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Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a

(blue mold disease/black shank disease/*Peronospora tabacina/Phytophthora parasitica*/systemic acquired resistance)

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ABSTRACT **Expression of pathogenesis-related protein 1a (PR-1a), a protein of unknown biochemical function, is induced to high levels in tobacco in response to pathogen infection. The induction of PR-1a expression is tightly correlated with the onset of systemic acquired resistance (SAR), a defense response effective against a variety of fungal, viral, and bacterial pathogens. While PR-1a has been postulated to be involved in SAR, and is the most highly expressed of the PR proteins, evidence for its role is lacking. In this report, we demonstrate that constitutive high-level expression of PR-1a in transgenic tobacco results in tolerance to infection by two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae*.**

Plants resist pathogen infection and associated disease development by a variety of mechanisms. Some of the best-characterized resistances work only against individual isolates of particular pathogens and exhibit considerable genetic diversity within a species (1). Other mechanisms are inducible and act more generally against a variety of pathogens (2). Systemic acquired resistance (SAR) is an induced defense response triggered by infection with a necrosis-causing pathogen (3) or by organic compounds such as salicylic acid (4) and 2,6-dichloroisonicotinic acid (5).

Establishment of SAR is correlated with the coordinate expression of nine gene families coding for proteins whose possible causal role in resistance has attracted considerable recent study (6, 7). Several of these so-called pathogenesis-related (PR) proteins possess anti-fungal activities *in vitro* and biochemical activity as chitinases, glucanases, or permatins (8–12). However, an *in vivo* role in disease resistance has not been demonstrated for any of the PR proteins.

PR-1, the most abundant of the PR proteins in tobacco, is induced ≈10,000-fold in infected tissue and accumulates to 1–2% of the total leaf protein (J.R., unpublished data), suggesting an active role for the protein in the disease response. Nevertheless, the protein has no known biochemical function and has not been shown to possess *in vitro* antimicrobial activity. Several investigators have expressed the PR-1 cDNA in transgenic plants, but no resistance was found against tobacco mosaic virus or alfalfa mosaic virus (13, 14). We have extended the study of PR-1 in transgenic plants by testing its activity toward several additional pathogens, including fungi, a bacterium, and another virus. Here we demonstrate that transgenic plants constitutively expressing PR-1a exhibited significant tolerance to two pathogenic oomycete fungi, thus providing evidence of a function for PR-1.

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MATERIALS AND METHODS

Transgenic Plants. A cDNA clone containing the entire coding region of the PR-1 gene (bp 916–1711) (15) was subcloned into the *Eco*RI site of pCGN1761 (16), a plant promoter/terminator cassette, between the enhanced 35S promoter and the *tml* 3′ terminator. The cassette containing the cDNA in a sense orientation was then subcloned into the *Xba* I site of the binary vector pCGN1540 (16, 17), resulting in plasmids pCGN1774A and pCGN1774C, which differ from each other with respect to the orientation of the *Xba* I fragment relative to the right and left T-DNA borders (see Fig. 1). One PR-1a construction, pCGN1764C, differs from the pCGN1774 series only by virtue of the binary vector used, pCGN783 (18). Chimeric gene constructions expressing several other PR protein cDNAs were generated in a similar manner, from essentially full-length cDNAs cloned into the pCGN1761 expression cassette, followed by insertion into binary plasmid pCGN1540 (see Table 1 for genes and references). The plasmids were transformed into *Agrobacterium tumefaciens* strain A136 (19), harboring a disarmed helper *vir* plasmid derived from pTiBo542 (20).

Leaf disks of *Nicotiana tabacum* cv. Xanthi nc were infected with the *A. tumefaciens* strain and selected for callus growth on kanamycin essentially as described (21). A single shoot (designated the T_1 generation) was regenerated from each leaf disk and grown in soil until seed set. Seed (T_2 generation) resulting from self-pollination of the regenerated transformants was scored for antibiotic resistance on MS medium (22) containing 150 mg of kanamycin per liter. T_2 seed populations expressing high levels of the engineered protein and that exhibited 3:1 (resistant/susceptible) segregation for the kanamycin marker were chosen for further analysis. Lines homozygous for the transgene were then identified by allowing 10 kanamycin-resistant T_2 progeny from each independent transformant to self-pollinate and set seed and by screening for plants whose seed (T_3 generation) were 100% kanamycin resistant. Only T_3 homozygous lines exhibiting a single active kanamycin-resistance locus, or F_1 lines generated from such parents (see Table 1), were used in the disease assays. None of the lines used in this study showed growth or morphological abnormalities.

