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Decreased Susceptibility to Viral Disease of β-1,3-Glucanase-Deficient Plants Generated by Antisense Transformation

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Antifungal class I β-1,3-glucanases are believed to be part of the constitutive and induced defenses of plants against fungal infection. Unexpectedly, mutants deficient in these enzymes generated by antisense transformation showed markedly reduced lesion size, lesion number, and virus yield in the local-lesion response of Havana 425 tobacco to tobacco mosaic virus (TMV) and of *Nicotiana sylvestris* to tobacco necrosis virus. These mutants also showed decreased severity of mosaic disease symptoms, delayed spread of symptoms, and reduced yield of virus in the susceptible response of *N. sylvestris* to TMV. The symptoms of disease in the responses of both plant species were positively correlated with β-1,3-glucanase content in a series of independent transformants. Taken together, these results provide direct evidence that β-1,3-glucanases function in viral pathogenesis. Callose, a substrate for β-1,3-glucanase, acts as a physical barrier to the spread of virus. Callose deposition in and surrounding TMV-induced lesions was increased in the β-1,3-glucanase-deficient, local-lesion Havana 425 host, suggesting as a working hypothesis that decreased susceptibility to virus resulted from increased deposition of callose in response to infection. Our results suggest novel means, based on antisense transformation with host genes, for protecting plants against viral infection. These observations also raise the intriguing possibility that viruses can use a defense response of the host against fungal infection—production of β-1,3-glucanases—to promote their own replication and spread.

INTRODUCTION

Interactions between plants and pathogens reflect an elaborate coevolution of recognition, defense, and counterdefense mechanisms. Infection of resistant strains of plants triggers a complex series of local biochemical and cellular events in the host, known as the hypersensitive reaction (HR) (reviewed in Collinge and Slusarenko, 1987). The HR is a stereotypic response, that is, the nature of the response is similar for viral, bacterial, and fungal pathogens. Some components of the HR appear to serve a general defense function independent of the inciting pathogen. Rapid cell death results in necrotic lesions at the site of infection. Antimicrobial compounds such as the phytoalexins are produced. Cell walls are modified by the deposition of lignin and the β-1,3-glucan callose. This process is thought to isolate the infected area and to help prevent spread of the pathogen (Bell, 1981). Other components of the HR, for example, the induction of antifungal β-1,3-glucanases and chitinases, appear to be tailored for defense against a particular class of pathogens, fungi (Schlumbaum et al., 1986; Mauch et al., 1988; Broglie and Broglie, 1993; Sela-Buurlage et al., 1993; Zhu et al., 1994).

Plant genes induced during the HR are commonly assumed to be part of the host's defense mechanisms. We provide evidence that induction of one class of these genes encoding β-1,3-glucanases could be a counterdefense mechanism employed by viral pathogens.

At least three structural classes of β-1,3-glucanases (glucan endo-1,3-β-glucosidase; EC 3.2.1.39) have been identified. The class I isoforms are basic proteins localized in the cell vacuole (Shinshi et al., 1988). The class II and class III isoforms are acidic proteins secreted into the extracellular space. Included in this group are the pathogenesis-related (PR) proteins PR-2, PR-N, PR-O, and PR-Q′ (Kauffmann et al., 1987; Payne et al., 1990) and two glycoproteins localized in the style of the flower (Ori et al., 1990). Finally, a distinct, intracellular class of "ersatz" β-1,3-glucanases is induced by virus infection in class I β-1,3-glucanase–deficient mutants of *Nicotiana sylvestris* and tobacco (*N. tabacum*) (Beffa et al., 1993).

β-1,3-Glucanases (or β-1,3-glucanase activity) have been implicated in several developmental and physiological processes, including cell division, pollen formation, and seed germination (Waterkeyn, 1967; Worrall et al., 1992; Vögeli-Lange et al., 1994a). There is also considerable evidence to suggest that these enzymes are part of both the constitutive and induced defense against pathogenic fungi (Mauch et al., 1988; Mauch

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and Staehelin, 1989; Keefe et al., 1990; Zhu et al., 1994; Jongedijk et al., 1995). These enzymes are thought to act directly by digesting β-1,3-glucans in fungal cell walls as well as indirectly by digesting fungal and host polysaccharides to produce elicitors capable of evoking the HR (Ebel and Scheel, 1992).

Although it has long been recognized that β-1,3-glucanases are induced as part of the local-lesion response to viral infection characteristic of resistant hosts (Moore and Stone, 1972; Hawker et al., 1974; Kauffmann et al., 1987; Vögeli-Lange et al., 1988; Ward et al., 1991a), a function for these enzymes in viral pathogenesis has not been established. Here, we report the unexpected finding that class I β-1,3-glucanase–deficient mutants generated by antisense transformation exhibit decreased susceptibility to necrotic virus infection both in the local-lesion, resistant response and in the systemic, susceptible response. Decreased symptoms in the local-lesion response were associated with a dramatic increase in callose accumulation in and around necrotic lesions; this finding suggests that β-1,3-glucanases may be important in regulating callose deposition during pathogenesis. Our results suggest a novel approach for obtaining virus-resistant plants and raise the intriguing possibility that viruses can use a component of the host's defense system against fungal infection to their own advantage.

