Characterization of Acquired Resistance in Lesion-Mimic Transgenic Potato Expressing Bacterio-Opsin

Mark S. Abad,¹ Salim M. Hakimi,¹ Wojciech K. Kaniewski,¹ Caius M. T. Rommens,¹ Vladimir Shulaev,² Eric Lam,² and Dilip M. Shah¹

¹Monsanto Company, 700 North Chesterfield Village Parkway, Chesterfield, MO 63198, U.S.A.; ²AgBiotech Center, Cook College, Rutgers University, P.O. Box 231, New Brunswick, NJ 08903-0231, U.S.A.

Received 7 January 1997. Accepted 28 March 1997.

The lesion-mimic mutants of certain plants display necrotic lesions resembling those of the hypersensitive response and activate local and systemic defense responses in the absence of pathogens. We have engineered a lesion-mimic phenotype in transgenic Russet Burbank potato plants through constitutive expression of a bacterio-opsin (bO) proton pump derived from *Halobacterium halobium*. Transgenic potato plants exhibiting a lesion-mimic phenotype had increased levels of salicylic acid and overexpressed several pathogenesis-related messenger RNAs, all hallmarks of systemic acquired resistance (SAR). The lesion-mimic plants also displayed enhanced resistance to the US1 isolate (A1 mating type) of a fungal pathogen, *Phytophthora infestans*, a causal agent of late blight disease. In contrast, little resistance was observed against the US8 isolate (A2 mating type) of this pathogen. Furthermore, a majority of the transgenic plants displaying the lesion-mimic phenotype had increased susceptibility to potato virus X. The tubers of these plants were not resistant to the bacterial pathogen *Erwinia carotovora*. These results indicate that expression of bO can result in the activation of defense responses in transgenic potato plants and show for the first time that bO expression can confer resistance to a pathogenic fungus. However, our results also demonstrate that like SAR, this “engineered” resistance is likely to be limited to certain pathogens and particular cultivars.

Plants are resistant to most pathogens, and only a few are capable of interacting compatibly with a plant and causing disease. Most encounters between plants and pathogens result in incompatible interactions attributable to passive, preformed structural barriers and secondary antimicrobial metabolites produced by plants or to active defense responses marshaled against pathogens by plants. Most incompatible interactions are correlated with the formation of necrotic lesions, termed hypersensitive response (HR), in plants at the site of pathogen attack. Although a full mechanistic description of HR is still lacking, it is clear that a number of biochemical changes take

place during HR. These include perturbations in ion fluxes, oxidative burst resulting in accumulation of reactive oxygen species, and biosynthesis of phytoalexins (Goodman and Novacky 1994). Additionally, a number of defense responses are activated locally around the pathogen infection site and distally in uninfected parts of the plant, culminating in a broad-spectrum resistance termed systemic acquired resistance (SAR) against a large number of pathogens (Enyedi et al. 1992; Ryals et al. 1994, 1996). There is mounting interest in harnessing the potential of SAR for broad-spectrum agronomic resistance to pathogens in transgenic crops.

The role of HR in plant defense is currently being investigated through studies on the lesion-mimic mutants of plants, which exhibit HR-like symptoms in the absence of pathogen infection (Dangl et al. 1996). These lesion-mimic mutants exhibit the biochemical markers associated with HR and display constitutive activation of defense genes locally around the lesions and systemically in tissues lacking the lesions. Such mutants of *Arabidopsis thaliana* exhibit resistance to a variety of bacterial and fungal pathogens (Greenberg et al. 1994; Dietrich et al. 1994).

Lesion-mimic phenotypes have also been engineered in plants via expression of a variety of transgenes (for reviews, see Mittler and Lam 1996 and Dangl et al. 1996). In tobacco, a lesion-mimic phenotype was engineered by expressing a *Halobacterium halobium* gene encoding a light-driven bacterio-opsin (bO) proton pump (Mittler et al. 1995). The transgenic plants formed lesions on lower leaves, contained higher systemic levels of salicylic acid (SA), overexpressed several mRNAs encoding pathogenesis-related (PR) proteins, and displayed enhanced disease resistance to two viral pathogens and to the bacterial pathogen *Pseudomonas syringae* pv. *tabaci*. Thus, it is of interest to determine whether bO expression activates lesion-mimic phenotype in important crop plants such as potato and whether it confers resistance to a range of pathogens.

In this study, we engineered a lesion-mimic phenotype in Russet Burbank potato through constitutive expression of the *H. halobium* bO gene. Since Russet Burbank potato is susceptible to a number of bacterial, viral, and fungal pathogens, we wanted to determine whether the bO-mediated lesion-mimic phenotype was correlated with the induction of the expression of pathogenesis-related genes and with the activation of resistance to a variety of pathogens. We report here on

Corresponding author: M. S. Abad;
E-mail: msabad@cmail.monsanto.com

the molecular characterization of the transgene-induced lesion-mimic phenotype in Russet Burbank potato and its resistance response to a variety of pathogens.

RESULTS

Transgenic potato plants expressing bO exhibit a lesion-mimic phenotype.

The bO gene was introduced into Russet Burbank potato under the control of the strong constitutive FMV 35S promoter by *Agrobacterium tumefaciens*-mediated transformation. Transgenic plants that expressed bO mRNA displayed a lesion-mimic phenotype composed of several morphological characteristics (Fig. 1, top right). Lesions appeared in a developmental pattern, first at the tips of the lowest leaves and progressively on higher leaves as the plants grew older, a pattern similar to that reported for transgenic tobacco expressing bO (Mittler et al. 1995). Lesions were also observed on stems and flowers (Fig. 1, bottom) of transgenic plants but not on tubers or roots. There was a range of symptom severity of the lesion-mimic phenotype. Plants from some transgenic lines had many lesions at a very young age, and those from other lines

showed no lesions at all. The lesions varied in size and shape. Thus, the expression of bO caused a significant perturbation in potato resulting in necrotic lesions.