Abbreviations: SAR, systemic acquired resistance; PR-1a, pathogenesis-related protein 1a.

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Blue Mold Disease Assay. *Peronospora tabacina* assays were performed on 6-week-old plants grown in a 1:1 soil/humus mixture (sterilized by autoclaving) in individual 4-inch pots. A spore suspension, stored frozen in small aliquots, was used for all assays. Spores were thawed, diluted to a concentration of 300 spores per ml, and sprayed on the upper surface of leaves. Inoculated plants were incubated in Plexiglas boxes at 20°C and 100% humidity. Incubation was in darkness for the first 24 hr of incubation, followed by a regime of 14-hr light/10-hr darkness. Disease ratings were scored as the amount of leaf surface infected, expressed as percentage total leaf area. All assays were scored blind on plants arrayed in a random design.

Black Shank Disease Assays. *Phytophthora parasitica* assays were also performed on 6-week-old plants grown as described above. Plants were watered, allowed to drain well, and then inoculated by applying 10 ml of a sporangia suspension (300 sporangia per ml) to the soil. Inoculated plants were then kept in a greenhouse maintained at 23–25°C in the day and 20–22°C at night. The wilt index used for the assay was as follows: 0, no symptoms; 1, some sign of wilting, with reduced turgidity; 2, clear wilting symptoms, but no rotting or stunting; 3, clear wilting symptoms with stunting, but no apparent stem rot; 4, severe wilting, with visible stem rot and some damage to root system; 5, as for 4, but plants near death or dead, and with severe reduction of root system (examples of rating scale are given in Fig. 4). All assays were scored blind on plants arrayed in a random design.

Statistical Analysis. Five lines representing four different PR-1a transgenic events were tested in the blue mold assay (six plants per line). Transgenic lines expressing other PR protein cDNAs (determined in previous screening not to be effective against these pathogens) were included to control for transformation and tissue culture effects. The black shank test included five independent PR-1a lines (six plants per line), as well as several non-PR-1a transgenic lines, and multiple groupings of control plants. Line genotypes are given in Table 1. Control and experimental groups for each disease test are described in the text and figure legends. Pairwise *t* tests were performed to compare different genotype and treatment groups for each rating date.

Transgenic Protein Expression. Levels of specific PR proteins in leaves were estimated by ELISA or immunoblot assay on leaf samples taken just prior to inoculation and frozen until assayed. PR-1 was assayed by a sandwich ELISA utilizing polyclonal and monoclonal antisera generated against tobacco PR-1a. All T_3 lines in our studies expressed their specific engineered protein(s) at levels at least as high as that observed in systemically resistant tissue.

Table 1. Description and reference list for transgenic and control tobacco lines

Line	Engineered gene	Ref.
1. Xanthi nc	None (no induction treatment)	
2. Induced Xanthi nc	None (chemically immunized)	
3. 1764C3-8	PR-1a (T_3 homozygote)	15
4. 1774C5-8	PR-1a (T_3 homozygote)	15
5. 1774C5-9	PR-1a (T_3 homozygote) (sib of line 4)	15
6. 1774A7-5	PR-1a (T_3 homozygote)	15
7. 1774A10-1	PR-1a (T_3 homozygote)	15
8. 1774A10-2	PR-1a (T_3 homozygote) (sib of line 7)	15
9. 1774A10-5	PR-1a (T_3 homozygote) (sib of line 7)	15
10. 1774C11-3	PR-1a (T_3 homozygote)	15
11. 1774A11-7	PR-1a (T_3 homozygote)	15
12. 1774C13-3	PR-1a (T_3 homozygote)	15
13. F_1-22	PR-1a × PR-2a	15, 23
14. F_1-2	PR-1a × basic chitinase	15, 24
15. F_1-3	PR-1a × basic glucanase	15, 25
16. F_1-7	PR-1a × PR-3b	15, 26
17. 1796C2-1	Basic glucanase −vtp (T_3 homozygote)	25
18. 1796C2-3	Sib of line 17	25
19. F_1-4	Basic chitinase × cucumber class III chitinase	24, 27
20. F_1-12	Cucumber class III chitinase × SAR8.2	27, 28
21. F_1-13	Basic chitinase × SAR8.2	24, 28
22. F_1-14	Basic glucanase × SAR8.2	25, 28
23. F_1-15	PR-3b × SAR8.2	26, 28
24. F_1-17	Cucumber class III chitinase × PR-Q′	27, 29
25. F_1-18	Basic chitinase × PR-Q′	24, 29
26. F_1-19	Basic glucanase × PR-Q′	25, 29
27. F_1-20	PR-3b × PR-Q′	26, 28
28. F_1-21	PR-Q′ × SAR8.2	28, 29
29. F_1-23	Cucumber class III chitinase × PR-2a	23, 27
30. F_1-24	Basic chitinase × PR-2a	23, 24
31. F_1-25	Basic glucanase × PR-2a	23, 25
32. F_1-26	PR-3b × PR-2a	23, 26
33. F_1-27	SAR8.2 × PR-2a	23, 28
34. F_1-28	PR-Q′ × PR-2a	23, 29
35. 3505C4-2	Basic class III chitinase (T_3 homozygote)	30
36. 1787C3-8	Basic glucanase (T_3 homozygote)	25
37. 1787C18-1	Basic glucanase (T_3 homozygote)	25
38. 1793C3-3	PR-2a (T_3 homozygote)	23
39. 1794C9-1	Basic chitinase −vtp	25

