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Coordinated Activation of Programmed Cell Death and Defense Mechanisms in Transgenic Tobacco Plants Expressing a Bacterial Proton Pump

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In plants, programmed cell death is thought to be activated during the hypersensitive response to certain avirulent pathogens and in the course of several differentiation processes. We describe a transgenic model system that mimics the activation of programmed cell death in higher plants. In this system, expression of a bacterial proton pump in transgenic tobacco plants activates a cell death pathway that may be similar to that triggered by recognition of an incompatible pathogen. Thus, spontaneous lesions that resemble hypersensitive response lesions are formed, multiple defense mechanisms are apparently activated, and systemic resistance is induced in the absence of a pathogen. Interestingly, mutation of a single amino acid in the putative channel of this proton pump renders it inactive with respect to lesion formation and induction of resistance to pathogen challenge. This transgenic model system may provide insights into the mechanisms involved in mediating cell death in higher plants. In addition, it may also be used as a general agronomic tool to enhance disease protection.

INTRODUCTION

Certain developmental pathways as well as some environmental insults result in the activation of a genetically defined programmed cell death (pcd) process (Schwartzman and Cidlowski, 1993). In plants, pcd is thought to be involved in anther, megagametophyte, and vascular tissue development, as well as in sex determination (DeLong et al., 1993; Chasan, 1994; Dietrich et al., 1994). The response of plants to certain avirulent pathogens that evoke a hypersensitive response (HR) is also thought to involve pcd (Dietrich et al., 1994; Greenberg et al., 1994; Lamb, 1994). During the HR, the recognition of a pathogen induces a rapid cell death process that results in the formation of a zone of dead cells around the site of infection. This HR lesion is believed to inhibit further spread of the pathogen and to generate a signal that activates host defense mechanisms and, in many cases, induces long-lasting systemic resistance to a broad spectrum of pathogens (Ross, 1961). Induction of such systemic resistance is termed systemic acquired resistance (SAR) and is accompanied by an increase in the rate of synthesis of several pathogenesis-related (PR) proteins and the accumulation of salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990; Ward et al., 1991). In certain instances, however, at least some of these host defense mechanisms can be activated in the absence of a pathogen. These include a variety of maize, barley, tomato, and Arabidopsis mutants that develop spontaneous lesions similar to lesions caused by a pathogen attack. These mutants are often termed "disease lesion mimics" and are thought to develop lesions that resemble necrotic disease symptoms or HR lesions in the absence of a pathogen (Neuffer and Calvert, 1975; Hoisington et al., 1982; Walbot et al., 1983; Wolter et al., 1993). The occurrence of these mutants, especially mutants that spontaneously develop lesions that resemble HR lesions (HR-type lesions), provides evidence for the involvement of a genetically defined pathway for cell death during the HR. Thus, plants may contain a pathway for cell death that can be spontaneously activated in the absence of a pathogen.

It has been demonstrated recently that in some of the Arabidopsis lesion mimic mutants the appearance of HR-type lesions is also coordinated with activation of host defense mechanisms, such as PR protein induction, phytoalexin production, and accumulation of SA (Dietrich et al., 1994; Greenberg et al., 1994). Interestingly, as many as 60 to 80 different loci specifying a lesion mimic phenotype were identified in maize (Pryor, 1987; Walbot, 1991). Such a large number of different loci may suggest that the spontaneous activation of the HR cell death program is not only the outcome of mutations in a specific pathogen recognition gene but may also be the result of mutations that alter cellular homeostasis. The unbalanced biochemical state induced by such a mutation may be misinterpreted by host cells for a pathogen infection, resulting in the triggering of the HR cell death pathway (Dietrich et al., 1994). Additional support for this model stems from reports

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that the perturbation of the ubiquitin-dependent protein degradation pathway (Bachmair et al., 1990; Becker et al., 1993) and expression of certain transgenes in tobacco plants (Takahashi et al., 1989; Elkind et al., 1990) induce lesion formation. However, in addition to lesion formation, these plants exhibit several other phenotypes, including abnormal or suppressed growth. This may indicate that perturbing cellular homeostasis by the expression of these transgenes and by inhibiting the ubiquitin system may have resulted in a general nonspecific effect on cellular metabolism. In addition, none of the genes responsible for the lesion mimic phenotype has been cloned. Therefore, in contrast to our understanding of pcd in animal cells, very little is known about the molecular events involved in the activation and the progression of pcd in higher plants.

As part of a long-term project aimed at enhancing the photosynthetic capacity of higher plants, we introduced a gene from *Halobacterium halobium* encoding the bacterio-opsin (bO) protein (Krebs and Khorana, 1993) into tobacco (cv Samsun NN). Functioning as a light-driven proton pump that utilizes a light spectrum different from that used by the photosynthetic apparatus of higher plants, the bO proton pump may increase the photosynthetic capacity of these plants when supplied with its chromophore, retinal. In this study, we report that expression of the *bO* gene in transgenic tobacco results in a phenotype that is very similar to that of some of the disease lesion mimic mutants. Lesions resembling HR lesions were formed, and multiple defense mechanisms were activated in the absence of a pathogen. Transgenic plants expressing the *bO* gene also exhibited heightened disease resistance against certain viral and bacterial pathogens. However, in contrast to other transgenes that induce lesions, *bO* expression did not result in abnormal or suppressed growth except in cases of very high overexpression. Therefore, these plants may provide a model system for studying the lesion mimic phenotype and the process of cell death activation in higher plants. We have further characterized the cellular events that accompany cell death in the differentiation of vascular tissue and in transgenically triggered or virus-induced cell death. We demonstrate that these processes in higher plants may involve the degradation of nuclear DNA, which is one of the hallmarks of apoptosis in animal cells (Wyllie et al., 1984).

RESULTS

Expression of the bO Protein in Higher Plants Results in a Lesion Mimic Phenotype

To utilize the *bO* gene for enhancing the photosynthetic properties of tobacco plants, the *bO* gene was fused to the soybean chlorophyll *a/b* binding protein transit peptide (Cab-t) in an attempt to direct the bO protein into the thylakoid membrane (Figure 1A). We generated different families of transgenic tobacco plants that vary in the level of expression of the *Cab-t–bO* fusion gene, presumably due to insertion of the gene into different regions of the plant genome and/or different copy numbers of the transgene (Figure 1B). Transgenic plants expressing the *bO* gene contained detectable levels of the mature bO protein (Figure 1C), indicating that the Cab-t–bO fusion protein was processed to its mature form in plants.

Introduction of the *bO* gene into the tobacco genome produced a phenotype very similar to that of some of the disease lesion mimic mutants (Figures 2A and 2B). Transgenic tobacco plants expressing the *bO* gene developed distinct lesions similar to pathogen-induced HR lesions (HR-type lesions) on leaves

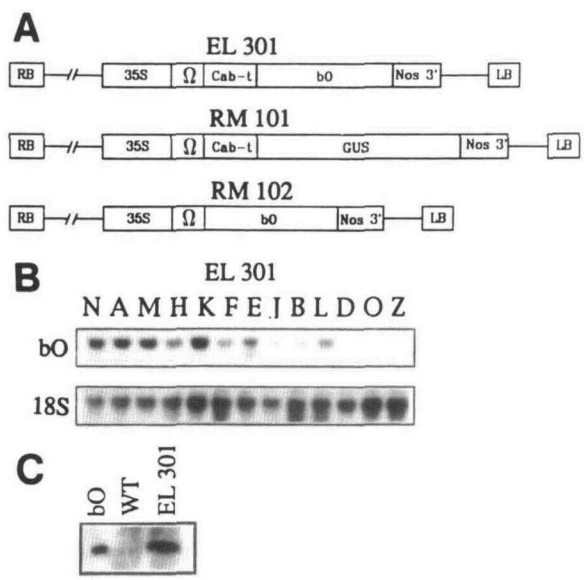

Figure 1. Expression of the *bO* Gene from *H. halobium* in Transgenic Tobacco Plants.

(A) The binary vector T-DNA constructs used for the study of *bO* expression in higher plants. EL 301, RM 101, and RM 102, individual construct names; RB and LB, left and right borders; GUS, β-glucuronidase coding sequence; Cab-t, chlorophyll *a/b* binding protein transit peptide coding sequence; Ω, translation-enhancing sequence; bO, bacterio-opsin coding sequence; Nos 3′, nopaline synthase transcription termination sequence.

(B) Independent transgenic lines with different expression levels of the *bO* transgene. Plants were subjected to RNA gel blot analysis to determine the presence of *bO* transcripts. Letters above the gel indicate independent transgenic lines expressing the *bO* gene. RNA was extracted from leaves of different transgenic plants expressing the *bO* gene. Total RNA (30 μg) was loaded in each lane, and hybridization was conducted with the probes indicated at left (bO and 18S rRNA).