The lesion-mimic phenotype in potato was closely associated with visible signs of stress. Some plants began to develop lesions in tissue culture after transformation, and those that had many lesions did not survive upon transfer to soil. Stunting was evident in transgenic plants grown in soil longer than 1 month and strongly correlated with the severity of the lesion-mimic phenotype. Another sign of stress derived from the lesion-mimic phenotype was excessive chlorosis and necrosis beginning on the lower leaves and progressively appearing on upper leaves as well (Fig. 1, top right). The plant shown in Figure 1, top right, represents an extreme phenotype and had been in soil for 1 month longer than the control plant (Fig. 1, top left). It has only compound leaves because the lower leaves have abscised. bO potato plants at 1 month after transfer to soil had these severe lesion-mimic symptoms only on the lowest leaves. Plants with the most severe phenotype lost most of their leaves by abscission, the lowest leaves falling off first, and died within 1 month. Transgenic plants with a milder lesion-mimic phenotype also lost their lower

Fig. 1. Lesion-mimic phenotype conferred to potato by the bacterio-opsin gene. A Russet Burbank plant (top left) and a bacterio-opsin transgenic plant (top right) were photographed approximately 1 and 2 months, respectively, after transfer to soil in a greenhouse. The stem (bottom left) and flowers (bottom right) of a bacterio-opsin transgenic plant also show development of lesions.

leaves soon after transfer from tissue culture to soil while they were still young. It was clear that the lesion-mimic phenotype in potato had a pleiotropically stressful effect on the physiology of the plants.

The environmental conditions under which the transgenic potato plants were grown influenced the extent of the lesion-mimic phenotype. Plants cultivated in a growth chamber programmed for optimal potato-growing conditions had much fewer lesion-mimic characteristics than plants of the same line grown in a greenhouse under drier and cooler conditions. The type of soil in which the transgenic plants were grown also had an effect on the severity of the phenotype. Lesion formation was less extensive in plants grown in a rich soil than in plants grown in a soil mixture that was less rich and more sandy. Although a small number of transgenic lines had no visible lesions, all of the lines, regardless of the severity of the lesion-mimic phenotype, had an irregular morphology and less vigor than the control lines at 3 months after transfer to soil. The correlation of more stressful growing conditions with lesion severity suggests that there is an inducible component to the extent of the lesion-mimic phenotype.

To investigate the nature of the lesions at the cellular level, we analyzed leaf tissue samples for cell death by staining them with trypan blue and observing them microscopically. Samples from the transgenic potato leaves contained many lesions with organized structures composed of many cells. Cells within developing lesions stained dark blue, and cells surrounding them were brilliantly autofluorescent (Fig. 2). Several variants of these lesions were observed. At one extreme, there were small numbers of cells that stained with trypan blue or were autofluorescent. At the other extreme, there were very well organized lesions, frequently occurring at the edges of leaves, composed of several layers of cells in progressive stages of cell death. The innermost cells of these lesions were stained with trypan blue and had a halo of autofluorescence, whereas outer layers of cells were opaque and encrusted. An intriguing observation was that some lesions were not associated with autofluorescence or trypan blue staining but were otherwise indistinguishable from those that did exhibit these characteristics of cell death. These observations indicate that the bO gene causes a complex lesion-mimic phenotype in potato similar to that described for bO-expressing tobacco plants and that environmental conditions can modulate the severity of this lesion-mimic phenotype.

Several pathogenesis-related genes are upregulated in bO potato plants.

To determine whether the lesion-mimic phenotype in potato is associated with the induction of PR genes, five bO transgenic lines (32, 37, 47, 56, and 66), the empty vector transgenic control line, and a nontransformed control line were chosen for PR gene-expression studies. The bO lines represented a range of lesion-mimic phenotype severity. RNA was prepared from tissue collected from upper leaves of plants grown in rich soil 1 month after transfer from tissue culture. At this age, the lesion-mimic phenotype of plants from lines 32, 37, 56, and 66 was restricted to a few lower leaves, and the tissue sampled for RNA contained no lesions. Conversely, plants of line 47 had many lesions on all leaves. The rank order of lesion severity in these five lines was $47 > 32 > 37 > 56 > 66$. RNA blot analysis with the bO gene as a probe

revealed that all bO lines expressed bO mRNA. Although no band was visible in the autoradiograph for line 66, a very faint hybridization signal was detected when the more sensitive technique of phosphorimager analysis was used. Additional evidence that line 66 contains the bO transgene was obtained by polymerase chain reaction analysis with bO-specific primers (data not shown).

Several PR genes encoding chitinase, glucanase, osmotin-like protein, and anionic peroxidase were analyzed for their expression in bO plants. RNA blots were probed with Ac19 (potato acidic chitinase), OSML (potato osmotin-like protein), GLU-A (tomato glucanase A), GLU-B (tomato glucanase B), and TAP (tomato anionic peroxidase) (Fig. 3). Increased expression of all five PR genes was observed in the bO lines, although the basal level of expression of the different PR genes in control lines varied significantly. Among the bO lines analyzed, line 47, which represents severely lesioned tissue, displayed the highest levels of bO and PR gene transcripts. For example, expression of acidic chitinase, Ac19, which is a reliable marker for SAR in potato (Kombrink et al. 1995), was elevated only three- to sixfold in lines 32, 37, 56, and 66 compared with approximately 48-fold elevation in line 47. Similar patterns of expression were observed for the other PR genes. Thus, the RNA blot analysis showed that the expression of known PR genes was upregulated in bO transgenic potato plants. PR genes were dramatically overexpressed in lesioned tissue. In lesionless leaves of bO transgenic potato plants, the PR genes were upregulated to a lesser extent that was not proportional to the expression level of the bO transgene. Also, different PR genes showed different rank order of expression levels in the lesionless leaves of different bO transgenic lines. The presence of bO mRNA was associated with the overexpression of known PR genes, and the extent of this overexpression correlated with the severity of the lesion-mimic phenotype.

bO potato plants have elevated levels of SA.