RESULTS

β-1,3-Glucanase-Deficient Mutants Show Decreased Susceptibility to Necrotizing Virus Infection

Knockout experiments were performed to identify functions of class I β-1,3-glucanases in viral pathogenesis. We used two host plants: the Havana 425 variety of tobacco, which gives a local-lesion, resistant response to tobacco mosaic virus (TMV) (Vögeli-Lange et al., 1988); and *N. sylvestris*, which gives a systemic, fully susceptible response to TMV and a local-lesion, resistant response to tobacco necrosis virus (TNV) (Lucas, 1975). The class I β-1,3-glucanase–deficient mutants TAG4.4 of Havana 425 tobacco and SAG2.3 of *N. sylvestris* have been described in detail elsewhere (Neuhaus et al., 1992; Beffa et al., 1993). In brief, they were obtained by Ti plasmid–mediated transformation with antisense constructions containing the coding sequence of the tobacco class I β-1,3-glucanase gene *GLA* under the control of the cauliflower mosaic virus 35S RNA promoter. Expression of class I but not class II or class III β-1,3-glucanases is effectively and specifically blocked in these antisense lines. Leaf tissues of homozygous transformants accumulated ~20-fold less class I isoform than did wild-type plants. Comparable inhibition was observed for induction of the class I isoforms by treatment with the stress hormone ethylene and by infection of SAG2.3 with TNV and TAG4.4 with TMV.

The effect of antisense transformation on the local-lesion response was investigated by use of TAG4.4 infected with TMV and SAG2.3 infected with TNV. Plants transformed with an empty vector and wild-type (i.e., untransformed) plants were used as controls. Infected and mock-infected plants were incubated for 7 days under conditions that gave the local-lesion response, and the leaves were then scored for disease symptoms. Three criteria were used to judge the severity of infection: necrotic lesion size, lesion number, and yield of infectious virus.

Reduction in Size and Number of Lesions

Figure 1 shows leaves of empty-vector–transformed and antisense Havana 425 plants 7 days after TMV infection. The empty-vector controls exhibited a typical local-lesion response (Figure 1A). Lesion size and number were greatly reduced in the antisense plant. In no case were lesions found on the leaves of mock-infected plants. The quantitative results obtained in four different experiments using several dilutions of inocula are presented in Table 1. Antisense transformants exhibited a substantial 30 to 80% reduction in the number of detectable lesions; this reduction was essentially independent of inoculum titer. There was also an ~50% reduction in lesion diameter relative to the control plants. Similar results were obtained when TNV was used to infect the *N. sylvestris* transformant SAG2.3, which contains another antisense construct (Table 1).

Reduction in Virus Yield

The titers of infectious TMV in extracts prepared from individual necrotic lesions on Havana 425 plants are shown in Table 2. The yield of virus per lesion from antisense plants was reduced ~10-fold relative to control plants. Moreover, the virus titers in mixtures of extracts prepared from lesions forming on the two types of plants were strictly additive, indicating that the observed differences are not due to factors present in the extracts tested. A similar reduction in virus yield was found for *N. sylvestris* plants infected with TNV (data not shown). For both hosts, the ~10-fold reduction in yield was considerably larger than the fourfold reduction in lesion area calculated from the lesion diameters shown in Table 1. Therefore, the decrease in virus yield cannot be accounted for solely by the decrease in lesion size.

Antisense Effect Is Not Due to Selective Inhibition of a Virus Subpopulation

Inspection of Figure 1A and the distributions of lesion sizes shown in Figure 2 indicate that lesions formed on TMV-infected, empty-vector Havana 425 plants fell into two classes, with median diameters of ~2.0 and 3.0 to 3.5 mm. In contrast, the lesions formed on antisense plants appeared to fall into a single class, with a median diameter of ~1.5 mm. These results, and comparable ones obtained with TNV-infected *N. sylvestris* plants (data not shown), raised the possibility that