(C) Accumulation of bO protein in transgenic tobacco plants expressing the *bO* gene. Protein from stems of wild-type (WT) and transgenic plants expressing the *bO* gene (WT and EL 301, 200 μg each) and from a suspension of purple membrane from a wild-type strain of *H. halobium* (bO, 0.1 μg) was analyzed for the presence of the bO protein by protein gel blot analysis using the bR-114 monoclonal anti-bO antibody.

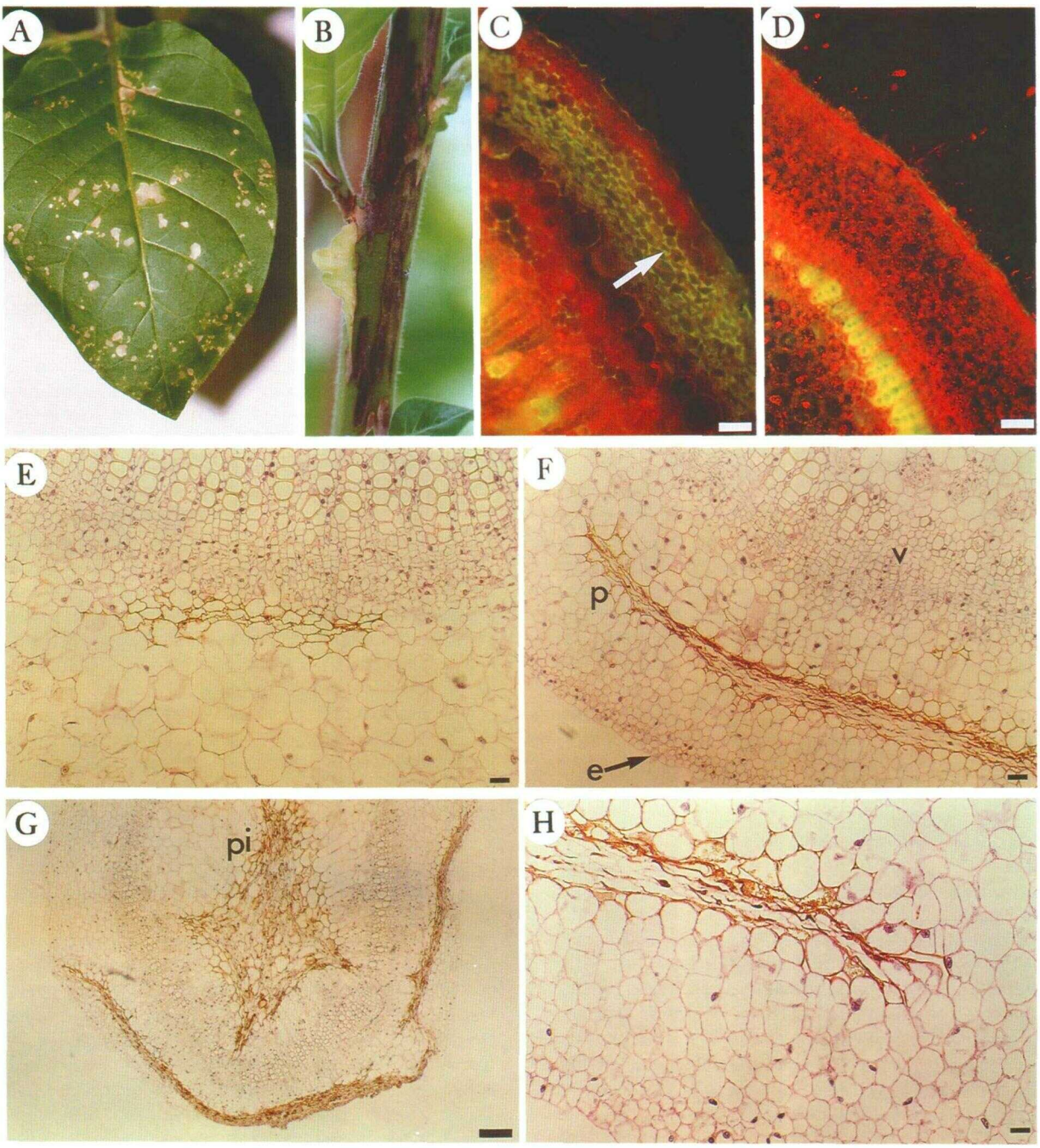

Figure 2. Expression of *bO* in Transgenic Tobacco Plants Induces a Disease Lesion Mimic Phenotype.

(A) and **(B)** Spontaneous lesions formed on leaves **(A)** or stems **(B)** of transgenic tobacco lines with a high level of *bO* expression.

(C) and **(D)** Accumulation of fluorescent material (observed with blue actinic light) in collapsed cells of a stem lesion **(C)** compared with a wild-type stem **(D)**. The collapsed cells are indicated with a white arrow. The red autofluorescence is due to chlorophyll.

(E) to **(G)** The propagation of lesion formation in stem sections. Lesions initiated **(E)** and propagated **(F)** through parenchymatic cell layers, finally resulting in the collapse of epidermis and pith cells **(G)**.

(H) Structural changes that occur in cells during formation of a lesion are shown at the border of a progressing lesion. These include thickening of cell walls, accumulation of granular material, and collapse of cells.

Plant material was prepared and mounted for microscopy as described in Methods.

Bars in **(C)**, **(D)**, and **(G)** = 0.2 mm; bars in **(E)** and **(F)** = 40 μm; bar in **(H)** = 20 μm. e, epidermis; p, parenchymatic cells; pi, pith; v, vascular tissue.

and stems. However, these plants did not exhibit growth or developmental abnormality. The number and size of spontaneous lesions were roughly correlated with the expression level of the *bO* transgene. Plants with high levels of *bO* transcript (plants N, A, M, and K; Figure 1B) developed severe symptoms both in tissue culture and in the greenhouse, moderate expressers (plants H, F, E, and L; Figure 1B) developed few lesions, and low expressers (plants J, B, D, O, and Z; Figure 1B) had no lesions. The formation of spontaneous lesions under aseptic growth conditions indicated that they do not result from pathogen infection.

The occurrence of spontaneous lesions correlated with the developmental stage of the plant. Thus, lesions usually occurred on older leaves and late in the life cycle of the plant. Furthermore, no lesions were found on shoots that were regenerated via tissue culture from lesion-containing leaves. However, regenerated plants did develop lesions later during the life cycle.

As a control, we examined transgenic tobacco plants transformed with a similar vector in which the *bO* gene was replaced with the β-glucuronidase (*GUS*) coding region (Figure 1A). These plants developed no lesions, although they showed very high levels of *GUS* expression. We also tested the *bO* gene with and without the Cab-t peptide in two other cultivars of tobacco (SR1 and Xanthi-nc nn; R. Mittler and E. Lam, unpublished data) and in Arabidopsis (L. Marques and E. Lam, unpublished data). In all cases, the *bO* gene induced spontaneous lesion formation in a similar fashion under aseptic conditions. These results indicate that the bO protein functions in at least two different plant species and does not require the Cab-t peptide for its lesion mimic phenotype. Hence, the action of *bO* in lesion formation may not require the localization of the mature bO protein to the chloroplasts.

Lesions that formed on stems of transgenic tobacco plants expressing the *bO* gene (Figures 2C to 2H) appeared as groups of collapsed cells that accumulated fluorescent material (compare Figures 2C and 2D). Lesions initiated and spread through parenchymatic cell layers (Figures 2E and 2F), resulting in the collapse of the epidermis and in some cases also the pith (Figure 2G). Several structural changes occurred in cells during the progression of lesions. These include accumulation of granular material, thickening of cell walls, and collapse of cells (Figure 2H). Formation of HR lesions in response to a tobacco mosaic virus (TMV) infection displayed similar structural changes. However, lesions usually initiated in cell layers that were located directly beneath the epidermis and/or in the vascular tissue (data not shown).