SA plays an important role in plant defense responses (Ryals et al. 1994, 1996). To determine whether the upregulated expression of PR genes in lesion-mimic potato was correlated with an increase in the levels of SA, we quantitated the levels of SA in the foliage of bO transgenic plants and control plants (Fig. 4). Leaves without lesions from transgenic plants of lines 32, 37, 56, 66 and from control lines were analyzed. In addition, heavily lesioned lower leaves of bO transgenic plants were analyzed, but to obtain enough mass of lesioned tissue for the analysis, the lower leaves of plants from several lines were pooled to represent this sample (the severity of the lesion-mimic phenotype in this tissue sample was similar to that of line 47 used for RNA analysis and to that of the plant shown in Figure 1, which is from a different line). The levels of free and total SA in plants from line 32 were approximately seven- and fourfold higher, respectively, than the levels in nontransgenic control Russet Burbank plants. Levels in plants from line 37 contained approximately fivefold more free SA and 1.2-fold more total SA than the nontransgenic control. Lines 56 and 66 showed a slight decrease in the levels of both free and total SA compared with control plants. The heavily lesioned foliar tissue contained levels of free SA that were about 60-fold higher and levels of total SA that were 10-fold higher than those of control plants. This experiment indicates

that the bO-mediated activation of PR genes in potato correlates with heightened SA levels in the transgenic tissues expressing higher bO levels. The levels of free and total SA are dramatically increased in lesioned tissues and moderately increased in lesionless systemic tissues in proportion to the severity of the lesion-mimic phenotype in the lower leaves of the bO transgenic potato plants.

bO plants display enhanced resistance to the A1 mating type of *Phytophthora infestans* but not to the A2 mating type.

In order to evaluate the disease resistance potential of the lesion-mimic potato, we challenged transgenic plants with fungal, viral, and bacterial pathogens and scored them for disease symptoms. On the basis of expression analysis of the bO

Fig. 2. Lesions that develop in bacterio-opsin potato show characteristics of programmed cell death. Photomicrographs were made after a foliar tissue sample of a bacterio-opsin transgenic potato was stained with trypan blue and illuminated with white light (top) and with ultraviolet light for autofluorescence detection (bottom).

Fig. 3. Bacterio-opsin transgenic potato plants upregulate the expression of pathogenesis-related mRNAs. Total RNA was isolated from upper and middle leaves of a Russet Burbank nontransgenic control plant (lane 1), an empty vector control plant (lane 2), plants from the bacterio-opsin transgenic lines 32 (lane 3), 37 (lane 4), 56 (lane 5), 66 (lane 6), and 47 (lane 7) and analyzed by Northern hybridization. Total RNA (30 µg) was loaded in each lane. The following hybridization probes were used: bacterio-opsin synthetic gene (bO), potato acidic chitinase II cDNA (Ac19), potato osmotin-like protein cDNA (OSML), tomato acidic glucanase cDNA (GLU-A), tomato basic glucanase cDNA (GLU-B), and tomato anionic peroxidase cDNA (TAP).

gene and of the endogenous PR genes, four lines (32, 37, 56, and 66) were chosen for disease analyses along with a transgenic control line lacking the gene and a nontransgenic control line. For these analyses, we used uniform plants that had grown in soil for 1 month and displayed lesion development only in the lowest leaves. Plants of this stature allowed for detection of fine differences in disease symptoms not only between transgenic plants and controls, but also between transgenic lines differing in their expression levels of bO.

P. infestans is a fungal pathogen of potato that causes late blight disease and exists as two mating types, A1 and A2. We obtained isolate US1 and isolate US8 to represent mating types A1 and A2, respectively, and both were used to challenge the bO lines. For the first analysis, whole-plant disease tests were performed with six uniform plants of each transgenic and control line. These plants were grown in the two soil types mentioned above and spray inoculated with spores of the US1 isolate. bO transgenic plants grown in both soil types showed significantly more resistance to the A1 mating type of *P. infestans* than the control lines when evaluated 5 days postinoculation (dpi) (Table 1). The plants from lines 56

Fig. 4. The levels of salicylic acid (SA) in bacterio-opsin transgenic potato plants are higher than those in nontransgenic control plants. The levels of both free SA (top) and total SA (bottom) are indicated for upper leaves of a nontransgenic Russet Burbank plant (RBID), upper leaves of an empty vector control plant (17227), upper leaves of plants from four different bacterio-opsin transgenic lines (32, 37, 56, and 66), and from pooled severely lesioned lower leaves of several bacterio-opsin transgenic plants of different lines (lesion). Values are the means of three samples, and the standard error is represented by an error bar.

Fig. 5. The phenotype of bacterio-opsin transgenic potatoes resistant to *Phytophthora infestans* US1 genotype. Top: bacterio-opsin transgenic potato plants (left front = line 56, and left back = line 66), empty vector (right front = line 17227), and nontransgenic controls (right back = Russet Burbank) were inoculated with sporangia of *P. infestans* and photographed 10 days postinfection. Bottom: leaves from uninoculated control Russet Burbank plant (upper left); uninoculated bacterio-opsin transgenic plant, line 37 (upper right); *P. infestans*-inoculated control Russet Burbank plant (lower left); and *P. infestans*-inoculated bacterio-opsin transgenic plant, line 37 (lower right) at 5 days postinfection.