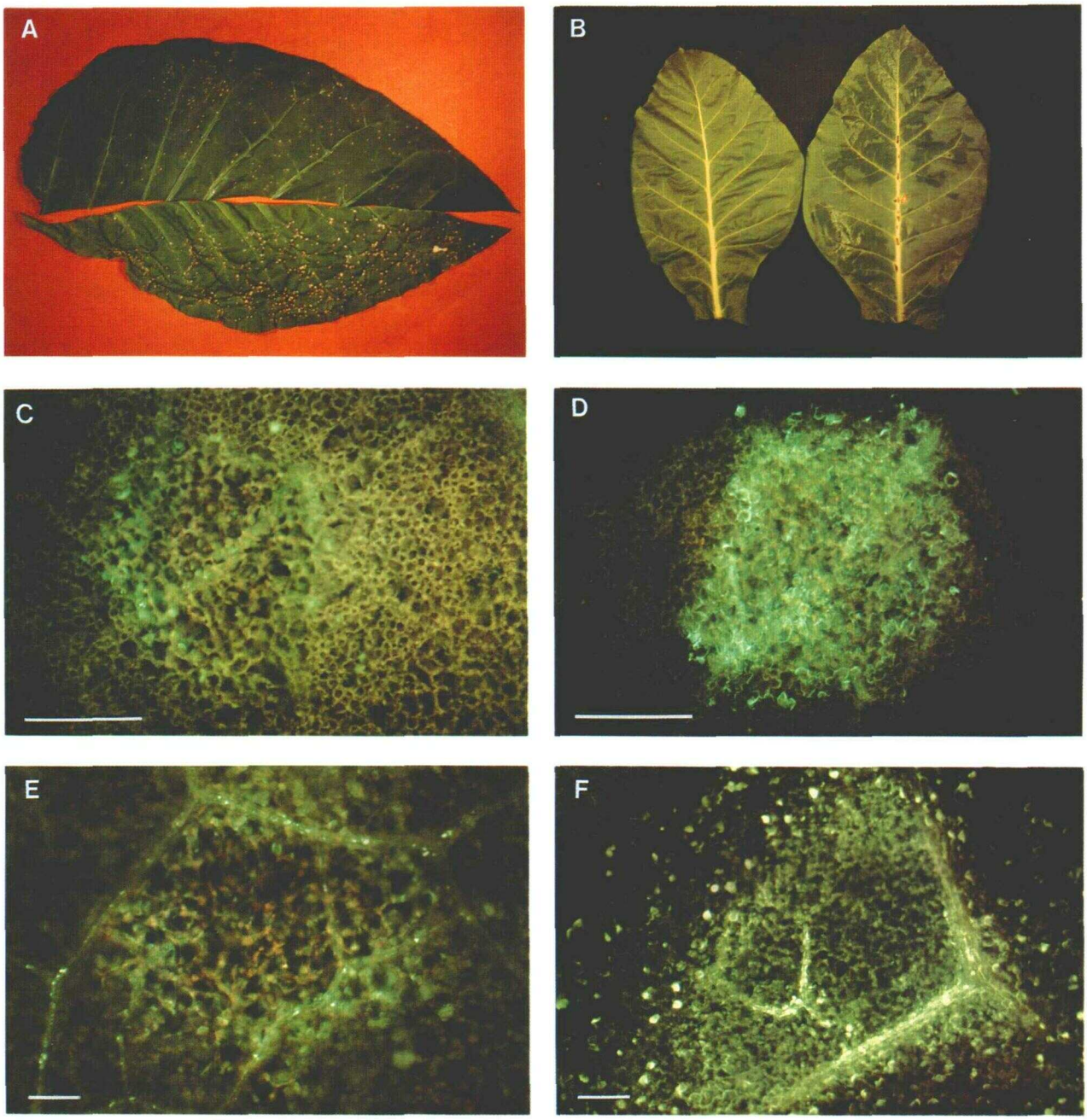

Figure 1. Development of Disease Symptoms after Infection of Havana 425 Tobacco and *N. sylvestris* with TMV.

(A) Empty-vector TCIB (lower half leaf) and antisense TAG4.4 (upper half leaf) transformants of Havana 425 tobacco 7 days after TMV infection. The leaves show the local-lesion response typical of a resistant host. Note the reduction of lesion size and number in the antisense host.
(B) Leaf number 15 (counting from the bottom of the plants) of antisense (left) and empty-vector (right) transformants of *N. sylvestris* 4 weeks after infecting leaf number 6 with TMV. Note that the antisense leaf does not show symptoms of mosaic disease.
(C) to **(F)** Leaf tissue of empty-vector (**[C]** and **[E]**) and antisense (**[D]** and **[F]**) transformants of Havana 425 tobacco stained for callose (yellow-white fluorescence). Microscopy of leaves 3 days after TMV infection is shown in **(C)** and **(D)** and of leaves 7 days after TMV infection is shown in **(E)** and **(F)**. The orange autofluorescence is typical of the HR response in tobacco. Bars = 100 μm.

Table 1. Effect of Antisense Transformation on the Local Lesion Response of Havana 425 Tobacco to TMV and *N. sylvestris* to TNV

Experiment[a]	Host Plant	Genotype	No. of Lesions		Lesion Size	
			No./Leaf[b]	% Reduction in Antisense[c]	Diameter (mm)[d]	% Reduction in Antisense[c]
1	Tobacco	Empty vector (TCIB)	48 ± 20			
		Antisense (TAG4.4)	10 ± 3	79.3		
2	Tobacco	Empty vector (TCIB)	521 ± 45			
		Antisense (TAG4.4)	102 ± 24	80.4		
3	Tobacco	Empty vector (TCIB)	1455 ± 269		3.08 ± 0.03	
		Antisense (TAG4.4)	431 ± 62	70.4	1.56 ± 0.03	49.4
4	Tobacco	Wild type	1258 ± 255		2.73 ± 0.04	
		Empty vector (TCIB)	1175 ± 219		2.87 ± 0.04	
		Antisense (TAG4.4)	755 ± 55	36.0	1.34 ± 0.03	53.3
5	*N. sylvestris*	Empty vector (SCIB)	51 ± 13			
		Antisense (SAG2.3)	26 ± 12	49.7		
6	*N. sylvestris*	Empty vector (SCIB)	352 ± 26		3.46 ± 0.03	
		Antisense (SAG2.3)	140 ± 17	60.2	1.68 ± 0.03	51.4

[a] Variation from experiment to experiment in lesion numbers reflects the different virus titers used.
[b] Mean ± SE for six to 16 leaves.
[c] Relative to empty-vector controls.
[d] Mean ± SE for 400 to 900 lesions counted.

heterogeneity in the virus preparation is responsible for the smaller size of lesions formed on antisense plants. To test this hypothesis, extracts prepared from lesions of empty-vector and antisense plants were used to inoculate both types of plants. The results in Table 2 show that independent of the source of the inoculum, the number of lesions formed was three- to fourfold lower on the antisense host than on the control host. Therefore, the reduced yield observed with antisense plants is due to reduced replication and spread of virus rather than to heterogeneity in the population of virus.