Formation of Spontaneous Lesions Is Coordinated with Activation of Host Defense Mechanisms

Because the appearance of spontaneous lesions indicated that the introduction of the *bO* gene into the tobacco genome may have activated some general plant defense mechanisms, we examined the expression levels of several PR proteins. Transgenic plants expressing the *bO* gene produced high levels of PR proteins in a constitutive manner both in tissue culture (Figure 3A) and under normal growth conditions (Figures 3B and 3C). Among these, PR-1 is usually synthesized in response to viral attack; PR-2 encodes β-1,3-glucanase, which may serve as an antibacterial/fungal enzyme; and PR-3 encodes the antifungal enzyme chitinase (Linthorst, 1991). Phenylalanine ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway involved in the biosynthesis of phenolic compounds (Bowles, 1990), also appeared to increase in its expression level in transgenic plants expressing the *bO* gene. Interestingly, PR-2 expression was higher in young leaves that did not contain lesions but was repressed in older leaves that contained lesions (Figure 3B). Several PR proteins were found in the intercellular fluid of leaf tissue from *bO*-expressing plants (Figure 3C). The pattern of synthesis of these PR proteins was similar to that found in TMV-infected Samsun NN wild-type plants, supporting the similarity observed between the spontaneous lesions and the TMV-induced lesions. This similarity also suggests that expression of the *bO* gene may trigger the same host defense mechanism that is naturally activated by a TMV infection. Upper leaves of *bO*-expressing plants that contained lesions on their bottom leaves synthesized several putative PR proteins (Figure 3C). These proteins were not found in the upper leaves of mock-infected plants (data not shown) and may be similar to PR proteins that are found in plant tissue undergoing SAR. In addition, as shown in Figure 3D, both the lower (lesion-containing) and upper (no lesions) leaves of *bO*-expressing plants contained very high levels of SA compared with wild-type tobacco. Because SA is thought to mediate the process of SAR in tobacco (Enyedi et al., 1992), this observation is consistent with a constitutive induction of PR proteins in *bO*-expressing plants.

The appearance of spontaneous lesions was accompanied by accumulation of autofluorescent compounds around the lesions and in the cell walls of collapsed cells within the lesions (Figures 4D to 4F). This may be indicative of cross-linked phenolics that serve to strengthen cell walls against an invading pathogen (Dietrich et al., 1994). Accumulation of autofluorescent material did not occur during necrotic cell death in response to wounding by dry ice (Figures 4J to 4L), suggesting that the *bO*-derived lesions were not simply the result of cellular injury but rather appear to be the outcome of activation of a defense mechanism similar to that activated during the formation of HR lesions (Figures 4G to 4I). Thus, introduction of the *bO* gene into the tobacco genome apparently resulted in the spontaneous activation of several components of the plant defense mechanism against an invading pathogen. These included the triggering of a cell death response similar to that activated during HR, induction of several PR proteins, and accumulation of autofluorescent material and high levels of SA. Activation of host defense mechanisms in the absence of a pathogen is similar to the activation of defense mechanisms in some of the lesion mimic mutants of Arabidopsis recently described by Greenberg et al. (1994) and Dietrich et al. (1994).

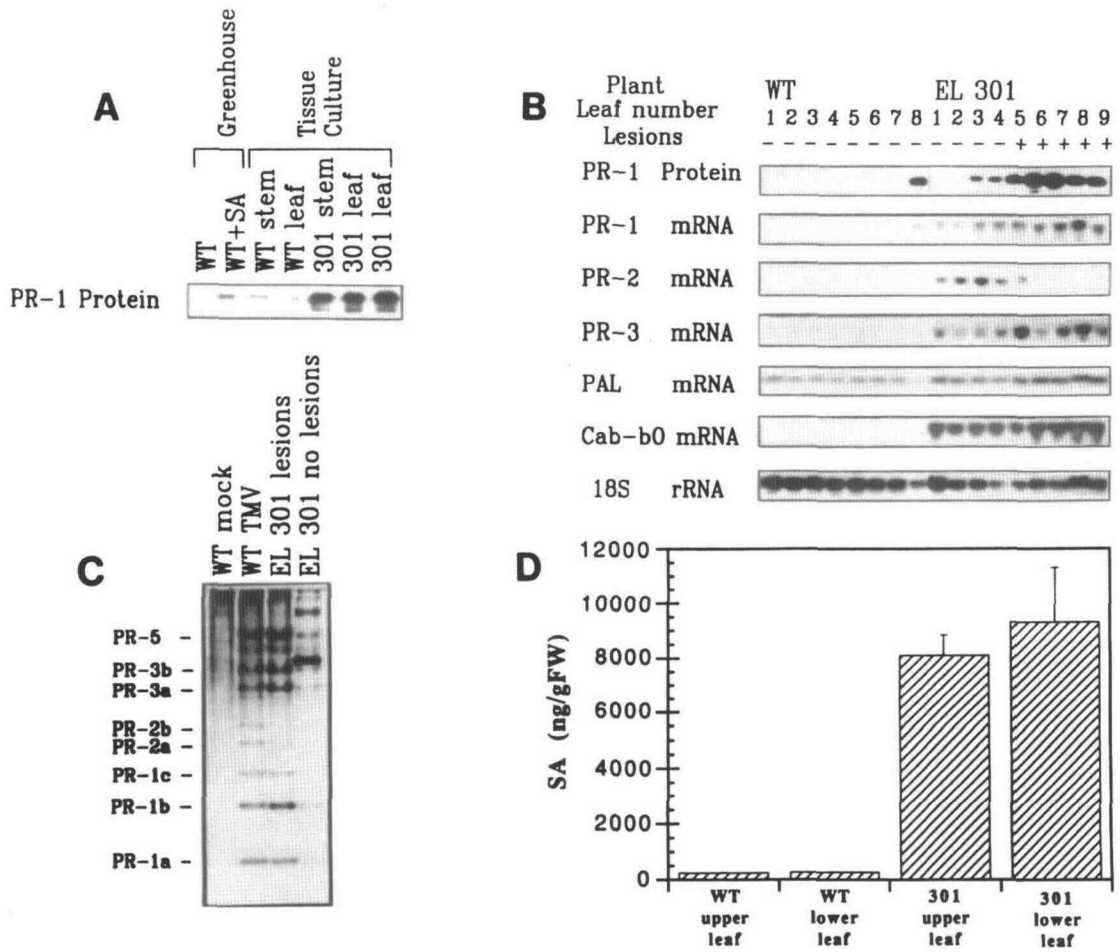

Figure 3. Constitutive Activation of Multiple Defense Mechanisms in Transgenic Tobacco Plants Expressing the *bO* Gene.

(A) Accumulation of the PR-1 protein in the stem (tissue culture, 301 stem) and leaves (tissue culture, 301 leaf) of three individual transgenic plants expressing *bO*. These plants were grown under tissue culture conditions and contained spontaneous lesions. The level of PR-1 protein is compared with that of stem and leaf of a tissue culture–grown wild-type (WT) Samsun plant (tissue culture, WT stem and WT leaf) and with greenhouse-grown (greenhouse, WT) and SA-treated (greenhouse, WT+SA) Samsun control plants. Leaves were collected from tissue culture–grown plants (wild-type Samsun NN and EL 301) and from control, greenhouse-grown Samsun NN plants and greenhouse-grown plants watered with 1 mM SA (48 hr post-SA application). Total protein was analyzed by protein gel blot for the abundance of the PR-1 protein with a monoclonal anti–PR-1 antibody.

(B) Expression of genes encoding PR proteins and PAL in transgenic tobacco expressing the *bO* gene. A comparison was made between the expression of PR proteins and PAL in leaves of a greenhouse-grown wild-type plant (WT, 1 to 8, top to bottom) and a transgenic tobacco plant expressing the *bO* gene (EL 301, 1 to 9, top to bottom). The occurrence of spontaneous lesions on the bottom leaves of the *bO*-expressing transgenic plant is indicated by a (+). Leaves obtained from top (leaf number 1) to bottom (leaf number 8 or 9) of wild-type (WT) and transgenic (R_1 progeny of EL 301 A; see Figure 1B for expression level of the *bO* gene in EL 301 A plants) Samsun NN greenhouse-grown plants were subjected to RNA and protein gel blot analysis. Total RNA (30 μg) was loaded in each lane, and hybridization was conducted with the probes indicated at left. PR-1, PR-2, and PR-3 transcripts were detected with the corresponding tobacco cDNAs as probes. The level of the *PAL* transcript was detected with a corresponding parsley cDNA as a probe. The PR-1 protein was detected via immunoblot analysis with a monoclonal anti–PR-1 antibody.

(C) Occurrence of PR proteins in the intercellular fluid of leaf tissue with spontaneous lesions (EL 301, with lesions) or without lesions (EL 301, no lesions). Leaves were obtained from EL 301 A plants that contained spontaneous lesions. The level of PR proteins is compared with intercellular fluid from mock-infected (WT mock) and TMV-infected (WT TMV) wild-type Samsun NN plants. Intercellular fluid was collected and analyzed according to Lawton et al. (1993).

(D) SA levels in leaves of control wild-type (WT) and transgenic (EL 301 A) plants expressing the *bO* gene. Plant material was collected from upper (no lesions) and lower (with lesions) leaves of 5-week-old *bO*-expressing plants and wild-type plants. Leaf tissue (0.3 g) was analyzed for total SA content as described in Methods. The data presented are the mean and standard deviation of five individual samples. gFW, grams fresh weight of tissue.