and 66 and the control lines grown in the soil mixture were photographed at 10 dpi (Fig. 5, top). The control plants were mostly consumed by this time, whereas the transgenic plants containing the bO gene were much more resistant to this fungal pathogen. In a separate experiment, one transgenic line and a nontransgenic control line were inoculated with spores of the US1 isolate; leaves were detached and photographed 5 dpi (Fig. 5, bottom). The disease progression of the A1 mating type of *P. infestans* on transgenic plants showed very interesting features. Both control plants and bO-expressing lines formed dark lesions initially upon infection. These lesions progressed in control plants to cover most of the leaf area and eventually led to defoliation and destruction of the whole plant. Although the lower leaves of bO plants already contained lesions, the lesion-free upper leaves developed lesions as a result of fungal infection, and these two types of lesions were indistinguishable. Some of the lesions that arose from fungal infection enlarged significantly but did not break out to consume the leaf as they did on controls (Fig. 5, bottom). Thus, fungal infection induced additional lesions in bO plants. Line 32 had the most severe lesion-mimic phenotype and upon infection remained the greenest of all the lines, although many lesions developed on infected leaves. Conversely, line 66 had the weakest lesion-mimic phenotype of all the bO transgenic lines and developed fewer induced lesions upon fungal infection. Line 66 also developed the most extensive chlorosis of all the lines upon infection with *P. infestans* isolate US1. Therefore, although the bioevaluation ranks line 66 as displaying the highest percentage of disease resistance, this ranking is based predominantly on the amount of area covered with lesions. Evaluation of other physiological responses to fungal infection, including development of chlorosis and loss of turgor, suggests that higher bO expression positively correlates with disease resistance. By 10 dpi, the control plants were mostly defoliated, whereas the bO plants were growing new apical shoot tissue free of disease. These results illustrate that the lesion-mimic phenotype in potato is associated with acquired resistance to the US1 isolate of *P. infestans*.

In order to determine whether the lesion-mimic phenotype could bestow protection against the more agronomically devastating US8 isolate of *P. infestans*, the bO transgenic plants were challenged with this pathogen, and disease symptoms were observed. Potato plants infected with the US8 isolate of *P. infestans* showed a very different disease progression than those infected with the US1 isolate. There were no discrete,

punctate lesions like those caused by infection with US1 spores. Instead, a very fast front of infected tissue developed unchecked and devoured the entire leaves within 4 dpi. Both control and bO lines were equally consumed in this short time by infection with the US8 isolate of *P. infestans*. There was no observable difference in the rate or quality of the infection on bO versus control plants; they were equally susceptible. Thus, the bO-induced lesion-mimic phenotype was not associated with acquired resistance to the US8 isolate of *P. infestans*.

In an attempt to observe differences in the progression of fungal infection of bO transgenic potato plants by the two different mating types, infected tissues were microscopically analyzed. Infected tissue was harvested at 24 and 48 h post-inoculation and subjected to the trypan blue staining procedure, which is specific for fungal structures and dead plant cells. Observational analysis was done with a light microscope with both white light and autofluorescing ultraviolet illumination. We observed normal fungal structures, hypersensitive single cell death, autofluorescence upon fungal attack, and lesions of various sizes. No differences were observed between bO transgenic tissue and control tissue infected with either isolate of *P. infestans*. At the cellular level, fungal infection of lesion-mimic tissue is not qualitatively different from that of control potato tissue within a 48-h postinoculation interval.

The bO gene does not impart acquired resistance to potato virus X.

Potato virus X (PVX) was used to challenge lesion-mimic plants to assess whether the bO gene could impart resistance in potato to a viral pathogen. Approximately 20 plants each from the control and transgenic bO lines were inoculated with PVX at a very low inoculum concentration. After 4 weeks, samples were taken from tissue distal from the inoculation site and analyzed for viral antigen by enzyme-linked immunosorbent assay specific for PVX. The disease resistance data is expressed as the ratio of the number of plants per line that showed strong viral titer to the total number of plants challenged (Table 2). Approximately 50% of the inoculated plants from the control lines were found to have high PVX titer. This represents our targeted percentage of infected plants and what is expected when such a low inoculum concentration is used. One-hundred percent of the plants from three of the four bO lines were infected and showed high viral titer. These results

Table 1. Bioevaluation of acquired resistance to *Phytophthora infestans* A1 mating type in lesion-mimic Russet Burbank potato^a

Potato line	Disease control (%)	
	Rich soil ^b	Soil mix ^c
Russet Burbank	0	0
Empty vector control	7	7
Line 32	54	77
Line 37	56	69
Line 56	56	63
Line 66	71	80

^a Six plants of each tested line were infected with the US1 isolate of *P. infestans* and rated 5 days postinfection for late blight disease symptoms.

^b Metromix 350.

^c Two parts Metromix 350, one part Redi-earth, and one part sand.

Table 2. Increased susceptibility of bacterio-opsin transgenic potato plants to potato virus X^a

Plant line	Infected/inoculated	Percentage infected ^b
Russet Burbank	9/19	47
Empty vector	8/20	40
FMV::bO		
Line 32	19/19	100*
Line 37	10/18	56
Line 56	20/20	100*
Line 66	19/19	100*

^a Bacterio-opsin transgenic potato, empty vector, and nontransgenic controls were inoculated with potato virus X and analyzed 1 month later by ELISA for virus in the upper leaves.

^b Asterisks denote values that are significantly different from the percentage of infected Russet Burbank plants as calculated with a chi-square test at 95% probability.

of the frequency of viral infection indicate that the bO gene not only failed to impart acquired resistance to the viral challenge, but actually caused the transgenic plants to have increased susceptibility to PVX.

The bO gene does not induce resistance to *Erwinia carotovora*.

To investigate the ability of the bO gene to confer resistance to tuber soft rot, tubers were challenged with the bacterial pathogen *Erwinia carotovora* pv. *carotovora* and evaluated for disease symptoms as described by Wu et al. (1995). Tuber slices were inoculated with a range of concentrations of the bacterial pathogen. After the slices were incubated for 4 days under humid conditions, the extent of the bacterially infected area was visually assessed, and an arbitrary rating system was used. Although some variability existed in the ratings of the bO tubers, there was no difference in infection between the transgenic tubers and the control tubers (data not shown). This analysis reveals that potato plants containing the bO gene are not resistant to *E. carotovora*.