Antisense Transformation Reduces and Delays Mosaic Disease Symptoms in a Susceptible Host

The results presented so far indicate that antisense β-1,3-glucanase transformation increases the resistance of local-lesion hosts to virus infection. It was of particular interest to determine whether antisense transformation is also effective in a fully susceptible host showing systemic infection. The experimental system we used was TMV infection of the susceptible host *N. sylvestris*. A single leaf near the top of young empty-vector (SCIB) and antisense (SAG2.3) plants with 10 to 12 leaves was infected with TMV. Disease symptoms and the spread of virus were assayed 1 month later. The empty-vector plants showed symptoms typical of mosaic disease (summarized in Lucas, 1975), that is, leaf mottling that spread progressively to younger leaves near the top of the plant (Figure 1B). After 4 to 5 weeks, almost all young leaves were affected. The severity of symptoms ranged from green mottling in mild infections to chlorosis and extensive necrosis in moderate and heavy infections.

The course of infection of antisense plants differed from that of the controls in several important ways. First, symptoms on leaves of antisense plants were consistently less severe than those on comparable leaves of control plants. Second, disease symptoms progressed to younger leaves more slowly in antisense plants (Table 3). One month after inoculation, the youngest control leaf with symptoms was leaf 17 of 19 leaves, whereas the youngest antisense leaf with symptoms was only leaf 11 of 19 leaves. Indeed, the youngest leaves of antisense

Table 2. Effect of Antisense β-1,3-Glucanase Transformation on the Yield of Virus from Individual Lesions on Leaves of Havana 425 Tobacco Infected with TMV

Experiment	Source of Virus[a]	Havana 425 Host	Virus Titer[b]
1	Empty vector	Wild type	284 ± 36
	Antisense	Wild type	34 ± 7
2	Empty vector	Wild type	267 ± 56
	Antisense	Wild type	33 ± 7
	Empty vector + antisense	Wild type	308 ± 12
3	Empty vector	Empty vector	329 ± 25
		Antisense	118 ± 14
	Antisense	Empty vector	48 ± 7
		Antisense	10 ± 2

[a] Obtained from local lesions 7 days after inoculation with TMV on leaves from TCIB (empty vector) and TAG4.4 (antisense) transformants.
[b] Given as the mean number of lesions on the indicated host per individual lesion assayed (± SE) for six to 12 samples.

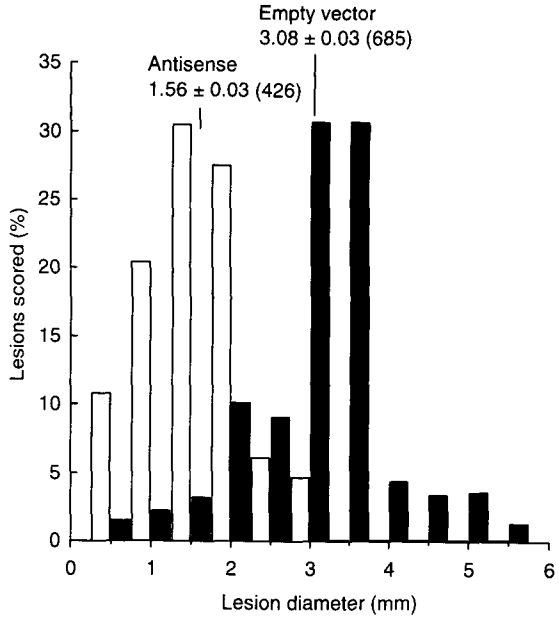

Figure 2. Distribution of Lesion Sizes.

Empty-vector TCIB (solid bars) and antisense TAG4.4 (open bars) transformants of Havana 425 tobacco were inoculated with TMV and scored for lesion diameter after 7 days. Values are the mean lesion size ± SE for the numbers of lesions measured, which are given within parentheses.

plants remained symptom free throughout the course of the experiment (Figure 1B). Finally, the yield of infectious virus from antisense plants was substantially lower than that from control plants. Moreover, essentially no infectious virus could be recovered from symptom-free young leaves, that is, leaf 17 of the antisense plants (Table 3). Taken together, these results indicate that antisense β-1,3-glucanase transformation markedly decreases the susceptibility of a susceptible host to TMV infection.

Disease Symptoms Are Correlated with Class I β-1,3-Glucanase Expression in Independent Transformants

We compared the disease response of seven independent antisense transformants of Havana 425 and *N. sylvestris* infected with TMV. Individual leaves from the different antisense transformants and empty-vector controls were assayed for β-1,3-glucanase content and then inoculated with virus. In the Havana 425 host, lesion size and lesion number were correlated with β-1,3-glucanase content (linear correlation of $P < 0.01$ and $P < 0.05$, respectively; Figure 3A). A similar correlation for the youngest leaf showing disease symptoms was found for the susceptible *N. sylvestris* host (linear correlation of $P < 0.02$; Figure 3B). These results confirm our findings with additional independent transformants and show that disease symptoms in both the local-lesion response and fully susceptible response increase with the β-1,3-glucanase content of the leaves at the time of inoculation.

Callose Deposition and β-1,3-Glucanase Accumulation in Havana 425 Transformants Showing a Local-Lesion Response

Callose, a potential substrate for β-1,3-glucanases that is deposited as part of the HR, is thought to restrict the systemic spread of virus from cell to cell via plasmodesmata (reviewed in Lucas et al., 1993). We compared the callose staining of TMV-infected leaves of control TCIB plants and antisense TAG4.4 plants. Representative photomicroscopy of tissues stained for callose with aniline blue fluorochrome are shown in Figures 1C to 1F. Callose gives a yellow-white fluorescence, and necrotic lesions exhibit orange autofluorescence typical of the HR (Mayama and Shishiyama, 1976). The intensity of callose staining of individual lesions was also quantitated and is shown in Figure 4A. No callose was detected in epidermal and mesophyll cells of uninfected leaves (data not shown). In control leaves, very low levels of callose were first detected when necrotic lesions appeared 3 days after infection (Figures 1C and 4A). The callose content per lesion rapidly increased ~15-fold by day 4 and then remained at the same level until

