transformed B-cell lymphoma lines and protects transformed cells from pcd (Korsmeyer, 1995). In animals, expression of proteins that belong to the Bcl-2 family inhibits pcd in a number of different cell lines and organisms (Boise et al., 1993; Korsmeyer, 1995). Proteins that belong to the Bcl-2 family may function in an antioxidant pathway to prevent pcd (Hockenbery et al., 1993; Kane et al., 1993). Because the inhibition of pathogen-induced pcd at low oxygen pressure suggested that some forms of active oxygen may be involved with this type of pcd, we were interested in testing whether expression of a Bcl-2 type anti-pcd protein in tobacco can inhibit pathogen-induced pcd.

As shown in Figures 7A and 7B, transgenic plants expressing the human Bcl-X_L protein (Boise et al., 1993) were produced. As shown in Figure 7C, inoculation of these plants with TMV revealed that expression of the Bcl-X_L protein did not suppress TMV-induced pcd. Infection of similar transgenic tobacco plants with bacteria revealed that expression of Bcl-X_L did not suppress bacterium-induced pcd (data not shown). Thus the Bcl-X_L protein, which is a negative regulator of pcd in many types of animal cells, does not appear to suppress pcd that is induced by TMV or bacteria in tobacco. Recent studies suggest that Bcl-2 does not always function as an antioxidant to prevent pcd (Jacobson and Raff, 1995; Shimizu et al., 1995). Thus, our findings with the Bcl-X_L protein may not be taken as an indication that reactive oxygen is not involved with pcd during the HR.

DISCUSSION

We studied the effects of low oxygen pressure on activation of plant defense mechanisms and pcd during the HR of tobacco to TMV and bacteria. We found that treatment with low oxygen pressure did not significantly affect the replication or viability of the viral or bacterial pathogens that elicit the HR (compare the level of the TMV coat protein between air and nitrogen in Figure 1C and the numbers of viable bacteria extracted from leaves treated with air or nitrogen in Figure 4B). Indeed, under conditions of low oxygen pressure, a variety of defense mechanisms was activated in TMV-infected leaves. These included the synthesis of PR proteins and the accumulation of SA (Figures 1 and 2). In contrast to the activation of these defense mechanisms, pcd was inhibited. Suppression of pcd in TMV-inoculated leaves at low oxygen pressure was not likely the result of a general inhibition in cellular metabolism because low oxygen pressure did not affect viral replication and activation of some defense mechanisms (Figures 1 and 2). Moreover, treatment with low oxygen pressure 36 hr after viral infection also resulted in inhibition of pcd (Figure 3), showing that this inhibition is not the result of a long-term treatment with low oxygen.

Inhibition of pcd at low oxygen pressure may have also resulted from the suppression of particular genes, which are only expressed under aerobic conditions. These genes may

be specifically required for the HR. However, the finding that pcd can occur at low oxygen pressure in leaves treated with 99.7% nitrogen–0.3% carbon dioxide (HR lesions appear, but they are reduced in size by ~60 to 75%) and the observation that treatment with low oxygen pressure at a late stage of the HR inhibits further pcd (Figure 3) suggest that this possibility is unlikely. Thus, suppression of pcd at low oxygen may result from the direct action of oxygen in signaling or mediating the execution of pcd.

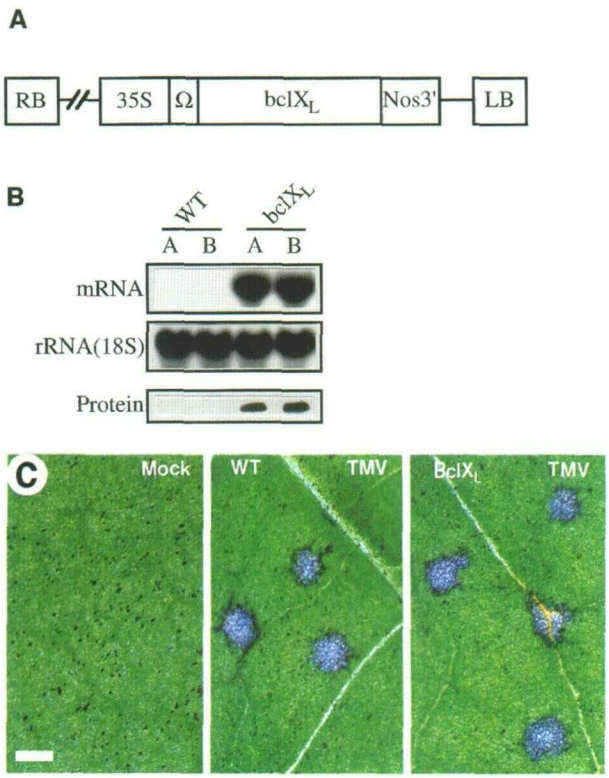

Figure 7. Expression of a Human cDNA Encoding the Anti-pcd Protein Bcl-X_L in Transgenic Tobacco Plants Does Not Suppress the HR.