DISCUSSION

The HR represents a general feature of many incompatible plant-pathogen interactions and is characterized by rapid collapse of tissue surrounding the pathogen infection site (Goodman and Novacky 1994). The HR is manifested in different forms, ranging from single cell death to a spreading necrotic lesion. Although it has been proposed that the HR plays a causal role in disease resistance (Heath 1980), its requirement for restricting pathogen growth is still an open question. There is currently strong interest in deciphering the genetic and molecular mechanisms underlying the HR (Dangl et al. 1996). The research efforts in several laboratories have focused on characterizing the so-called lesion-mimic mutants of plants to unravel the mechanisms underlying the HR and to examine its role in disease resistance. Such mutants have been reported in several plant species such as tomato, *Arabidopsis*, and maize. Some of these mutants in *Arabidopsis* have been reported to activate resistance responses spontaneously, resulting in heightened local and systemic resistance to a variety of pathogens (Greenberg et al. 1994; Dietrich et al. 1994). These mutants likely define genes whose wild-type functions are involved in activating plant defense responses. Several examples of transgenic plants exhibiting lesion-mimic phenotypes have been reported (for reviews, see Mittler and Lam 1996 and Dangl et al. 1996). The expression of certain plant and nonplant transgenes apparently triggers the activation of cell-death pathways resulting from the perturbation of cellular metabolism. The cell-death phenotype is often accompanied by the activation of pathogen-resistance pathways. For example, heterologous expression of a yeast invertase in tobacco has been shown to lead to SAR to potato virus Y along with induction of several PR mRNAs and development of HR-like lesions. It has been proposed that hexose sensing in the secretory pathway is essential for mediating the activation of defense-related genes (Herbers et al. 1996). A similar scenario was described for the ectopic expression of cholera toxin in tobacco. These transgenic plants were also stimulated to express defense-response genes and showed increased resistance to a bacterial pathogen, although in the absence of a lesion-

mimic phenotype (Beffa et al. 1995). It was speculated that the toxin ribosylated a G-protein regulator and caused a change in the cellular homeostasis of the transgenic plants. It is conceivable that the ectopic expression of a proton pump can similarly impact cellular metabolism, since cytosolic protons have been identified as secondary messengers in elicitor-induced defense responses (Mathieu et al. 1994). The fact that these very different types of effectors all induce a similar type of defense response in planta suggests that a defense response may be some kind of default physiological status triggered when the plant cell encounters any of a variety of stresses.

In our study, we examined the possibility of engineering the SAR state in Russet Burbank potato via a lesion-mimic phenotype. We demonstrated that high-level constitutive expression of the prokaryotic-derived bO proton pump (Mittler et al. 1995) leads to the development of a very strong lesion-mimic phenotype in the absence of pathogen infection. Lesions developed on leaves, stems, and flowers but not on tubers or roots. As in tobacco, the bO-induced lesions in potato resembled those in the initiation mutants of *Arabidopsis* (Dietrich et al. 1994) in that the growth of the lesions was determinate. Although the nature of the lesions observed was similar to that of bO-expressing tobacco plants (Mittler et al. 1995), we noticed that the potato plants with the lesion-mimic phenotype were compromised in their growth. Even potato plants expressing low levels of bO apparently showed some stunting compared with the control plants. Similarly, tobacco plants expressing the bO gene from the strong constitutive FMV 35S promoter were also considerably stunted (M. S. Abad, *unpublished observation*), much more so than tobacco expressing the bO gene from the CaMV 35S promoter (Mittler et al. 1995).

As in tobacco, the bO-induced lesion-mimic phenotype in Russet Burbank potato was also associated with the activation of a number of defense mechanisms that are characteristic of SAR (Ryals et al. 1994, 1996). Severely lesioned tissue pooled from four bO potato lines contained dramatically increased levels of both free and total SA compared with control plants. Tissue without lesions from two of the four bO potato lines had elevated levels of both forms of SA. It is a significant observation that all the bO potato lines analyzed displayed increased resistance to *P. infestans* isolate US1 in lesionless tissue, regardless of their SA levels. This indicates that a systemic SA increase is not required for resistance to this fungal pathogen. A similar finding was made in a study in which arachidonic acid was used to induce SAR in potato against an A1 mating type isolate of *P. infestans* (Coquoz et al. 1995). It is interesting to speculate that the signal that leads from bO transgene expression to disease resistance may not pass through SA in potato and that SA accumulation may be an indirect consequence of the lesion-mimic phenotype. Future studies in which a salicylate hydroxylase gene is used in combination with either the bO gene or arachidonic acid may indicate whether SA plays any role in acquired resistance to *P. infestans* in potato.

PR genes encoding an acidic chitinase, acidic and basic glucanases, an osmotin-like protein, and an anionic peroxidase were found to be upregulated in bO transgenic potato tissue. The cDNA probes used for expression analysis in this study represent the protein-coding sequences of known PR genes and are not gene specific. It is important to note that some of these genes or homologously related genes have also been shown to be inducible by abiotic stimuli (Bol et al. 1996; Park

et al. 1992). In particular, regulatory elements of genes encoding an osmotin-like protein and a tomato anionic peroxidase have been shown to convey wound-inducible expression of β -glucuronidase from gene fusions in transgenic plants (Mohan et al. 1993; Zhu et al. 1995). This could mean that additional signaling pathways that induce the expression of other classes of stress genes in addition to PR genes are activated in bO potato plants. Lesion-mimic potatoes also displayed enhanced resistance to the US1 isolate of *P. infestans*. Some of these responses have been documented in a bona fide potato SAR model (Kombrink et al. 1995). For example, resistance to *P. infestans* can be induced in different cultivars of potato after plants are challenged with either *P. infestans* or *Pseudomonas syringae* pv. *maculicola*, and this resistance is correlated with systemic activation of genes encoding acidic and basic forms of potato chitinases and glucanases. The activation of PR genes in bO transgenic potato also correlates with expression of bO and the extent of the lesion-mimic phenotype.