Table 3. Effect of Antisense β-1,3-Glucanase Transformation on Disease Symptoms in TMV-Infected *N. sylvestris*

		Leaf Number[a]		TMV Titer[b]		
Host	Leaves/Plant[c]	Leaves Inoculated	Youngest Leaf with Symptoms	Leaf 17	Leaf 11	Leaf 9
Empty vector	18.5 ± 0.3	6.7 ± 0.2	16.5 ± 0.4	3,150 ± 527	121,000 ± 16,900	322,000 ± 40,700
Antisense	18.5 ± 0.2	7.1 ± 0.2	11.1 ± 0.2	0.5 ± 0.3	2,590 ± 655	118,000 ± 12,700

[a] The mean leaf number (± SE) counting from the bottom of 11 or 12 plants 4 weeks after inoculation.
[b] The mean number of lesions formed on wild-type Havana 425 tobacco per gram fresh weight of leaf tissue (± SE) for six plants 4 weeks after inoculation.
[c] The mean number (± SE) for 11 or 12 SCIB and SAG2.3 plants 4 weeks after inoculation.

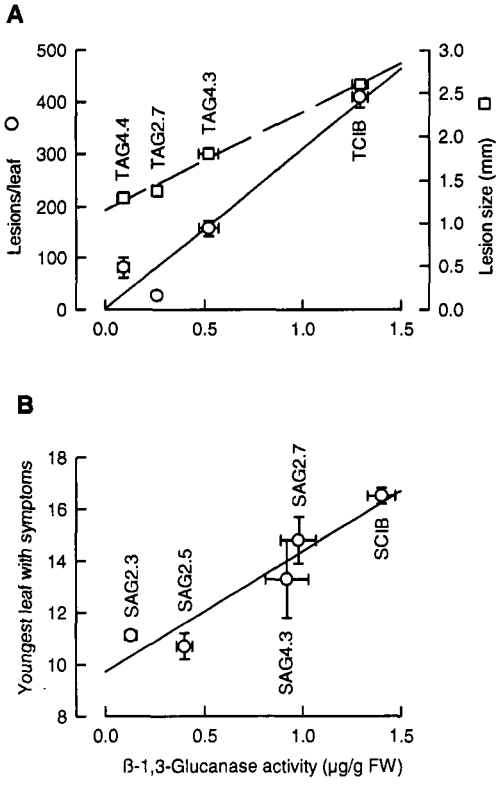

Figure 3. Correlation of Disease Symptoms with β-1,3-Glucanase Content in Independent Transformants.

(A) The local-lesion response of Havana 425 tobacco inoculated with TMV and scored for lesion number (○) and lesion size (□) after 7 days. The independent transformants tested are indicated.
(B) The systemic response of *N. sylvestris* inoculated with TMV and scored for symptoms of mosaic disease on leaves 1 month after inoculation of leaf 7. The independent transformants tested are indicated. Data are expressed as mean values; error bars, ±SE for three to 12 plants; solid and dashed lines, least-squares fit of the data points; FW, fresh weight. β-1,3-Glucanase activity was measured in extracts prepared from leaves just before inoculation with virus.

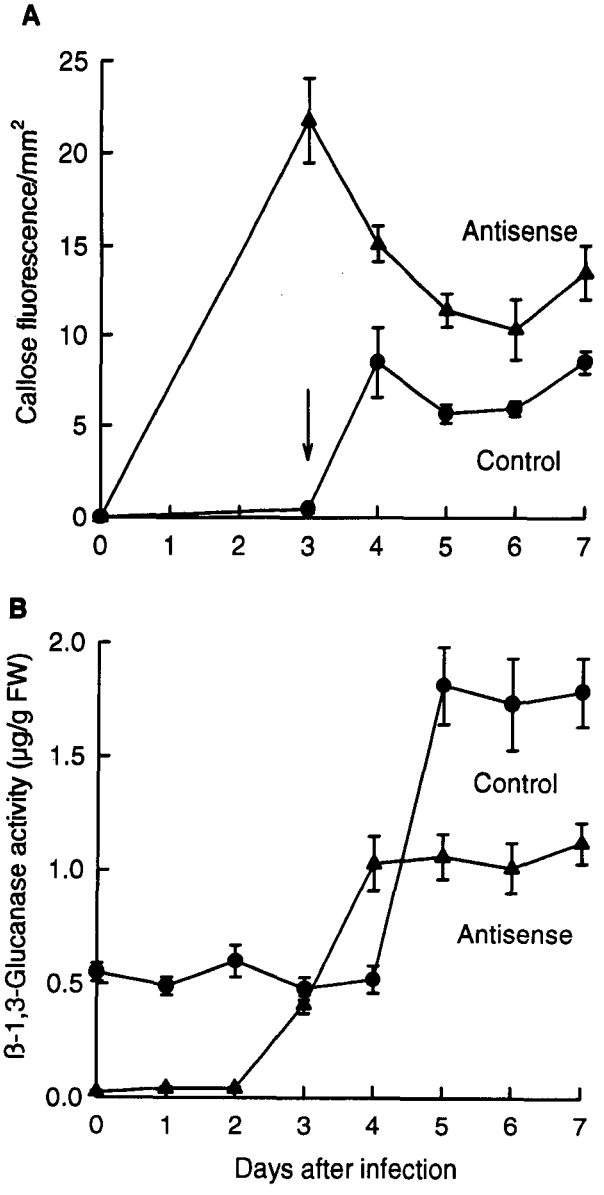

Figure 4. Time Course of Callose and β-1,3-Glucanase Accumulation.