cDNAs for basic glucanase or basic chitinase were engineered to remove the C-terminal vacuolar targeting peptide (−vtp), thus allowing secretion of the proteins to the extracellular space (31).

RESULTS

Development and Initial Evaluation of PR-1a Transgenic Tobacco. To evaluate the role of PR-1a in SAR, transgenic tobacco plants were created that expressed the coding sequence of the major PR-1 isoform (PR-1a) under control of a strong, constitutive promoter (Fig. 1). From 32 independent primary transformants, 13 were advanced based on a high level of PR-1a expression and seed segregation for kanamycin resistance consistent with a single locus insertion of the transgene. A homozygous line (Table 1, line 7) derived from one of the transformants was initially tested for resistance to diseases caused by the following pathogens: tobacco mosaic virus, potato virus Y, *Cercospora nicotianae, P. parasitica*

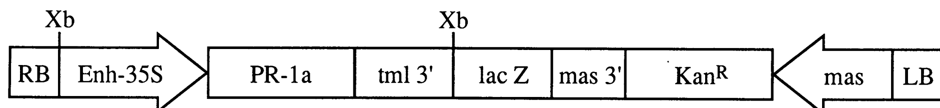

FIG. 1. Schematic of T-DNA in plasmid pCGN1774A. LB, left T-DNA border sequence; RB, right border; Xb, *Xba* I site; Enh-35S, enhanced 35S promoter; mas, mannopine synthase promoter; mas 3′, mannopine synthase 3′ untranslated sequence; Kan^R, neomycin phosphotransferase II gene conferring kanamycin resistance; lacZ, DNA sequence encoding the β-galactosidase α fragment; tml 3′, *tml* gene 3′ untranslated sequence.

var. *nicotianae, Pseudomonas syringae* pv. *tabaci*, and *P. tabacina*. Symptoms in the transgenic line did not differ from controls with respect to most of the diseases tested. However, significant delays of infection and substantial reductions of disease symptoms were found on plants inoculated with the two oomycete pathogens *P. tabacina* (causal agent of blue mold disease) and *P. parasitica* var. *nicotianae* (causal agent of black shank disease).

Effects of PR-1a on Blue Mold Disease. Preliminary evidence was obtained for an effect of PR-1a on tolerance to blue mold disease in three independent tests with line 7. To determine whether this effect was due to the PR-1a gene product and not a result of somoclonal variation or some epigenetic effect of the transformation process, the evaluation was expanded to include other independently derived PR-1a lines, as well as transgenic lines expressing other genes. Lines 7 and 9 were siblings from the same primary transformant; lines 5, 10, and 11 were derived from three other independent transformation events. Also included were four F_1 heterozygous lines derived from crossing line 7 with transgenic tobacco expressing PR-2a glucanase (line 13), class I chitinase (line 14), class I glucanase (line 15), or PR-3b chitinase (line 16), and 18 transgenic tobacco lines expressing other genes (lines 17–34). None of the non-PR-1a lines had shown indications of blue mold tolerance in first-round tests. A positive control in the experiment was nontransformed Xanthi nc tobacco treated with a synthetic immunizing chemical (5) that induces the entire repertoire of SAR-related genes (line 2). A negative control was untreated nontransformed tobacco (line 1).

Disease was rated for each line 7 (Fig. 2*A*) and 9 (Fig. 2*B*) days after inoculation. For statistical analysis, the data were grouped into four sets: PR-1a homozygotes, PR-1a heterozygotes, non-PR-1a lines (including nontransformed control), and chemically immunized nontransformed plants. On average after 7 days, 48% of the leaf area of the control lines was infected by the fungus (Table 2). In contrast, the PR-1a homozygotes and PR-1a heterozygotes were on average 28% and 35% infected, respectively. This represented a symptom reduction at 7 days of 42% for the homozygotes and 27% for the heterozygotes compared to controls. Pairwise *t* test comparisons showed that reductions in disease severity were significant for both PR-1a homozygotes and heterozygotes (P = 0.99). At day 9, disease severity ratings for homozygous PR-1 transgenic plants had increased to 76% of controls, while the heterozygous lines were 79% of controls, both still significantly different from the negative control group (P = 0.99).