Interestingly, however, the activation of defense mechanisms in the bO plants was not sufficient to afford significant resistance to the more aggressive US8 isolate of *P. infestans*. This illustrates the limitations of the bO-induced SAR pathway to provide broad-spectrum disease resistance in Russet Burbank. This is further supported by the observation that SAR induced in Russet Burbank potato biotically by using *P. syringae* pv. *maculicola* or abiotically by using SA or 2,6-dichloroisonicotinic acid also provided effective resistance to the US1 isolate but not to the US8 isolate of *P. infestans* (C. Rommens, *personal communication*). It is likely that the aggressiveness of the US8 isolate of *P. infestans* overcomes resistance afforded by SAR or that it suppresses the SAR pathway in Russet Burbank potato. Under our experimental conditions, the US8 isolate totally overcame Russet Burbank in 4 dpi compared with approximately 10 dpi for the US1 isolate. The antifungal activities induced in bO potato plants may also be active against the more aggressive US8 isolate but are too low in either abundance or potency to effectively control the disease progression of this fungal pathogen in planta. It is also possible that the US1 isolate is controlled in bO potato by additional factors that are induced upon fungal infection with this isolate but not with the US8 isolate.

The bO potato plants also failed to provide any resistance to PVX. The bO tobacco plants also were not resistant to PVX (data not shown) but were previously shown to have enhanced resistance to the tobacco mosaic and tobacco necrosis viruses. In our experiments with PVX infection of bO potato plants, we made the interesting observation that the bO potato plants were in fact more susceptible to this viral pathogen than the control plants were. A similar relationship between virus susceptibility and defense gene expression has been described in tobacco, although the opposite direction was used to show the relationship in this example (Beffa et al. 1996). Transgenic plants engineered to suppress expression of β -1,3-glucanase via antisense technology were found to be more resistant to the tobacco mosaic and tobacco necrosis viruses. It was suggested that callose degradation by β -1,3-glucanase causes a breach in the barrier of the necrotic lesion to viral spread. However, bO transgenic tobacco plants also have upregulated expression of β -1,3-glucanase (Mittler et al. 1995), indicating different mechanisms of pathogenicity for these different viruses. Since the SAR pathway induced in tobacco by the

ectopic expression of yeast invertase conferred enhanced resistance to potato virus Y as measured by reduced multiplication in systemic leaves (Herbers et al. 1996), it would be interesting to determine whether the bO potato plants would similarly display enhanced resistance to this virus. Also, the bO gene may bestow increased resistance only against viruses that cause necrosis by themselves. It is important to note the absence of reports in the literature describing whether biotically or chemically induced SAR in potato is able to confer resistance to infection by PVX. It is possible that the SAR phenotype in potato activated by such agents would also result in heightened susceptibility of the treated plants to PVX. If not, we may conclude that the bO phenotype includes physiological responses to viral infection different from or in addition to SAR alone. Further work is needed to carefully characterize the general virus-resistance phenotype of potato plants that have been induced to express SAR.

E. carotovora pv. *carotovora*, the bacterial pathogen that causes soft rot in tubers, infects both bO transgenic tubers and control tubers with similar voracity. This could mean that any systemic signaling of acquired resistance in bO plants does not penetrate the tubers. Conversely, any such signal is not effective in tubers against this bacterial pathogen. It is important to mention that we have found no reports of phenotypes resistant or susceptible to this pathogen in potatoes induced for SAR. However, there is one report of SA-induced resistance to this bacterial pathogen in tobacco leaves (Palva et al. 1994), but the physiology of this system is sure to be variant from that of potato tubers.

The limited disease resistance afforded by the bO gene and its deleterious effect on the growth of transgenic potato plants indicate that its utility in engineering agronomically useful broad-spectrum resistance to pathogens of Russet Burbank potato is limited at best. However, this study is the first to demonstrate that an agronomically important fungal pathogen can be controlled by the expression of this gene in planta. This work also demonstrates that another solanaceous plant species besides tobacco is capable of bO-induced cell death that leads to acquired pathogen resistance. Other plant-pathogen systems may be more amenable to the application of this technology in two ways: i) more effective control of the pathogen by the induced defense responses and ii) less stunting and fewer detrimental phenotypic characteristics. In addition to the agronomic applications of this technology, the generation of a dominant lesion-mimic phenotype in Russet Burbank potato may one day serve as a very useful tool for the comparative analysis of programmed cell death and its consequences in potato versus other plant model systems. The induction of a nuclease activity and DNA fragmentation have recently been reported to be concomitant with programmed cell death in bO tobacco plants (Mittler and Lam 1995). The investigation of similar phenomena in potato may shed further light on the various aspects of programmed cell death in plants.

MATERIALS AND METHODS

Construction of the bO expression vector and transformation of potato.

A synthetic gene encoding *H. halobium* bO (Nassal et al. 1987) was used to create an expression vector in which transcription of bO was driven by the figwort mosaic virus promoter

(Richins et al. 1987; Sanger et al. 1990) and curtailed by the nopaline synthase transcription terminator. Polymerase chain reaction was used to amplify the complete bO coding sequence with the primers MSA 180 (5'-GCG CGC GAA TTC ATC GAT AAG CAA ATC CAA AAA ATG CAA GCT CAA ATT ATC GGA CGT CCG GAA TGG ATC TGG CTA GCT CTG GGC-3') and MSA 172 (5'-CGC CG GAA TTC TCA TCA AGA GGT AGC GGC CGC ACC GTC ACC CGC GG-3'), generating a DNA fragment of approximately 800 bp. This fragment was inserted into a binary vector, pMON17227, between the aforementioned genetic elements within the transfer DNA borders. The resulting bO expression vector, pMON23331, contained a glyphosate resistance selectable marker gene (Barry et al. 1992). pMON23331 was introduced into potato (*Solanum tuberosum* cv. Russet Burbank) as described by Newell et al. (1991). The control lines were generated by transforming potato with pMON17227 alone. Five transgenic lines were chosen for subsequent analysis on the basis of the extent of the lesion-mimic phenotype and the expression of the bO transgene at the RNA level. For minituber production, plants were cultivated in a greenhouse at 22°C. All plants used for analyses were generated from vegetative cuttings of axenically grown transgenic Russet Burbank potato plantlets.

Fungal isolates.