(A) The binary vector T-DNA construct used for the study of Bcl-X_L expression in higher plants. RB and LB, right and left borders; Ω, translation enhancing sequence; bclX$_L$, the full-length open reading frame encoding the human B-cell lymphoma X anti-pcd gene; Nos3', nopaline synthase transcription termination sequence.

(B) RNA and protein gel blot analyses using antisera raised against Bcl-X_L and cDNA probes for human Bcl-X_L showing expression of Bcl-X_L in two independent transgenic tobacco plants (lanes A and B). WT, wild type.

(C) Formation of visible HR lesions in transgenic plants, indicating that the expression of Bcl-X_L does not suppress the HR. No lesions developed on mock-infected wild-type (WT) or Bcl-X_L–expressing plants. Construction of binary vectors, production of transgenic tobacco plants, analysis of transgenic plants by RNA and protein gel blots, and infection with TMV were performed as described in Methods. Bar = 2.5 mm.

Our findings (Figures 1 to 3) suggest that the recognition of TMV by tobacco cells (genotype NN) triggers a pathway that results in the activation of two distinct processes with different requirements for atmospheric oxygen: activation of defense mechanisms, as measured by induction of SA synthesis and PR proteins, and pcd. The induction of PR proteins and SA synthesis in the absence of pcd, as measured by the leakage of ions and the appearance of visible lesions, suggests that the extensive cell death accompanying the HR may not be required for the activation of these defense mechanisms.

In addition to suppressing TMV-induced pcd, low oxygen pressure also inhibited bacterium-induced pcd (Figures 4 and 5). However, in contrast to the inhibition of TMV-induced pcd (Figure 3), the inhibition of bacterium-induced pcd occurred only if low oxygen pressure was applied immediately after infection (Figure 5). Thus, unlike TMV-induced pcd, in which oxygen was required through most parts of the cell death process (Figure 3), oxygen may only be required during the first 3 hr after infection with bacteria (Figure 5). This suggests that in bacterium-induced pcd, oxygen is involved in generating an early signal for activation of pcd, whereas in TMV-induced pcd, it may be required throughout the cell death process. Alternatively, this difference may reflect the faster activation of signal transduction and cell death induced by bacteria. Thus, bacteria that induce visible HR lesions within 12 to 16 hr after infection may only require oxygen for a short period of time compared with TMV, which induces lesions within 36 hr.

Recent studies suggest that H_2O_2, a form of ROS, is a key mediator of pathogen-induced pcd during the HR (Levine et al., 1994, 1996; Tenhaken et al., 1995). Because treatment of cells with low oxygen pressure is likely to cause a decrease in the level of ROS (Jacobson and Raff, 1995; Shimizu et al., 1995), the inhibition of pcd at low oxygen pressure (Figures 1 to 5) may support the suggestion that ROS are involved in signaling or mediating cell death.

The requirement for atmospheric oxygen during the first 3 hr after infection with bacteria (Figure 5) coincides with the activation of specific oxidases and the rapid production of H_2O_2 during the so-called oxidative burst thought to play a key role in the induction of the HR (Levine et al., 1994, 1996; Tenhaken et al., 1995). Previous reports suggest that pathogen-induced cell death is accompanied by an increase in ROS and lipid peroxidation (summarized in Goodman and Novacky, 1994; May et al., 1996; Rusterucci et al., 1996). We also found an increase in the level of malonyldialdehyde, a by-product of lipid peroxidation during the HR (data not shown), and an increase in the steady state level of transcripts encoding certain ROS-scavenging enzymes during the HR (Figure 6). The HR is known to be accompanied by an increase in oxygen uptake (Weintraub et al., 1960). However, it is not clear whether this phenomenon reflects an increase in respiration, polyphenol oxidase activity, or production of ROS and lipid peroxidation. It is possible that ROS produced by plant cells during the HR to TMV, and bacteria may be involved in signaling or mediating pcd, because low oxygen pressure was found to suppress

this process. However, further studies are required to address this issue (Baker and Orlandi, 1995; Glazener et al., 1996).