(A) Empty-vector TCIB (●) and antisense TAG4.4 (▲) Havana 425 transformants were inoculated with TMV on day 0. Regions of the leaf with local lesions were fixed and stained with aniline blue. The fluorescence due to callose associated with individual lesions was measured. No fluorescence due to callose could be detected by visual inspection before the appearance of lesions on day 3 (arrow).
(B) Extracts from replicate tissue samples were prepared and assayed for β-1,3-glucanase activity. When lesions were present, starting on day 3, individual lesions were assayed. FW, fresh weight.
Data are presented as mean values of callose fluorescence in arbitrary units (for at least 10 individual lesions) and of β-1,3-glucanase activity in class I β-1,3-glucanase equivalences (for 12 individual lesions). Error bars, ±SE.

the end of the experiment on day 7 (Figures 1E and 4A). Throughout this period, callose was localized primarily as discrete spots surrounding the necrotic lesion (Figures 1C and 1E).

In antisense leaves, high levels of callose (~40-fold higher than in control leaves) were found in the first lesions, which appeared 3 days after infection (Figures 1D and 4A). Thereafter, the callose content decreased with time but always remained higher than in control leaves. Callose was present both as discrete spots surrounding lesions and as a diffuse mass within the lesions themselves (Figures 1D and 1F). These results indicate that antisense β-1,3-glucanase transformation enhances the callose response to TMV infection in the resistant Havana 425 host.

The time course of β-1,3-glucanase accumulation in TMV-infected TCIB and TAG4.4 leaf tissues is shown in Figure 4B. β-1,3-Glucanase activity was measured in 9-mm-diameter discs excised from the inoculated region of the leaves and is expressed on a fresh-weight basis. Once lesions appeared 3 days after inoculation, 9-mm-diameter discs centered on individual lesions were assayed. In control leaves, the β-1,3-glucanase activity remained at a constant, basal level for the first 4 days after infection. Between days 4 and 5, enzyme activity increased by approximately fourfold and then remained constant until the end of the experiment on day 7. In the antisense leaves, basal activity was below the detection limit. β-1,3-Glucanase was first detected on day 3 when lesions appeared, and it remained at a constant, elevated level from day 4 to day 7. We confirmed that this activity was due to induction of antibody-insensitive ersatz β-1,3-glucanase (Beffa et al., 1993; data not shown). Induction of ersatz isoform activity started 2 days earlier but was less than the induction of antibody-sensitive class I to class III β-1,3-glucanases in control leaves. Comparison of the data in Figures 4A and 4B obtained in the same experiment shows that more β-1,3-glucanase and less callose accumulated in control plants than in antisense plants. Moreover, the rapid decline in the callose content of antisense leaves after day 3 was correlated with the induction of ersatz β-1,3-glucanase activity.

DISCUSSION

β-1,3-Glucanases Are Important in Viral Pathogenesis

The knockout experiments described here provide a direct demonstration that β-1,3-glucanases function in viral pathogenesis. We show that deficiencies in class I β-1,3-glucanases decrease the susceptibility of *N. sylvestris* to TMV and TNV infections and of Havana 425 tobacco to TMV infection. This conclusion is based on several lines of evidence. First, antisense β-1,3-glucanase transformation specifically and effectively blocked induction of class I β-1,3-glucanases, but not induction of the other known β-1,3-glucanases, in response to infection with the combinations of viruses and host plants tested for susceptibility (Beffa et al., 1993). Second, antisense transformation further increased the resistance to infection of local-lesion hosts, as determined by substantial decreases in lesion size, lesion number, and virus yield from individual lesions. Third, antisense transformation also protected susceptible plants against infection, as evidenced by reduced severity of mosaic disease symptoms, delayed spread of symptoms in the host, and reduced virus yield. Finally, reduction of disease symptoms was correlated with reduction of β-1,3-glucanase content in several independent transformants of two plant species, making it likely that the observed effects were due to β-1,3-glucanase deficiency rather than to the site of transgene insertion or random mutation.

Earlier evidence that β-1,3-glucanases function in viral pathogenesis is limited, indirect, and contradictory. β-1,3-Glucanase activity, measured with the algal polysaccharide laminarin as substrate, is strongly induced in a variety of host plants by viruses as part of the local-lesion response (Moore and Stone, 1972; Hawker et al., 1974). This activity is due to several classes of structural isoforms, which are highly conserved in evolution, differ in specific activity, and are located in different cellular compartments (reviewed in Meins et al., 1992).

There are suggestions that β-1,3-glucanases defend plants against viruses. For example, a protein induced by TMV infection of tobacco reported to have antiviral activity and immunological similarity to human interferon-β is similar in sequence to class I β-1,3-glucanases (Edelbaum et al., 1991). Fungal β-1,3-glucans, which might be generated by β-1,3-glucanase digestion of fungal cell walls, have been reported to have antiviral activity (Wood et al., 1971; Kopp et al., 1989). On the other hand, Kearny and Wu (1984) have proposed that high β-1,3-glucanase levels promote virus infection. Although they were unable to find a correlation between lesion size and β-1,3-glucanase activity in several host plants, more recent studies based on measuring specific β-1,3-glucanases support their view. For example, the virus-encoded movement protein is thought to promote TMV spread by increasing the effective aperture of plasmodesmata. Movement protein did not modify secondary plasmodesmata of young tobacco leaves in which class I β-1,3-glucanases were not detectable (Felix and Meins, 1986; Ding et al., 1992).