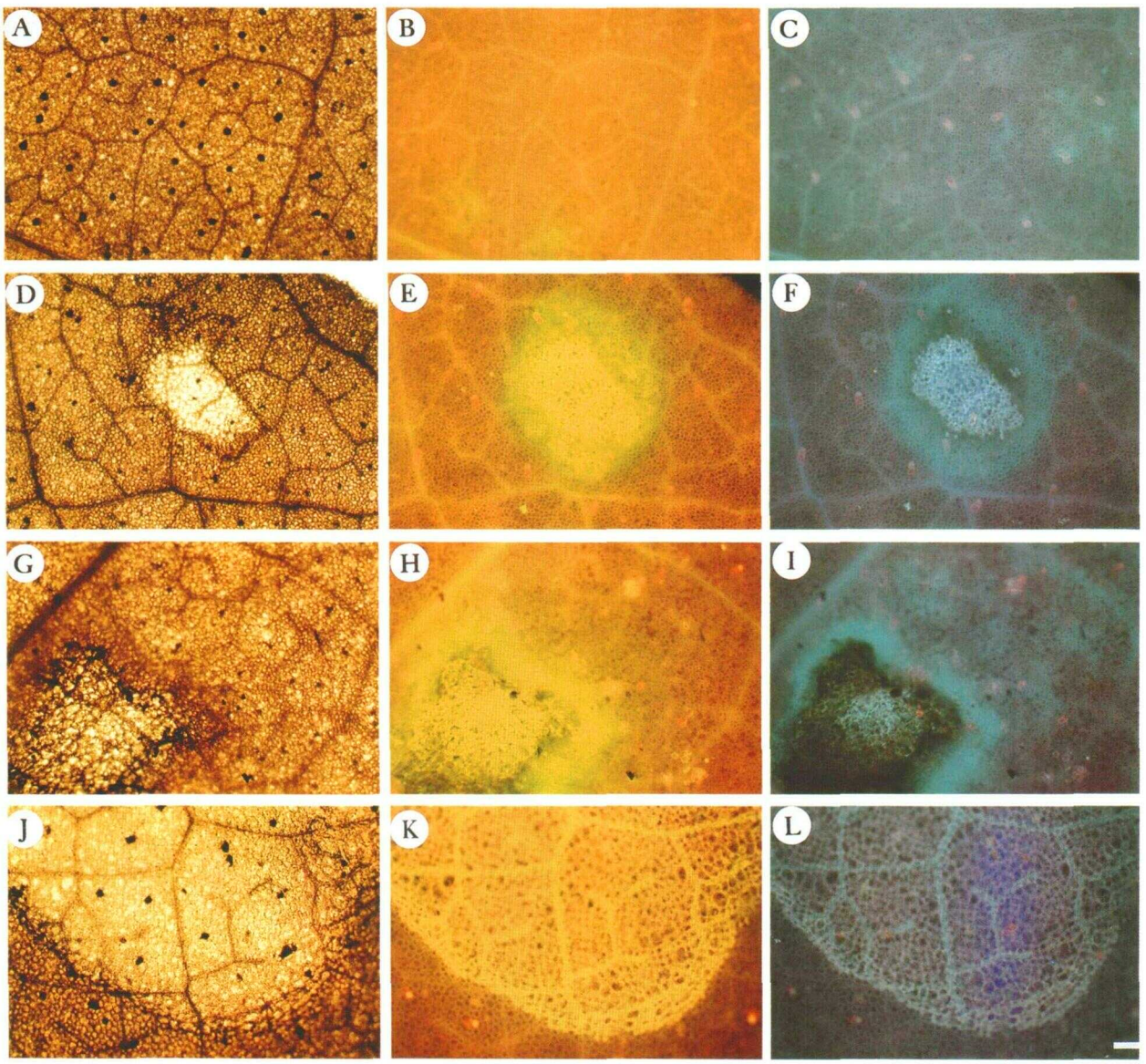

Figure 4. Accumulation of Autofluorescent Material in the Cell Walls of Spontaneously Formed Lesions and TMV- or Injury-Induced Lesions.

(A) to **(C)** Leaves of wild-type plants.
(D) to **(F)** Leaves of transgenic plants expressing the *bO* gene showing a spontaneous lesion.
(G) to **(I)** Leaves of TMV-infected wild-type plants showing a TMV-induced HR lesion.
(J) to **(L)** Leaves of wild-type plants showing a dry ice–induced injury.
Leaves were observed from the top side. Plant tissue was cleared, mounted, and photographed with white-light settings in **(A)**, **(D)**, **(G)**, and **(J)**, UV-light settings in **(B)**, **(E)**, **(H)**, and **(K)**, or blue-light settings in **(C)**, **(F)**, **(I)**, and **(L)**. Conditions for clearing the tissue and epifluorescence microscopy are described in Methods.
Bar in **(L)** = 0.2 mm.

Induction of Systemic Resistance against Viral and Bacterial Pathogens

Several of the lesion mimic mutants exhibited a heightened disease resistance against a variety of pathogens (Wolter et al., 1993; Dietrich et al., 1994; Greenberg et al., 1994). To ascertain whether the constitutive activation of plant defense mechanisms in *bO*-expressing plants confers similar disease protection, we challenged the upper leaves of *bO*-expressing plants with TMV and tobacco necrosis virus (TNV). These

transgenic plants contained lesions on their bottom leaves and high SA levels in their upper and lower leaves and were therefore thought to have acquired systemic resistance in the absence of a pathogen. Transgenic tobacco plants expressing the *bO* gene developed very few HR lesions in response to a TMV challenge (Figures 5A and 5B) and successfully blocked TNV necrotic symptoms (Figures 5C and 5D). These results are very similar to the response of SAR-induced tobacco plants upon subsequent pathogen infection (Ross, 1961; Ward et al., 1991). Thus, the formation of few HR lesions upon TMV challenge and the blocking of TNV necrotic symptoms are indicative of a heightened disease resistance state induced by expression of the *bO* gene. We further tested whether the formation of only a few HR lesions on the infected leaf of *bO*-expressing plants was the outcome of successful inhibition of virus replication. As shown in Figure 6A, *bO*-expressing plants were superior to wild-type plants in their ability to block the replication of TMV in the infected leaf. This inhibition of TMV replication may explain the formation of very few HR lesions on plants with induced systemic resistance. The amount of TMV particles in the infected leaves of these plants may be insufficient to trigger the HR.

We further tested the resistance of *bO*-expressing plants to a bacterial challenge. Transgenic plants expressing the *bO* gene exhibited a higher degree of resistance to the bacterial pathogen *Pseudomonas syringae* pv *tabaci*. As shown in Figures 5E and 6B, plants expressing the *bO* gene successfully blocked both the appearance of disease symptoms and the growth of bacteria.

These results indicate that the heightened disease resistance exhibited by *bO*-expressing tobacco plants is similar to pathogen-induced SAR (Ross, 1961; Ward et al., 1991). This observation suggests that expression of *bO* in higher plants may be misinterpreted by the plant for a pathogen challenge and that the *bO* gene action may be similar to that of some of the mutations that cause the lesion mimic phenotype (Dietrich et al., 1994; Greenberg et al., 1994).

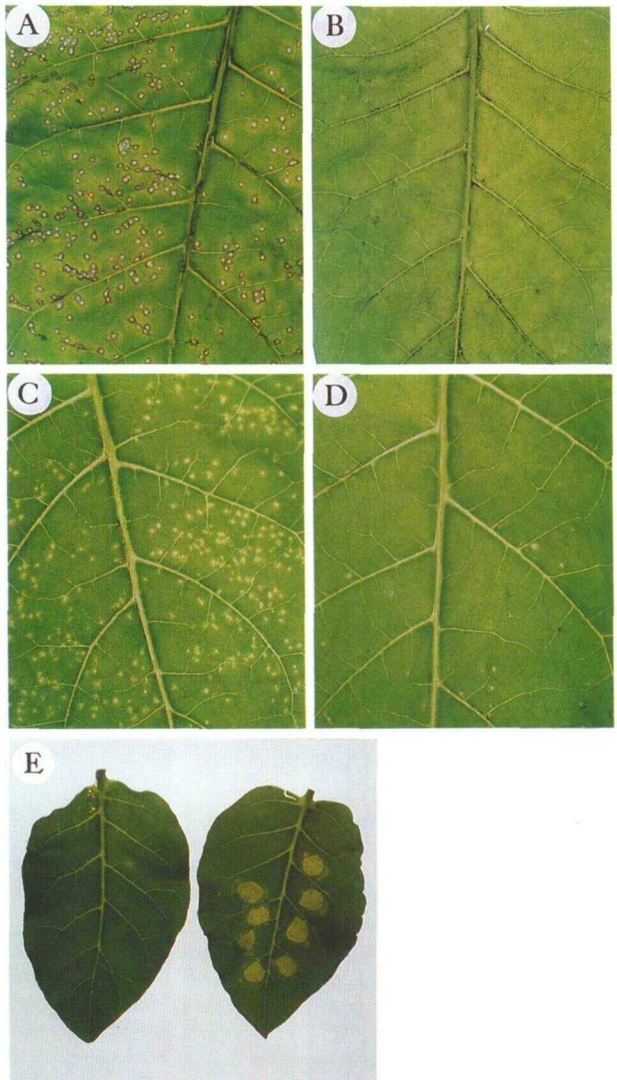

Figure 5. Heightened Disease Resistance of *bO*-Expressing Transgenic Tobacco Plants to Viral and Bacterial Pathogens.