Two different isolates of *P. infestans* were used for disease tests. A culture of the US1 isolate, kindly provided by W. Stevenson (University of Wisconsin, Madison), was previously identified as belonging to the A1 mating type. The US8 isolate of this fungal pathogen was collected in August 1995 from a blighted potato field in Washington State. Pure fungal cultures were isolated by placing infected tissue directly under fresh potato tuber slices in sterile petri dishes. Mycelia grew through the tuber slices and sporulated within 7 to 10 days. Sporangia and mycelia were aseptically transferred to fresh media on small agar blocks. Axenic fungal cultures were subsequently maintained on rye A agar medium (Difco, Detroit, MI). The US8 isolate was identified by W. Stevenson as mating type A2. Cellulose acetate electrophoresis analysis of phosphoglucose isomerase allozymes of the US8 isolate indicated the closest match to the *P. infestans* strain ME93-2A.

Fungal inoculation of plants and disease assessment.

Sporangia of *P. infestans* were suspended in cold (4°C) deionized distilled water at approximately 10⁴ sporangia per milliliter, and potato leaf surfaces were inoculated with these suspensions with a spray gun (model EGA-502, DeVilbiss, Toledo, OH). Each challenged potato line was represented by six plants that were grown in soil for 4 to 5 weeks and had three to four shoots with five to seven leaves per shoot. Inoculated plants were placed in a humid growth chamber at 17°C in the dark for approximately 40 h to ensure infection and then transferred to a growth chamber at 18°C for development of late blight symptoms. Disease severity was assessed at 4 and 7 dpi by estimating the area of true leaf tissue covered by late blight symptoms.

Inoculation with and immunodetection of PVX.

PVX (Berk 1970) used for resistance experiments was a field isolate from Washington State. Russet Burbank control and transgenic plants were inoculated with virus at 5 µg/ml

and assayed for susceptibility according to previously described protocols (Hemenway et al. 1988, Lawson et al. 1990). Systemically infected plants that reported values of virus titer in excess of 100 ng per ml of extract were rated positive.

E. carotovora soft rot susceptibility assay.

Minitubers of transgenic and control Russet Burbank plants were sliced and inoculated with suspensions of *E. carotovora* pv. *carotovora* as described by Lapwood et al. (1984) and visually evaluated for disease progression.

Trypan blue staining and autofluorescence analysis.

Potato tissue samples were harvested before and after infection with *P. infestans* and stained with trypan blue to microscopically visualize programmed cell death as described previously (Keogh et al. 1980). Photomicrographs were taken at 400× illuminated with either white light to visualize trypan blue staining or with ultraviolet light for observations of autofluorescence.

RNA analysis and derivation of hybridization probes.

Potato tissue samples were collected from plants that had grown in soil for 1 month after transfer from tissue culture. Total RNA was extracted from 2 g of foliar tissue per sample with Trizol reagent (Gibco-BRL, Bethesda, MD) according to the vendor's instructions. Northern analysis was performed according to previously described protocols (Sambrook et al. 1989). In addition to autoradiographs, radioactive membranes were analyzed with a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). The bO coding sequence from pSBO (Nassal et al. 1987) was used in Northern hybridization as a probe for the transgene. Potato acidic chitinase was provided by E. Kombrink (Kombrink et al. 1995). Tomato acidic glucanase and tomato basic glucanase were provided by J. van Kan (van Kan et al. 1992). Tomato anionic peroxidase cDNA was provided by P. E. Kolattukudy (Roberts and Kolattukudy et al. 1989). A Russet Burbank version of osmotin-like protein coding sequence was generated by reverse transcription polymerase chain reaction from RNA of arachidonic acid-treated plants and oligonucleotide primers based on a published sequence from *Solanum commersonii* (Zhu et al. 1995).

SA determination.

Both free and total SA levels of control and transgenic bO potato were quantified by high-performance liquid chromatography from 0.5-g leaf tissue samples. The protocol for extraction and determination of SA, with correction for SA recovery, was previously described (Yalpani et al. 1993).

ACKNOWLEDGMENTS

The horticultural support from Glenda DeBrecht was instrumental in generating uniform potato plants and soil formulations. Stephanie Castro provided excellent support for *Phytophthora infestans* maintenance and infection of potato plants. The Ceregen potato transformation team performed the *Agrobacterium*-mediated transformation and tissue culture maintenance of Russet Burbank. Ilya Raskin provided support for the SA determination experiments. Jerry Anderson provided excellent technical assistance for the ELISA detection of PVX. Kathy Swords shared unpublished data on *Phytophthora infestans*-potato interactions. Barbara A. Rodriguez-Thomas verified the bO expression plasmid constructs. Jeffery L. Dangl shared a review manuscript before publication. We thank Kenneth Barton for critically reading the manuscript.