Virus infection induces accumulation of the stress hormone ethylene (Boller, 1988). Treatment of tobacco plants with ethylene increases the class I β-1,3-glucanase content of leaves (Felix and Meins, 1987) and promotes lesion growth after viral infection (Pritchard and Ross, 1975). Combinations of the growth hormones auxin and cytokinin down-regulate class I β-1,3-glucanase and block ethylene-dependent induction of the enzyme in cultured tobacco cells and leaf explants (Felix and Meins, 1987; Vögeli-Lange et al., 1994b). Pretreatment of tobacco leaves with combinations of auxin and cytokinin before TMV infection can inhibit lesion formation (Simons et al., 1972; Kasamo and Shimomura, 1977).

A Working Hypothesis: β-1,3-Glucanase Deficiency Decreases Virus Susceptibility by Promoting Callose Deposition

The mechanism for the enhanced virus resistance of class I β-1,3-glucanase–deficient plants is not known. We cannot at present rule out the possibility that the ersatz β-1,3-glucanases (Beffa et al., 1993) specifically induced in these plants have an antiviral function or that β-1,3-glucanase deficiency results in subtle morphological changes that alter the efficiency of experimental infection. Another possibility is that reduced susceptibility is due to systemic acquired resistance induced by β-1,3-glucanase deficiency. This is unlikely because class II

β-1,3-glucanases, which are a specific marker for systemic acquired resistance (Ward et al., 1991b), were not constitutively expressed in the uninfected antisense transformants used (Beffa et al., 1993). Our working hypothesis is that enhanced resistance to virus in the local-lesion host is linked to increased callose deposition resulting from decreased production of β-1,3-glucanase. Callose is frequently deposited at the plasma membrane–cell wall interface within minutes after pathogen invasion (reviewed in Kauss, 1985). This effect is often more pronounced in resistant than in susceptible plants (e.g., Hächler and Hohl, 1984; Skou et al., 1984; Bonhoff et al., 1987). Treatments that delay the HR and local-lesion formation in TMV infection also decrease the rate of callose deposition (Wu and Dimitman, 1970). Thus, it is likely that callose serves as a physical barrier to contain viruses at the site of infection in the local-lesion host.

Histological studies of the local-lesion response show that aniline blue staining is more intense and earlier in antisense transformants than in control plants and extends into the necrotic lesion itself. Although the specificity of this stain and the effect of the physical state of polysaccharides on fluorescence intensity are uncertain, aniline blue staining is traditionally accepted as an indication of callose (reviewed in Stone and Clarke, 1992). Based on this criterion, infected, β-1,3-glucanase–deficient mutants accumulate more callose than do infected control plants. This effect, which is particularly conspicuous early in infection, is correlated with reduced β-1,3-glucanase activity relative to that of control plants. Moreover, induction of the ersatz β-1,3-glucanase activity in antisense plants is accompanied by a rapid drop in callose content. The amount of callose deposited is thought to depend on its rate of synthesis and rate of degradation by β-1,3-glucanases (Kauss, 1985). Our working hypothesis is that β-1,3-glucanase deficiency impairs callose degradation, which in turn increases steady state callose deposition in response to infection and reduces lesion size and virus yield.

The callose hypothesis might also explain the decreased susceptibility of β-1,3-glucanase–deficient mutants to systemic infection. Plasmodesmata, which are the major route of virus spread from cell to cell, are surrounded by collars containing callose (reviewed in Lucas et al., 1993). Transport of virus through plasmodesmata is facilitated by virus-encoded movement proteins, which increase permeability to the viral nucleoprotein complex. With increasing deposition of callose, the cytoplasmic annulus joining adjacent cells becomes constricted and the size exclusion limit for polymers moving through the plasmodesmata is reduced. This appears to be a reversible process that is dependent on the rates of callose synthesis and callose hydrolysis mediated by β-1,3-glucanases. We propose that β-1,3-glucanase deficiency might diminish the capacity of the movement protein to "dilate" plasmodesmata by reducing callose degradation and hence restricting systemic spread of virus.

One objection to our hypothesis is that the class I β-1,3-glucanases are localized in the cell vacuole, whereas the putative substrate, callose, is deposited outside the cell. Recent studies have shown that certain vacuolar proteins can be alternatively targeted to the cell wall (Kjemtrup et al., 1995), and secretion of class I β-1,3-glucanases into the medium of tobacco cells in suspension cultures has been reported (Kunze et al., 1995). The location of class I β-1,3-glucanases in virus-infected tissues has not been established by immunohistological procedures. The intriguing possibility that these enzymes might be rerouted to the cell wall during viral pathogenesis is still open.

Role of β-1,3-Glucanases in Plant-Pathogen Interactions

β-1,3-Glucanases are induced in the resistant host as part of a stereotypic response to infection by pathogenic or potentially pathogenic viruses, bacteria, and fungi (Meins and Ahl, 1989). There is strong indirect evidence that the class I β-1,3-glucanases can protect plants against fungus infection (Mauch et al., 1988; Zhu et al., 1994; Jongedijk et al., 1995). These enzymes also accumulate to high concentrations in epidermal leaf cells of uninfected plants. This is thought to be a constitutive defense against early stages in the invasion of leaves by fungi (Mauch and Staehelin, 1989; Keefe et al., 1990). It is of interest in this regard that viruses such as TMV require wounding of the epidermis to enter plants, that is, early events in virus infection are associated with cells rich in β-1,3-glucanase. Taken together, these findings and our conclusion that β-1,3-glucanases can promote virus spread lead us to speculate that the same pathogenesis-related protein can have opposite effects with different classes of pathogens. Apparently, necrotic viruses can use a defense mechanism of plants against fungi to their own advantage.