(A) Wild-type tobacco leaf infected with TMV.
(B) Equivalent leaf of a *bO*-expressing (R_1 progeny of EL 301 A) plant infected with TMV.
(C) Control wild-type leaf infected with TNV.
(D) Equivalent leaf of a *bO*-expressing (R_1 progeny of EL 301 A) plant infected with TNV.
(E) *bO*-expressing (left) and wild-type (right) Samsun NN tobacco leaves infected with *P. s. tabaci*. Each leaf was infiltrated with 500 colony-forming units at eight different sites.
Fully expanded young leaves were infected with equal amounts of TMV strain U1 or TNV strain A in 5 mM potassium phosphate buffer, pH 7, or mock infected with the same 5 mM phosphate buffer. Plants were kept under continuous light and photographed 72 hr postinfection. No lesions developed on mock-infected leaves of wild-type and transgenic EL 301 Samsun NN plants. Conditions for infection with *P. s. tabaci* are described in Methods.

Induction of Cell Death Is Inhibited by High Temperatures

In *N* gene–containing cultivars, the formation of TMV-induced lesions and the activation of host defense responses are inhibited at high temperatures (Malamy et al., 1992). Therefore, these TMV-resistant cultivars can be systemically infected by TMV at 30°C. Upon shifting of the infected plants to 22°C, the inhibition of SA accumulation, PR protein gene expression, and activation of HR cell death are removed and massive cell death occurs. To determine whether the formation of spontaneous lesions is also inhibited by high temperatures, we grew transgenic plants expressing the *bO* gene at 30°C together with wild-type and TMV-infected wild-type Samsun NN plants. Formation of both TMV- and *bO*-induced lesions, as measured by increase in membrane leakage, was inhibited by high temperatures (Figure 7A). The inhibition of *bO*-derived lesions was

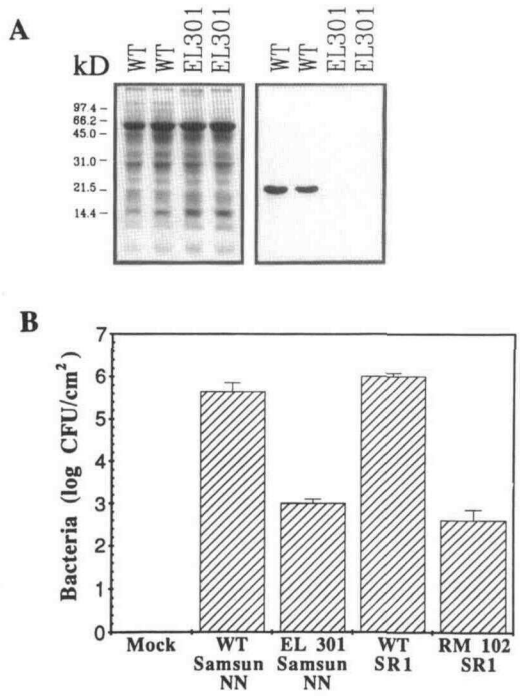

Figure 6. Inhibition of TMV Replication and Growth of Pathogenic Bacteria in Transgenic Plants Expressing the *bO* Gene.

(A) A protein gel (left) and a corresponding immunoblot (right) probed with an anti–TMV coat protein antibody, demonstrating inhibition of TMV replication in the infected leaf of two individual *bO*-expressing plants (EL 301) compared with two wild-type (WT) Samsun NN plants. Total protein taken from TMV-infected leaves 72 hr postinfection of two individual wild-type and transgenic EL 301 (R_1 progenies of EL 301 A) plants was subjected to immunoblot analysis with an anti–TMV coat protein antibody. Molecular mass markers are given at left in kilodaltons.
(B) Inhibition of *P. s. tabaci* growth in leaves of *bO*-expressing Samsun (EL 301) and SR1 (RM 102; see Figure 1 for vector description) plants, indicating an increased disease resistance compared with the corresponding wild-type plants (WT Samsun, WT SR1). No bacterial growth was found in mock-inoculated plants. The data presented are the mean and standard deviation from five individual leaves. Conditions for pathogen infection and analysis are described in Methods. CFU, colony-forming unit.

not the result of a decrease in *bO* expression, because the level of both *bO* transcript (Figure 7B) and bO protein (data not shown) was similar between plants grown at 22 and 30°C. Activation of the cell death process occurred in both transgenic and TMV-infected wild-type plants upon shifting from 30 to 22°C (Figure 7A). Activation of cell death was coordinated with induction of PR-1 and accumulation of SA (Figures 7B and 7C). This coordinated activation of defense mechanisms and cell death upon the temperature shift in both *bO*-expressing and TMV-infected wild-type plants is further evidence that the action of *bO* in transgenic plants may be similar to that of a lesion mimic gene and that both *bO* and the TMV pathogen may activate a similar cell death pathway that is inhibited at high

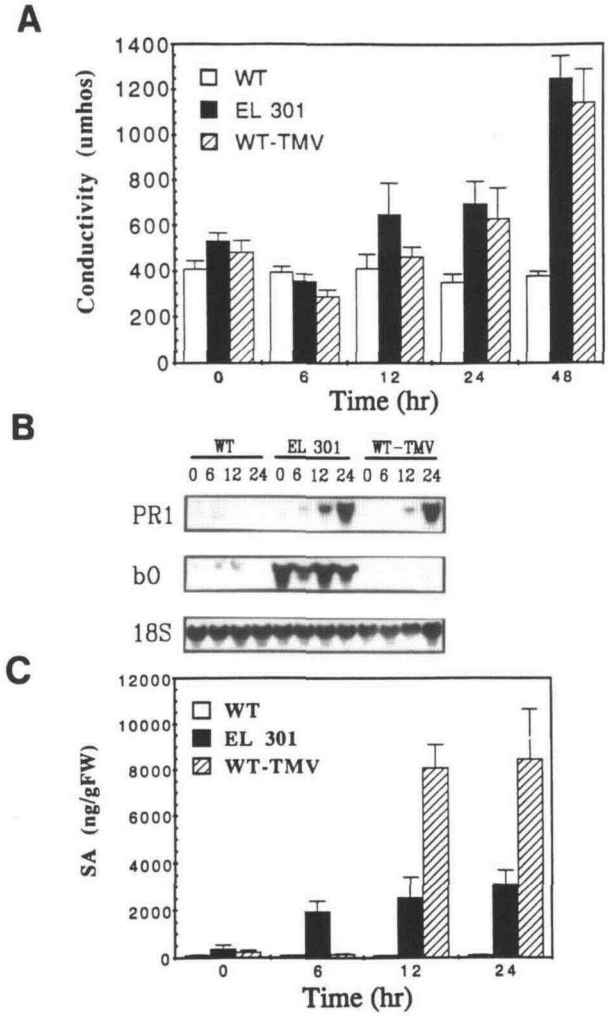

Figure 7. Activation of Cell Death and Defense Mechanisms in Transgenic Plants Expressing *bO* Is Inhibited at High Temperatures.

(A) Formation of lesions during the shift of transgenic *bO*-expressing (EL 301), TMV-infected wild-type (WT-TMV), and wild-type (WT) Samsun NN plants from 30°C (0 hr) to 22°C (6 to 48 hr). Formation of lesions was determined by measuring the leakage of ions from leaf discs as described in Methods. Conductivity is expressed in micromhos (μmhos).
(B) and **(C)** Accumulation of PR-1 transcripts in **(B)** and SA in **(C)** during the temperature shift of transgenic *bO*-expressing (EL 301), TMV-infected wild-type (WT-TMV), and wild-type (WT) plants. gFW, grams fresh weight of tissue.
Fully expanded young leaves were collected and analyzed for SA accumulation and PR-1 induction as previously described (Figure 3). Plants were infected with TMV as previously described (Figure 5) and grown at 30°C for 3 weeks. TMV-infected Samsun NN plants grown at 30°C showed distinct mosaic symptoms indicative of TMV infection. The data presented for ion leakage in **(A)** and SA in **(C)** are the mean and standard deviation of three individual leaves.

temperatures. Interestingly, accumulation of SA preceded lesion formation in *bO*-expressing plants. This finding may indicate that the presence of lesions is not required for the triggering of certain signals for defense response activation, such as SA accumulation. It is interesting to note that inhibition of pcd at high temperatures has also been reported in animal cells (Mailhos et al., 1993).