The level of PR-1a accumulated in all of the lines was determined by ELISA. At the time of inoculation, none of the control lines expressed detectable amounts of protein (<1 μg/g dry wt), while the PR-1a homozygotes contained amounts of PR-1 ranging from 40 to 70 μg/g dry wt. Chemically induced control plants expressed the protein at ≈120 μg/g dry wt.

Effects of PR-1a on Black Shank Disease. Five homozygous PR-1a lines (lines 3, 4, 6, 8, and 12) were tested for resistance to *P. parasitica* var. *nicotianae*. Controls included five sets (six plants each) of untransformed Xanthi nc plants (line 1),

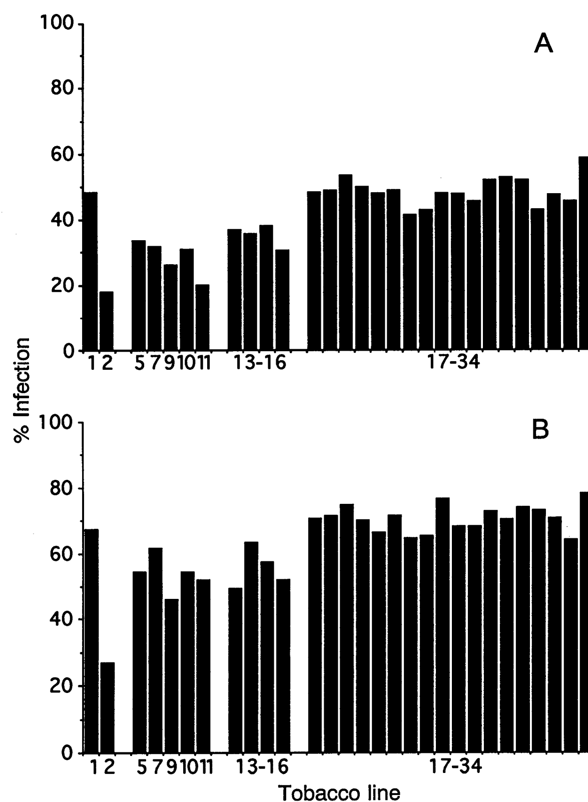

FIG. 2. Blue mold disease ratings. Plants were scored for percentage of total leaf area infected 7 (*A*) and 9 (*B*) days after infection. Each bar represents mean of six sibling plants from a single line. Line designations are described in Table 1.

five transgenic lines homozygous for other PR genes (lines 35–39), and two sets (six plants each) of chemically treated Xanthi nc plants (line 2). Disease severity ratings for black shank disease were based on a six-point wilt index. At 3 days after inoculation, the differences in disease symptoms were evident in the PR-1a transgenics and chemically induced controls compared to negative controls. Fig. 3 shows two wild-type Xanthi nc plants compared to two transgenic PR-1a plants on day 8 postinfection. The photograph illustrates clearly the progression of the disease and the easily observable differences in disease rating scores. Fig. 4 shows mean wilt index as a function of time after infection with *P. parasitica*. At the end of the test, the mean rating for PR-1a transgenic plants was 32% lower than controls. Pairwise *t* testing indicated no difference between non-PR-1a transgenics and Xanthi nc controls and no difference between PR-1a transgenics and chemically induced Xanthi nc throughout the test. However, there was a significant difference (P = 0.90 or greater) between the negative controls and PR-1a transgenics on each rating date. For day 7 and later, the differences in disease severity between the tolerant and control lines were highly significant (P = 0.99).

PR-1a protein levels in leaf material on the day of inoculation ranged from 10 to 30 μg/g dry wt in transgenic PR-1a

Table 2. Downy mildew disease ratings

Group	Line(s)	Average % infected leaf area		% disease reduction	
		Day 7	Day 9	Day 7	Day 9
Negative controls	1, 17–34	48 ± 3.0	70 ± 1.8	—	—
Chemically induced	2	18 ± 11.3	27 ± 11.8	63	61
PR-1a homozygotes	5, 7, 9–11	28 ± 6.1	53 ± 5.5	42	24
PR-1a heterozygotes	13–16	35 ± 7.4	55 ± 5.2	27	21

Results are means ± SE; P = 0.90.