Plants can be protected against virus infection by sense and antisense transformation with selected genes of viral origin (reviewed in Wilson, 1993). Our findings suggest to us an alternative approach based on inactivating components of the host's response to viruses by antisense transformation with genes of plant origin.

METHODS

Plant Materials and DNA Transformation

Nicotiana sylvestris, *N. tabacum* cv Havana 425, and their transgenic derivatives were grown from seed in a greenhouse. The *N. sylvestris* transformants SAG2.3 and SCIB, the Havana 425 tobacco transformants TAG4.4 and TCIB, as well as the plasmids and procedures used to obtain additional transformants, have been described previously (Neuhaus et al., 1992; Beffa et al., 1993). The independent *N. sylvestris* lines SAG2.3, SAG2.5, and SAG2.7 and the Havana 425 line TAG2.7 were transformed with the antisense vector pAGL2. Plasmid pAGL2 contains the coding region from positions +27 to +608 of a class I tobacco β-1,3-glucanase gene in reverse orientation regulated by cauliflower mosaic virus 35S RNA expression signals and a bacterial neomycin phosphotransferase *nptII* gene as a plant-selectable kanamy-

cin resistance marker. The independent *N. sylvestris* line SAG4.3 and the Havana 425 lines TAG2.7, TAG4.3, and TAG4.4 were transformed with the antisense vector pAGL4. Plasmid pAGL4 is a variant of pAGL2 containing the entire 1111-bp coding region plus 184 bp of 3′ untranslated sequence of the β-1,3-glucanase gene in reverse orientation. The *N. sylvestris* line SCIB and Havana 425 line TCIB used as controls were transformed with the empty vector pCIB200. The lines SAG2.3, TAG4.4, SCIB, and TCIB are monogenic transformants homozygous for the transgene. The other transgenic plants used are kanamycin-resistant progeny of the primary transformants indicated that show reduced levels of β-1,3-glucanase activity.

Viral Infection

For local-lesion infections, the second and third horizontal leaves from the top of ~80-cm-tall Havana 425 plants and ~50-cm-tall *N. sylvestris* plants were infected with common strains of tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), respectively, as described previously (Vögeli-Lange et al., 1988; Beffa et al., 1993). Virus suspensions were diluted to give from 50 to 1500 lesions per leaf. The size detection limit for lesions was ~0.2 mm. For systemic infection of *N. sylvestris* with TMV, leaf 6 or 7, counting from the bottom of plants with 10 to 12 leaves, was inoculated with suspensions giving roughly 1000 lesions when assayed on Havana 425 tobacco. Mock-infected leaves were used as controls.

Assays for Infectious Virus

In assays of the local-lesion response, discs of tissue were cut with a radius of 4.5 mm from the center of individual lesions. Each leaf disc included the entire lesion. Three discs from each leaf were pooled, frozen on dry ice, stored at −70°C, and then homogenized in two volumes of 70 mM Sorensen's phosphate buffer, pH 7.0. After clarification by centrifugation in an Eppendorf microcentrifuge (Netheler and Hinz, Hamburg, Germany) for 5 min at room temperature, the extracts were diluted 1:50 and used to infect Havana 425 leaves as described above. Virus titer is expressed as the number of lesions obtained on the host 7 days after infection per lesion assayed. Six to 12 samples, each from a leaf at the same position on a different plant, were assayed. In assays of systemic infection, the indicated leaf was harvested and the midvein removed. The remainder of the leaf was frozen on dry ice, stored at −70°C, and then homogenized in 10 mL of Sorensen's buffer per gram of fresh weight of tissue. After clarification by centrifugation (5000*g* for 10 min at room temperature), the extracts were diluted if necessary to obtain a convenient number of lesions and used to infect Havana 425 leaves. Virus titer is expressed as the number of lesions obtained on the host after 7 days per gram of fresh weight of the leaf sampled.

Measurement of β-1,3-Glucanase Activity

Discs (9 mm in diameter) of leaf tissue without visible lesions and discs with a radius of 4.5 mm from the center of lesions were frozen on dry ice and stored at −70°C. Extracts were prepared as described by Beffa et al. (1993) and assayed for β-1,3-glucanase activity by the radiometric assay of Keefe et al. (1990), using ^{3}H-laminarin as the substrate. Enzyme activity is expressed in microgram equivalents of authentic tobacco class I β-1,3-glucanase (Felix and Meins, 1985) used as a standard.

Callose Staining and Quantitation

Callose was stained by a modification of the aniline blue fluorochrome method (Currier and Strugger, 1956). In brief, discs of leaf tissue with a radius of 4 mm from the center of lesions, when present, were fixed in 1% (v/v) glutaraldehyde, 5 mM citric acid, 90 mM Na_2HPO_4, pH 7.4, decolorized by boiling for 3 min in water and extracting for 25 min in 95% ethanol, and then stained with 0.1% (w/v) water-soluble aniline blue in 67 mM Sorensen's phosphate buffer adjusted to pH 12 with 1 N KOH. Fluorescence due to callose was measured in fields centered on individual lesions with use of an MPV3 microdensitometer equipped with a Ploemopak filter system D (excitation filter, 355 to 425 nm; dichroic mirror, 455 nm; barrier filter, 460 nm; Leitz, Wetzlar, Germany). Relative callose content is expressed per square millimeter of lesion-containing areas showing callose fluorescence. Areas were measured using a Quantimet 500+ (Leitz).

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