Detection of Nuclear DNA Degradation during pcd in Higher Plants

Degradation of nuclear DNA is one of the hallmarks of apoptosis. We have therefore utilized an assay based on the detection of 3′ hydroxyl groups of degraded nuclear DNA (Tunel assay; Gavrieli et al., 1992). This assay can be applied for analysis of nuclear DNA degradation in sections of fixed and embedded tissue. As a control for detecting nuclear DNA degradation in higher plants, we studied the development of tracheids during the differentiation of the vascular system in pea roots. During this developmental process, tracheids undergo a sequence of changes in cell morphology and physiology. Procambium-derived xylem elements enlarge and lay down a patterned cell wall in a spiral arrangement. These changes are followed by lignification of cell walls and activation of a developmentally controlled cell death process that involves the loss of the nucleus. Finally, the cell elongates to its mature form (the various morphological changes and activation of pcd in this model system will be described elsewhere). Using the process of tracheid differentiation as a positive control for detection of nuclear DNA degradation in higher plants, we have applied the Tunel assay for the detection of cell death in *bO*- and TMV-induced lesions.

We detected degradation of nuclear DNA in both developing tracheids (Figures 8A to 8C) and in cells located in and around *bO*- or TMV-induced lesions (Figures 8D to 8I). Degradation of nuclear DNA in developing tracheids occurred before elongation of the mature element and coincided with the expected developmental time point for cell death (R. Mittler and E. Lam, unpublished data). Detection of DNA degradation during *bO*-triggered and TMV-induced cell death was performed with tobacco stems obtained from the 24- and 48-hr time points of the temperature shift experiment (Figure 7). During cell death, DNA degradation was detected in cells located in and around lesions that were formed by the activation of the cell death pathway following a temperature shift from 30 to 22°C (Figures 7A and 8E to 8I). Wild-type control tobacco plants (shifted and unshifted, from 30 to 22°C; data not shown), as well as TMV-infected and *bO*-expressing transgenic plants at 30°C (Figures 8D and 8G), exhibited no or very low detectable staining for nuclear DNA degradation. The detection of nuclear DNA degradation during *bO*-induced and TMV-triggered cell death was coordinated with an increase in the activity of several endonucleases (R. Mittler and E. Lam, unpublished data). This observation further suggests that nuclear DNA degradation may be involved during pcd in higher plants. Characterization of the mode of DNA degradation during TMV-induced HR and the regulation of endonuclease activation may shed light on the regulation of this cell death process in plants.

Mutational Analysis of the bO Protein Function in Plants

Bacterio-opsin is a light-driven proton channel that requires the presence of the chromophore retinal to function (Krebs and Khorana, 1993). However, it is not known whether retinal or a closely related molecule is present in higher plants. It is possible that the bO protein, when expressed in plants, functions as an active or a passive proton channel. To address the question of bO protein function and to test if this function may involve some proton-pumping properties of the bO protein, we expressed a mutated form of bO that has a single amino acid substitution (Asp to Ala) at position 85 (D85A; Figure 9A). This amino acid substitution renders the bO protein incapable of active proton pumping due to an alteration in the putative charge-translocating path through the "proton channel" (Otto et al., 1990). Importantly, this mutant preserves the ability to reconstitute with retinal to form a pigment that is very similar to wild-type bacteriorhodopsin. Thus, the protein-folding properties of this mutant are likely not affected significantly (Krebs and Khorana, 1993). Transgenic plants expressing this D85A mutant did not develop spontaneous lesions (data not shown), did not exhibit elevated levels of PR-1 (Figure 9B), and in contrast to *bO*-expressing plants, did not show heightened disease resistance (Figure 9C). However, transgenic plants expressing the D85A mutant did synthesize the mutated bO protein to similar levels as *bO*-expressing plants (data not shown). These results suggest that the bO protein may require some active or passive proton-pumping properties for eliciting its lesion mimic phenotype.

DISCUSSION

Transgenic plants expressing the *bO* gene exhibited many characteristics of a lesion mimic mutant. These include the spontaneous formation of lesions that resemble HR lesions, the activation of host defense mechanisms in the absence of a pathogen, and the induction of heightened disease resistance similar to pathogen-induced SAR (Dietrich et al., 1994; Greenberg et al., 1994). Similar to lesion mimic mutants, the formation of spontaneous lesions in *bO*-expressing plants was developmentally regulated and inhibited at high temperatures (Hoisington et al., 1982; Wolter et al., 1993).

The coordinated inhibition at high temperatures of lesion formation and defense response activation in *bO*-expressing and TMV-infected plants (Figure 7) provides further evidence that the *bO* gene may activate a pathway similar to that activated by an incompatible pathogen. These results also suggest that at least one of the components involved in this pathway

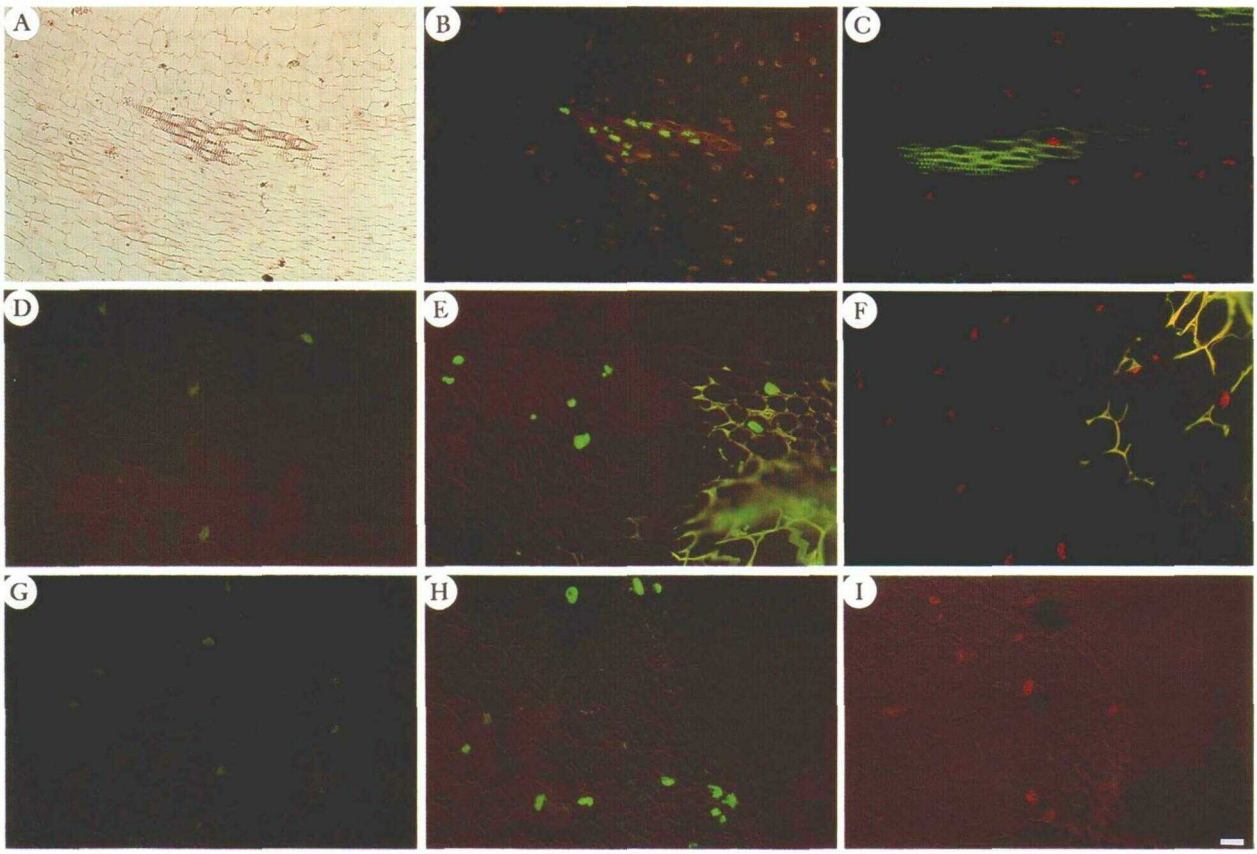

Figure 8. Detection of Nuclei Undergoing Programmed Cell Death in Higher Plants.

(A) to **(C)** Detection of nuclear DNA degradation in developing tracheids. Tracheids undergoing cell death in **(A)** were stained with the ApopTag in situ apoptosis detection kit as shown in **(B)** and as described in Methods. Control sections in **(C)** were stained similarly except for the absence of terminal deoxynucleotidyl transferase.