The suggestion that H_2O_2 is part of a signal transduction pathway leading to the induction of PR proteins and systemic resistance has been controversial (Chen et al., 1993; Bi et al., 1995; Green and Fluhr, 1995; Leon et al., 1995a; Neuenschwander et al., 1995). The fact that induction of SA synthesis and PR proteins can occur at low oxygen pressure may suggest that a pathway not requiring ROS is involved. However, it is also possible that during the interaction with TMV at low oxygen pressure, enough H_2O_2 is produced in plant cells to signal the induction of SA synthesis and PR proteins. In support of the putative role of ROS as a signal to induce PR proteins (Chen et al., 1993; Green and Fluhr, 1995), PR-1a was observed to be slightly induced in mock-infected plants treated with pure oxygen (Figure 1A). This treatment is most likely to cause oxidative stress. Indeed, the steady state level of transcripts encoding the antiperoxidative enzyme APX was found to increase in leaves treated with pure oxygen (data not shown).

Pathogen-induced cell death in plants exhibits some morphological and biochemical features found in animal cells undergoing pcd (Mittler and Lam, 1995; Levine et al., 1996; Reyerson and Heath, 1996; Wang et al., 1996; R. Mittler, L. Simon, and E. Lam, submitted manuscript). However, this study demonstrates that the HR in tobacco uses a mechanism that may be distinct from that responsible for some cases of pcd in animal cells. Thus, in contrast to some examples of pcd or apoptosis in animals (Jacobson and Raff, 1995; Shimizu et al., 1995), cell death during TMV-induced and bacterium-triggered HR in tobacco is suppressed at low oxygen pressure (Figures 1 to 5) and is not inhibited by Bcl-X_L (Figure 7), a protein that inhibits pcd or apoptosis in animal cells (Boise et al., 1993).

METHODS

Plant Material and Infection with Pathogens

Fully expanded leaves of 6- to 7-week-old *Nicotiana tabacum* cv Xanthinc NN 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 rubbing the leaves with carborundum, as previously described (Mittler et al., 1995). Plants were kept at 22 to 24°C under continuous illumination provided by cool-white fluorescent lamps (200 μmol m^{-2} sec^{-1}). Sixty hours after inoculation, the leaves were sampled, photographed, and analyzed. The hypersensitive response (HR) to TMV was also induced by infecting tobacco plants at 30°C and shifting of TMV-infected plants to 23°C, as previously described (Mittler and Lam, 1995). The HR to bacteria was induced by infecting leaves of tobacco plants grown at 22 to 24°C with *Pseudomonas syringae* pv *phaseolicola* (NPS3121), as described by Lindgren et al. (1986). Bacteria were also grown in minimal medium, as described by Huynh et al. (1989) and He et al. (1993). As a control for programmed cell death (pcd) induced by bacteria, leaves were infiltrated with a *Hrp*$^-$ derivative of NPS3121 (NPS4000). Plants infected with bacteria were kept at 22 to 24°C under continuous illumination. Twenty-four hours after infection, bacteria were extracted from infected leaves, as previously described (Mittler et al., 1995), and plated, as described by Lindgren et al. (1986). All experiments were repeated at least twice with similar results. Fixation of leaf tissues and examination of mounted sections (10 μm thick) by light microscopy were performed as previously described (Mittler et al., 1995).

Treatment of Tobacco Leaves at Low Oxygen Pressure

Pathogen- or mock-infected leaves were enclosed in gas-tight, temperature-controlled (22°C), transparent chambers (total volume of 1 L), as described by Shulaev et al. (1995); the rest of the plant remained exposed to ambient air. The atmosphere within the chambers was replaced by flushing them with a steady flow (60 mL/min) of ambient air, pure oxygen (high oxygen pressure), or oxygen-free nitrogen (low oxygen pressure). The oxygen level within the chamber was measured with a Perkin-Elmer 8500 gas chromatograph by using a Supelco (Bellefonte, PA) molecular sieve 5A column.

Measurement of Ion Leakage from Leaf Discs

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 604; VWR Scientific, New York, NY).