FIG. 3. *P. parasitica*-infected tobacco plants. The two untreated Xanthi nc plants on the left show wilting and stunting (symptoms of black shank disease). One was rated as 3 (far left) and the other as 2.5 (second from left). One PR-1a transgenic plant (third plant from left) showed slight wilting and was rated as 1. The other PR-1a plant (far right) was rated as 0 (no symptoms). Infected plants were photographed 8 days after inoculation.

plants, while control plants contained <1 μg/g dry wt. Chemically induced plants averaged 15 μg of PR-1a per g dry wt.

We considered the possibility that expressing PR-1a as a transgene might have caused induction of the other PR proteins in tobacco, which would then lead to resistance. Therefore, we analyzed expression of PR-2, PR-3, PR-4, and PR-5 in uninoculated homozygous PR-1a lines by RNA blot analysis. mRNAs encoding the other PR proteins were expressed only at very low levels, indistinguishable from uninduced nontransgenic control lines (data not shown), indicating that expression of PR-1a alone was sufficient to lead to the observed tolerance.

DISCUSSION

Our results provide clear evidence of a biological role for the PR-1 proteins in disease tolerance. High-level constitutive expression in transgenic plants of PR-1b, a closely related isoform of PR-1a, has previously been reported to have no effect on disease development by tobacco mosaic virus or

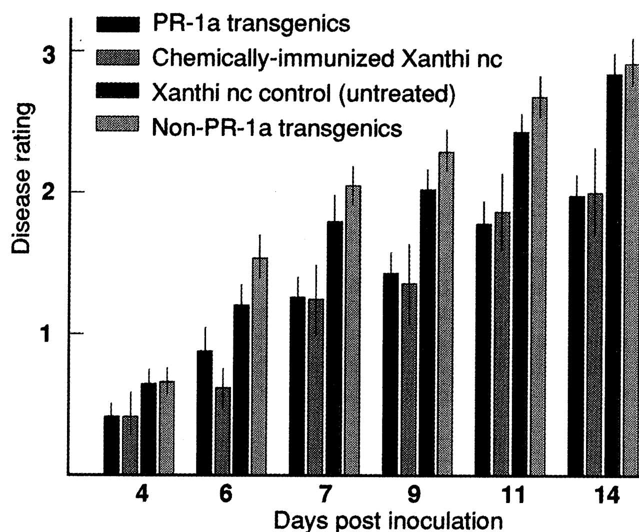

FIG. 4. Black shank disease ratings. Bars represent mean ratings for data groups at each rating date. Data groups were as follows: PR-1a transgenics, dark blue; chemically induced Xanthi nc, light blue; untreated Xanthi nc, dark red; non-PR-1a transgenics, light red. Error bars represent SEM ($P = 0.90$).

alfalfa mosaic virus (13, 14). We have shown that PR-1a does not have a measurable effect on diseases caused by tobacco mosaic virus, potato virus Y, or a range of other pathogens, but it does reduce significantly the disease severity caused by infection with the two oomycete pathogens tested.

Although the observed blue mold tolerance was significant for both rating dates, the level of protection in PR-1a heterozygotes decreased somewhat by 9 days. In both the homozygotes and heterozygotes, the protection from disease was less than in the chemically immunized control. In the blue mold test, PR-1a levels in chemically induced plants were ≈2-fold higher than in transgenic PR-1a lines. In the black shank assay, in which the PR-1a transgenes and chemically induced controls gave equal protection, chemically induced plant PR-1a levels fell within the range of transgenic plants. These observations are consistent with PR-1a acting alone, providing tolerance proportional to the amount of protein. The diminished tolerance in heterozygote PR-1a plants could also be explained by such a model. We did not see a strong correlation of protein level to disease rating of individual lines (data not shown). However, given the small sample size within a line, the inherent variation in the disease assays makes such comparisons difficult.

The exact nature of the disease tolerance provided by constitutive PR-1a expression is unknown. PR-1a may exert a direct fungicidal effect that could account for the decreased disease development on transgenic plants. Alternatively, PR-1a may slow the pathogen's establishment or aid in its recognition (or both), thereby allowing the plant to activate additional defense responses that limit spread of the disease.

Oomycete fungi represent some of the most economically important plant pathogens. The downy mildews, of which tobacco blue mold is an example, are especially damaging diseases that may account for worldwide crop losses estimated as high as $50 billion per year. Moreover, the oomycete fungi contain little or no chitin in their cell walls and therefore are unlikely to be affected by chitinases expressed in transgenic plants (32). Thus, PR-1 represents an activity that may be useful for genetically engineering crops resistant to oomycete pathogens.

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