(D) to **(I)** Detection of nuclear DNA degradation during cell death in *bO*- and TMV-induced lesions. In **(D)** and **(G)**, stem sections of *bO*-expressing **(D)** and TMV-infected **(G)** Samsun NN plants grown at 30°C are shown; the sections show no staining for DNA degradation. In **(E)** and **(H)**, detection of nuclear DNA degradation in stem sections containing *bO*- and TMV-induced lesions, respectively, is shown. Stems were sampled 48 hr after the temperature shift of the plants from 30 to 22°C (see Figure 7A). No nuclear DNA degradation was detected in wild-type plants grown at 30°C or grown at 30°C and shifted to 22°C (data not shown). **(F)** and **(I)** show lesion-containing sections similar to those used for **(E)** and **(H)**, except for the absence of the terminal deoxynucleotidyl transferase enzyme.

Degradation of nuclear DNA is indicated by bright green fluorescence in **(B)**, **(F)**, and **(H)**. Nuclei that do not contain degraded DNA are indicated by red fluorescence (counterstained with propidium iodide). Plant material was fixed, embedded, sectioned, and analyzed for nuclear DNA degradation as described in Methods.

Bar in **(I)** = 30 μm.

is temperature sensitive. It is not likely that this temperature-sensitive component is involved in pathogen recognition (the *N* gene) because spontaneous lesions formed on SR1 plants that do not contain the *N* gene were also inhibited at 30°C (data not shown). In addition, upon temperature shift, lesion formation and defense mechanism activation occurred more rapidly in *bO*-expressing plants than in TMV-infected wild-type plants (Figure 7). This may suggest that in contrast to the host–pathogen system that involves recognition and activation of HR, *bO* expression may involve only activation. Our data suggest that *bO* expression may trigger the plant defense mechanism at a step that is independent of or downstream from the *N* gene product but upstream from a heat-sensitive step that may be involved in the cell death process.

Lesion mimic mutants were classified into two major groups: initiation mutants and feedback or propagation mutants (Walbot et al., 1983; Dietrich et al., 1994; Greenberg et al., 1994). Initiation mutants form lesions whose growth is determinate and therefore result in the formation of lesions with a defined border. These mutants are thought to be defective in regulating the trigger of the HR and may either lack a negative regulator of HR activation (recessive mutations *lsd3* and *lsd5*, see Dietrich

et al., 1994) or constitutively activate an HR signaling component (dominant mutations, Neuffer and Calvert, 1975; *lsd2* and *lsd4*, see Dietrich et al., 1994). Propagation mutants form lesions that spread indeterminately, eventually resulting in the death of the entire leaf. These mutants are presumed to be defective in down-regulating the process of lesion progression (recessive mutations *acd1*, see Greenberg and Ausubel, 1993; *lsd1*, see Dietrich et al., 1994; *acd2*, see Greenberg et al., 1994). Transgenic plants expressing the *bO* gene resemble dominant initiation mutants. The dominant nature of the *bO* transgene also supports the assumption that *bO* action results in the constitutive activation of a pathogen response signal. Because the *bO* gene induced spontaneous lesion formation in several different tobacco cultivars (Samsun NN, Xanthi-nc nn, and SR1; R. Mittler and E. Lam, unpublished data) and in transgenic Arabidopsis plants (L. Marques and E. Lam, unpublished data), it is unlikely that it functions by mimicking the infection of a specific pathogen. The expression of the *bO* gene may therefore affect the activation of the cell death pathway at a relatively basic level that is common to different plants.

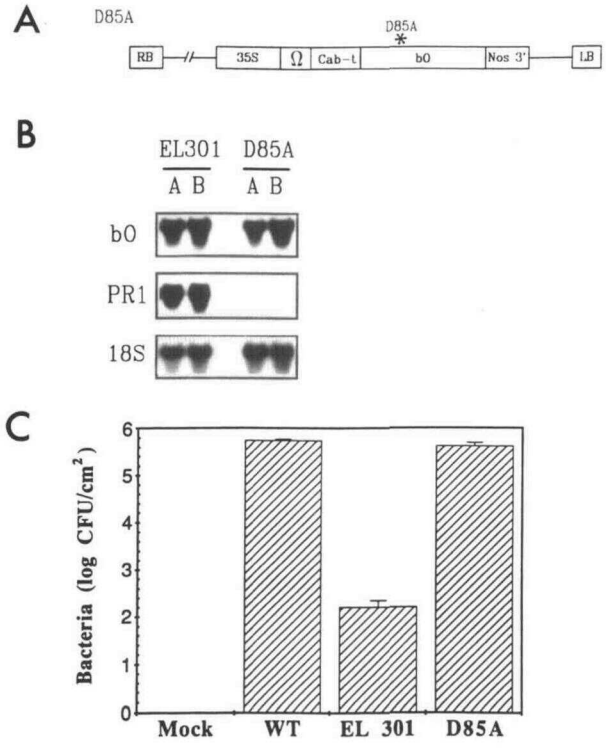

Figure 9. Expression of a Mutated *bO* Gene in Transgenic Tobacco Plants Does Not Cause Spontaneous Lesions and Systemic Resistance.

(A) The binary vector T-DNA construct used for expressing the bO D85A mutant. RB and LB, left and right borders; Cab-t, chlorophyll *a/b* binding protein transit peptide coding sequence; Ω, translation-enhancing sequence; bO, bacterio-opsin coding sequence; Nos 3′, nopaline synthase transcription termination sequence.

(B) In contrast to SR1 plants expressing the *bO* gene (EL 301; lanes A and B represent two independent transgenic SR1 plants), expression of the D85A gene (D85A; lanes A and B represent two independent transgenic SR1 plants) does not result in PR-1 induction.

(C) Expression of D85A does not induce heightened disease resistance to the bacterial pathogen *P. s. tabaci*. The data presented are the mean and standard deviation of four individual leaves. WT, wild type. The level of *bO* and PR-1 transcripts and 18S rRNA was analyzed by RNA gel blots as described for Figure 3. Resistance to *P. s. tabaci* was assayed as described for Figure 6 and in Methods.

Interestingly, ectopic expression of the *bO* gene resulted in the formation of distinct lesions. Formation of distinct lesions was also observed in several of the dominant lesion mimic mutants and in transgenic plants expressing certain transgenes under a constitutive promoter (Walbot et al., 1983; Takahashi et al., 1989; Dietrich et al., 1994). These results may indicate that although a signal for the activation of HR cell death is present in many of the cells of these plants, the activation of the death process is dependent on certain cellular conditions. These may be controlled by the level of certain hormones or may be coordinated by certain cell cycle proteins or other cellular signals. Therefore, at a particular time point, only certain cells in the leaves of these plants may be able to initiate the cell death program and induce the formation of distinct lesions.

It has been previously suggested that some of the dominant lesion initiation mutants perturb cellular homeostasis. The resulting unbalanced biochemical state may be misinterpreted by the host cells as a pathogen infection and trigger the HR. Because bacteriorhodopsin is a light-driven proton pump, its expression in higher plants may result in an artificial increase in proton-pumping activity that may perturb the natural ionic homeostasis of the cell. It is not known, however, whether retinal, which is required for the active pumping of protons by bO, is present in higher plants, although retinal-based photoreceptors were found in unicellular algae (Kreimer, 1994). *H. halobium* cells deficient in retinal biosynthesis accumulate mature bO protein, and the introduction of the *bO* gene into fission yeast does not result in cell death (Hoffmann et al., 1994). These results suggest that, at least in bacteria and fission yeast, stable *bO* expression does not cause leakage of protons. Interestingly, expression of the D85A mutant, which is incapable of active proton pumping, does not result in a lesion mimic phenotype. This finding suggests that the bO protein may require some properties that involve proton translocation across membranes for eliciting its action as a lesion-inducer gene. It may be possible that expression of the *bO* gene in higher plants alters cellular homeostasis through unbalanced translocation of protons. Such perturbation of cellular homeostasis may in turn trigger a cell death pathway. It should be noted that the triggering of the HR in tobacco cells by certain bacterial pathogens requires the activation of a plasma membrane K^+ efflux/net H^+ uptake exchange (Atkinson and Baker, 1989). In addition, the induction of this altered biochemical balance depends on a plasmalemma H^+-pumping ATPase activity. Induction of proton influx is also associated with early events in the elicitation of defense responses by several pathogen elicitors (Felix et al., 1991; Nurnberger et al., 1994). Therefore,

alteration of the proton pumping homeostasis by the *bO* gene may induce similar altered biochemical balance or mimic an early event in the elicitation of defense responses causing the spontaneous triggering of the cell death pathway. Interestingly, dominant mutations at the *mec-4* locus of *Caenorhabditis elegans* result in the activation of cell death and the degradation of touch receptor neurons (the *mec-4* gene encodes a protein with some similarities to ion channels; Driscoll and Chalfie, 1991). Therefore, it appears that in at least some cases perturbing the homeostatic ion/proton balance may result in the activation of cell death.