LITERATURE CITED

- Barry, G., Kishore, G., Padgett, S., Kolacz, K., Re, D., Weldon, M., Taylor, M., Parker, G., Eichholtz, D., Hayford, M., Klee, H., Fincher, K., Rozman, R., Didier, D., Dellannay, X., DeBoer, D., and Hallas, L. 1992. Commercialization of glyphosate herbicide resistant crops for improved weed control. (Abstr.) J. Cell. Biochem. 16(suppl. F):202.
- Beffa, R., Szell, M., Meuwly, P., Pay, A., Vogeli-Lange, R., Metraux, J.-P., Neuhaus, G., Meins, F., Jr., and Nagy, F. 1995. Cholera toxin elevates pathogen resistance and induces pathogenesis-related gene expression in tobacco. EMBO J. 14:5753-5761.
- Beffa, R. S., Hofer, R.-M., Thomas, M., and Meins, F., Jr. 1996. Decreased susceptibility to viral disease of β -1,3-glucanase-deficient plants generated by antisense transformation. Plant Cell 8:1001-1011.
- Berks, R. 1970. Potato virus X. No. 4 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
- Bol, J. F., Buchel, A. S., Knoester, M., Baladin, T., Van Loon, L. C., and Linthorst, H. J. M. 1996. Regulation of the expression of plant defence genes. Plant Growth Regul. 18:87-91.
- Coquoz, J.-L., Buchala, A. J., Meuwly, Ph., and Métraux, J.-P. 1995. Arachidonic acid induces local but not systemic synthesis of salicylic acid and confers systemic resistance in potato plants to *Phytophthora infestans* and *Alternaria solani*. Phytopathology 85:1219-1224.
- Dangl, J. L., Dietrich, R. A., and Richberg, M. H. 1996. Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell 8:1793-1807.
- Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryals, J. A., and Dangl, J. L. 1994. *Arabidopsis* mutants simulating disease resistance response. Cell 77:565-577.
- Enyedi, A. J., Yalpani, N., Silverman, P., and Raskin, I. 1992. Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc. Natl. Acad. Sci. USA 89:2480-2484.
- Goodman, R. N., and Novacky, A. J. 1994. The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. American Phytopathological Society, St. Paul, MN.
- Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. 1994. Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense functions. Cell 77:551-563.
- Heath, M. C. 1980. Reaction of nonsusceptible to fungal pathogens. Annu. Rev. Phytopathol. 18:211-236.
- Hemenway, C., Fang, R.-X., Kaniewski, W., Chua, N.-H., and Tumer, N. 1988. Analysis of the mechanism of protection in transgenic plants expressing the PVX coat protein gene. Page 281 in: J. Cell. Biochem. Suppl. 12C.
- Herbers, K., Meuwly, P., Frommer, W. B., Metraux, J.-P., and Sonnewald, U. 1996. Systemic acquired resistance mediated by the ectopic expression of invertase: Possible hexose sensing in the secretory pathway. Plant Cell 8:793-803.
- Keogh, R. C., Deverall, B. J., and McLeod, S. 1980. Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. Trans. Br. Mycol. Soc. 74:329-333.
- Kombrink, E., Büchter, R., Wegener, S., and Scheel, D. 1995. Systemic acquired resistance in potato. Pages 483-491 in: Modern Fungicides and Antifungal Compounds. Proc. Int. Symp., 11th. H. Lyr, P. E. Russell, and H. D. Sisler, eds. Intercept, Andover, England.
- Lapwood, D. P., Read, P. J., and Spokes, J. 1984. Methods for assessing the susceptibility of potato tubers of different cultivars to rotting by *Erwinia carotovora* subspecies *atroseptica* and *carotovora*. Plant Pathol. 33:13-20.
- Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technology 8:127-134.
- Mathieu, Y., Jouanneau, J.-P., Thomine, S., Lapous, D., and Guern, J. 1994. Cytosolic protons as secondary messengers in elicitor-induced defence responses. Biochem. Soc. Symp. 60:113-130.
- Mittler, R., and Lam, E. 1995. Identification, characterization, and purification of a tobacco endonuclease activity induced upon hypersensitive response cell death. Plant Cell 7:1951-1962.
- Mittler, R., and Lam, E. 1996. Sacrifice in the face of foes: Pathogen-induced programmed cell death in plants. Trends Microbiol. 4:10-15.
- Mittler, R., Shulaev, V., and Lam, E. 1995. Coordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. Plant Cell 7:29-42.
- Mohan, R., Bajar, A. M., and Kolattukudy, P. E. 1993. Induction of a tomato anionic peroxidase gene *TAP1* by wounding in transgenic tobacco and activation of *TAP1*-GUS and *TAP2*-GUS chimeric gene fusions in transgenic tobacco by wounding and pathogen attack. Plant Mol. Biol. 21:341-354.
- Nassal, M., Mogi, T., Karnik, S. S., and Khorana, H. G. 1987. Structure-function studies on bacteriorhodopsin. III. Total synthesis of a gene for bacterio-opsin and its expression in *Escherichia coli*. J. Biol. Chem. 262:9264-9270.
- Newell, C. A., Rozman, R., Hinchey, M. A., Lawson, E. C., Haley, L., Sanders, P., Kaniewski, W., Tumer, N. E., Horsch, R. B., and Fraley, R. T. 1991. *Agrobacterium* mediated transformation of *Solanum tuberosum* L. cv. 'Russet Burbank'. Plant Cell Rep. 10:30-34.
- Palva, T. K., Hurtig, M., Saindrenan, P., and Palva, E. T. 1994. Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. Mol. Plant-Microbe Interact. 7:356-363.
- Park, R.-D., Lee, C.-M., and Jhon, D.-Y. 1992. Differential induction of chitinase isozymes in bean leaves under stress conditions. Korean Biochem. J. 25:101-106.
- Richins, R. D., Scholthof, H. B., and Shepard, R. J. 1987. Sequence of the figwort mosaic virus DNA (Caulimovirus group). Nucleic Acids Res. 15:8451-8466.
- Roberts, E., and Kolattukudy, P. E. 1989. Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated highly anionic peroxidase. Mol. Gen. Genet. 217:223-232.
- Ryals, J., Uknes, S., and Ward, E. 1994. Systemic acquired resistance. Plant Physiol. 104:1109-1112.
- Ryals, J., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y., and Hunt, M. D. 1996. Systemic acquired resistance. Plant Cell. 8:1809-1819.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sanger, M., Daubert, S., and Goodman, R. M. 1990. Characterization of a strong promoter from figwort mosaic virus: Comparison with the analogous 35S promoter from cauliflower mosaic virus and the regulated mannopine synthase promoter. Plant Mol. Biol. 14:433-443.
- van Kan, J. A. L., Joosten, M. H. A. J., Wagemakers, C. A. M., van den Berg-Velthuis, G. C. M., and de Wit, P. J. G. M. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol. Biol. 20:513-527.
- Wu, G., Shortt, B. J., Lawrence, E. B., Levine, E. B., Fitzsimmons, K. C., and Shah, D. M. 1995. Disease resistance conferred by expression of a gene encoding H_2O_2 -generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357-1368.
- Yalpani, N., Shulaev, V., and Raskin, I. 1993. Endogenous salicylic acid levels correlate with accumulation of pathogenesis-related proteins and virus resistance in tobacco. Phytopathology 83:702-708.
- Zhu, B., Chen, T. H. H., and Li, P. H. 1995. Activation of two osmotin-like protein genes by abiotic stimuli and fungal pathogen in transgenic potato plants. Plant Physiol. 108:929-937.