Field Inversion Gel Electrophoresis

Field inversion gel electrophoresis was performed as described by R. Mittler, L. Simon, and E. Lam (submitted manuscript). Briefly, tissue samples (0.2 g) were ground to a fine powder in liquid nitrogen. The powder was resuspended in 1 mL of 1 × ET buffer (10 mM Tris-HCl, pH 8.0, 50 mM EDTA) preheated to 50°C and mixed with 1 mL of 1.4% (w/v) low-melting-point agarose (Bethesda Research Laboratories) in 1 × ET preheated to 50°C. Agarose plugs were poured in a Bio-Rad standard mold. Plugs were incubated for 3 hr in 10 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 1% sarcosyl, and 1 mg/mL proteinase K (Promega) at 52 to 54°C with gentle agitation. Plugs were then washed with 1 × ET and stored at 4°C in 1 × ET. Field inversion gel electrophoresis was performed with a Bio-Rad CHEF Mapper apparatus, using 1% agarose (Bethesda Research Laboratories) gels and 0.5 × TBE buffer (1 × TBE is 45 mM Tris-borate, 1 mM EDTA, pH 8.0). Electrophoretic separation was performed with a 6 V/cm voltage gradient at an angle of 120° and a linear ramping factor with alternating pulse time of 5.3 to 20.5 sec over the course of 18 hr. After electrophoretic separation, the gels were stained with ethidium bromide and photographed. A λ DNA ladder (Promega) was used to determine the size of DNA fragments.

RNA Isolation and Analysis

Total RNA was isolated as previously described (Mittler et al., 1995) and subjected to RNA gel blot analysis. RNA gel blots were first hybridized with the various PR probes (PR-1a, PR-2, and PR-3), a probe

for alcohol dehydrogenase (Adh), a probe for cytosolic ascorbate peroxidase (APX), or a probe for cytosolic superoxide dismutase (CuZnSOD), and then with a probe for the 18S rRNA (Mittler and Zilinskas, 1994). RNA gel blot hybridization and membrane washing were performed using Duralose-UV membranes and QuickHyb solution (Stratagene), as suggested by the manufacturer. PR-1a, PR-2, and PR-3 transcripts were detected with the corresponding tobacco cDNAs as probes (gift of D. Klessig, Rutgers University), the level of transcripts encoding Adh with a corresponding tomato cDNA as a probe (gift of T. Chase, Rutgers University), the level of transcripts encoding cytosolic APX with a corresponding tobacco cDNA, and the level of transcripts encoding cytosolic CuZnSOD with the corresponding pea cDNA as probes (gift of B. Zilinskas, Rutgers University).

Protein Isolation and Immunodetection

Immunodetection of PR-1a and the TMV coat protein was performed by protein blot analysis of total leaf protein with a chemiluminescence detection system (Renaissance kit; Du Pont). The monclonal anti–PR-1a antibody was a gift of D. Klessig, and the anti-TMV coat protein antiserum was a gift of N. Turner (Rutgers University).

Salicylic Acid Determination

Salicylic acid (SA) was extracted from leaf samples (0.3 g) and quantified by HPLC. Total SA (the sum of free and glucose-conjugated SA) was determined and corrected for SA recovery as previously described (Yalpani et al., 1993).

Construction of Transgenic Tobacco Plants

The cDNA encoding the human Bcl-X_L protein was a gift from C.B. Thompson (University of Chicago). The human Bcl-X_L cDNA was fused to a synthetic Ω translation-enhancing sequence and inserted downstream from the cauliflower mosaic virus 35S promoter (Figure 7A). The 35S-Ω-(Bcl-X_L)-Nos 3′ construct was inserted into a T-DNA pBI100 backbone plasmid (Clontech, Palo Alto, CA) and mobilized into tobacco plants (Samsun NN) via *Agrobacterium tumefaciens*–mediated transformation (Fraley et al., 1985). Individual transformants were screened for the expression level of the transgene by RNA and protein gel blots using the human Bcl-X_L cDNA and anti–human Bcl-X_L antiserum (gift from C.B. Thompson). Fully expanded young leaves of five independent primary transformants were infected with TMV or bacteria, as described above. Plants were kept at 22 to 24°C under continuous illumination (200 μmol m^{-2} sec^{-1}) and analyzed 24 or 48 hr after infection.

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