In animal cells, pcd plays a key role in many developmental pathways and in response to several environmental and hormonal cues. Higher plants seem to utilize cell death in a similar manner for both developmental processes and in response to stress induced by a pathogen challenge. In addition, the activation of some cell death processes in animal cells involves the removal of negative regulators of pcd (Bissonnette et al., 1992). Plants seem to have similar negative regulators for cell death, and the removal of these may explain some of the phenotypes exhibited by the recessive disease lesion mimic mutants (Dietrich et al., 1994; Greenberg et al., 1994). Dominant mutations, such as *mec-4*, were also found to mediate cell death in animal cells. These may function in a manner similar to that of the dominant disease lesion mimic mutants in plants and the *bO* gene in this study.

Apoptosis is a type of pcd that is characterized by a set of distinct morphological changes that includes nuclear DNA degradation and the appearance of apoptotic bodies. In addition, unlike necrotic death that causes swelling of cells, apoptotic death results in the collapse of the dying cell (Schwartzman and Cidlowski, 1993). Here, we demonstrate that the process of cell death in higher plants may have several characteristics of apoptosis, including degradation of nuclear DNA (Figure 8) and collapse of cells (Figure 2).

Because the *bO* gene functions as a lesion inducer in at least two different plant species and because induction of systemic resistance, similar to SAR, is expected to confer disease protection against a variety of viral, fungal, and bacterial pathogens (Lawton et al., 1993), the *bO* gene may be useful as a general agronomic tool to enhance disease protection in different cultivars against a variety of pathogens. Application of the *bO* gene may involve screening for transgenic lines that express the *bO* gene at a moderate level, causing the formation of few spontaneous lesions yet conferring disease protection. Alternatively, inducible expression of the *bO* gene by a regulated promoter may allow controlled activation of the plant defense mechanism, thus minimizing the time for which the defense mechanism will be turned on.

The transgenic *bO* system may also provide insights into the mechanisms involved in the activation of cell death in higher plants. Our results and those of others (Takahashi et al., 1989; Bachmair et al., 1990; Elkind et al., 1990; Dietrich et al., 1994) suggest that perturbation of cellular homeostasis in higher plants may result in the activation of a cell death pathway. Such perturbation may be the outcome of an environmental insult, a pathogen infection, or expression of a particular transgene. It is important, however, to point out that activation of cell death does not always correlate with induction of pathogen resistance. This is demonstrated by Arabidopsis mutants such as *acd1* (Greenberg and Ausubel, 1993). Although the process for cell death activation remains unclear at present, *bO*-expressing tobacco plants may serve as a valuable model system for studying different mechanisms that may be involved in the activation of at least one pcd pathway in higher plants.

METHODS

Construction of Transgenic Plants

The chlorophyll *a/b* transit peptide (*Cab-t*) coding region was amplified by polymerase chain reaction from total genomic soybean DNA and fused to the bacterio-opsin (*bO*) gene (gift of G. Khorana, Massachusetts Institute of Technology, Cambridge, MA; Nassal et al., 1987). The *Cab-t–bO* fusion gene was fused to a synthetic Ω translation-enhancing sequence and inserted downstream from the cauliflower mosaic virus 35S promoter (Figure 1A). The 35S–Ω–*Cab-t–bO–Nos* (nopaline synthase) 3′ construct was inserted into a T-DNA pBI100 backbone plasmid (Clontech, Palo Alto, CA) and mobilized into tobacco (*Nicotiana tabacum* cv Samsun NN) plants via *Agrobacterium tumefaciens*–mediated transformation (Fraley et al., 1985). Individual transformants were screened for the expression level of the *bO* transgene by RNA gel blot analysis.

RNA Isolation and Analysis

Leaves were collected, frozen in liquid nitrogen, and ground to a powder. RNA was extracted as described by Nagy et al. (1987) and subjected to RNA gel blot analysis. RNA gel blots were hybridized first with the *bO*, phenylalanine ammonia-lyase (*PAL*), or the various PR probes (PR-1, PR-2, and PR-3) and then with a probe for the 18S rRNA. RNA blot hybridization and membrane washing were performed using Duralose-UV membranes and QuickHyb solution (Stratagene), as suggested by the manufacturer. PR-1, PR-2, and PR-3 transcripts were detected with the corresponding tobacco cDNAs as probes (gift of D. Klessig, Rutgers University, NJ), and the level of *PAL* transcript was detected with a corresponding parsley cDNA as a probe (gift of K. Hahlbrock, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany).

Protein Isolation and Immunodetection

Immunodetection of PR-1, bO, and tobacco mosaic virus (TMV) coat protein was performed by protein blot analysis of total leaf protein with a chemiluminescence detection system (Renaissance kit; Du Pont). Monclonal anti–PR-1 antibody was a gift of D. Klessig, monoclonal anti–bO antibody was a gift of G. Khorana, and anti–TMV coat protein serum was a gift of N. Turner (Rutgers University, NJ). Detection of PR proteins in the intercellular fluid of *bO*-expressing or TMV-infected tobacco plants was performed according to Lawton et al. (1993).

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).

Pathogen Infection and Analysis

Fully expanded young leaves were infected with equal amounts of TMV strain U1 or tobacco necrotic virus (TNV) strain A in 5 mM potassium phosphate buffer, pH 7, or mock infected with the same 5 mM phosphate buffer by gently rubbing the leaves with carborundum. Plants were kept at 22 or 30°C under continuous light. Lesions were photographed 72 hr postinfection. No lesions developed on mock-infected leaves of wild-type and transgenic *bO*-expressing Samsun NN plants. The progression of lesion formation in *bO*-expressing and TMV-infected plants after a temperature shift from 30°C to 22°C was assayed by measuring ion leakage from leaf discs obtained at different time points after the shift. For each measurement, five leaf discs (7-mm diameter) were floated abaxial side up on 6 mL of distilled water for 4 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). Measurements for each time point were performed in triplicate. Inoculation of plants with *Pseudomonas syringae* pv *tabaci* (ATCC 11528) was performed by infiltrating the leaves with a 1-mL syringe without a needle. Seventy-two hours after infiltration of leaves with bacteria (500 or 15,000 colony-forming units per centimeter in sterile distilled water), the leaves were surface sterilized with 20% bleach–0.1% Tween 20 for 1 min and washed three times with sterile distilled water. Leaf discs (10-mm diameter) were taken from the infection site, ground in sterile distilled water, diluted, and plated on nutrient agar. No bacterial growth was observed with leaf discs obtained from mock-inoculated plants that were infiltrated with sterile water.

Histochemistry and Microscopy

For the detection of autofluorescent material, plant tissue was either sectioned and mounted on slides (untreated) or cleared by boiling the tissue for 2 min in alcoholic lactophenol (95% ethanol–lactophenol, 2:1), rinsing in 50% ethanol, and finally rinsing with water. Autofluorescence was observed with a Nikon EF-D optiphot epifluorescence microscope (Nikon, Tokyo, Japan) using either UV (excitation filter, 330 to 380 nm; dichroic mirror, 400 nm; and barrier filter, 435 nm) or blue (excitation filter, 420 to 490 nm; dichroic mirror, 510 nm; and barrier filter, 520 nm) light settings. For structural studies and detection of nuclear DNA degradation, 1-week-old pea root tips (10-mm long) and 2- to 3-mm-thick tobacco stem sections were fixed in 10% formaldehyde–5% acetic acid–45% ethanol for 3 hr, dehydrated through a graded ethanol series (25, 50, 75, and 100% for 20 min at each step), and incubated overnight in 100% ethanol (alternatively, tissue was stained with 0.5% eosin in 95% ethanol overnight and washed twice, 10 min each, in 100% ethanol). The dehydrated tissue was then taken through a graded xylene series (25, 50, 75, and 100% in ethanol for 1 hr at each step). Finally, the tissue was embedded in paraffin (Paraplast+; Fisher Scientific, Pittsburgh, PA) by a paraffin-graded series (25, 50, 75, and 100%, in xylene, for 3 hr at each step, at 59°C). Tissues were infiltrated in 100% paraffin overnight at 59°C and sectioned on a Reichert-Jung 2040 retractable rotary microtome (Leica, Nussloch, Germany) at a thickness of 5 μm. For structural studies, sections were mounted on slides, deparaffinized, stained with hematoxylin (Fisher Scientific) according to the manufacturer's instructions, and observed by light microscopy. For the detection of nuclear DNA degradation, sections were mounted on ProbeOn+ slides (Fisher Scientific) and incubated at 45°C overnight. Sections were then deparaffinized, washed with PBS buffer, digested with proteinase K (20 μg/mL) for 20 min at 25°C, washed with distilled water, and stained for apoptotic cell death with the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD), using fluorescein-bound anti-digoxigenin-dUTP antibodies. Slides were then counterstained with propidium iodide/antifade (Oncor). Nuclei undergoing apoptotic cell death were observed with a Nikon EF-D optiphot epifluorescence microscope using an excitation filter of 420 to 490 nm, dichroic mirror of 510 nm, and barrier filter of 520 nm.

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