

Response of Transgenic Cucumber and Carrot Plants Expressing Different Chitinase Enzymes to Inoculation with Fungal Pathogens

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ABSTRACT

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Three lines of cucumber cv. Endeavor, each transformed with a chitinase gene originating from petunia (acidic), tobacco (basic), or bean (basic) using *Agrobacterium tumefaciens*, were compared with nontransgenic plants for response to inoculation with *Alternaria cucumerina*, *Botrytis cinerea*, *Colletotrichum lagenarium*, and *Rhizoctonia solani*. In both growth chamber studies using whole plants and in vitro inoculations conducted with detached leaves, no differences in disease development (rate and final levels) were detected between transgenic and nontransgenic plants. Carrot cvs. Nanco and Golden State transformed with two chitinase genes (from petunia and tobacco) were also evaluated for response to inoculation with the pathogens *Alternaria radicina*, *B. cinerea*, *R. solani*, *Sclerotium rolfsii*, and *Thielaviopsis basicola*. A detached petiole inoculation method was used to compare nontransgenic and transgenic plants. The rate and final extent of lesion development after 7 days were significantly ($P = 0.01$) lower in the transgenic plants expressing the tobacco (basic) chitinase gene upon inoculation with *B. cinerea*, *R. solani*, and *S. rolfsii*, but not in plants expressing the petunia (acidic) chitinase gene. There were no detectable differences with *A. radicina* or *T. basicola* in either group of transgenic plants. These results demonstrate the in planta efficacy of a basic chitinase protein in providing enhanced tolerance of carrot to three fungal pathogens; however, the efficacy of chitinase gene transformation as a strategy for enhancing disease tolerance in plants can be influenced by the plant species used, the type of chitinase protein expressed, and the characteristics of the fungal pathogen.

Additional keywords: antifungal, hydrolytic enzymes

Higher plants express a group of proteins, the pathogenesis-related (PR) proteins, upon infection by pathogenic microorganisms, which are reported to have a role in defense against pathogen development (4,23,42). Among these proteins are a group of hydrolytic enzymes, the chitinases, which have been extensively studied to determine their roles in plants that are challenged by fungal pathogens (3,11,13,15,35). Inoculation with various pathogens such as fungi, bacteria, and viruses, physical wounding, and application of chemical compounds, have all been reported to induce the production of plant chitinases, suggesting that the response also may be stress-induced (35). Two general groups of chitinases are known to occur in plants, i.e., acidic and basic, depending on their isoelectric points. Acidic (extracellular) chitinases have been postulated to lyse hy-

phae of invading fungal pathogens during the early stages of pathogenesis and to release fungal cell wall fragments, which in turn may activate other defense-related mechanisms (3,15,26). The basic (vacuolar) forms may affect the hyphae following plant cell collapse, when the vacuolar contents are released into the extracellular space (3,26). Differences in antifungal activity have been reported between the acidic and basic chitinases, depending on the characteristics of the groups within which they are placed (6,40).

The expression of genes encoding antifungal proteins in transgenic plants has been suggested as a strategy to enhance resistance to pathogenic fungi (3,12,21,42). Such expression could verify the role of these proteins in plant defense. Chitinase genes from some plant species, and from a bacterium (*Serratia marcescens*), have already been expressed in several transgenic plants, including tobacco (*Nicotiana tabacum*) (6-8,17,19,24,28,40,44,47), *N. sylvestris* (29,44), *N. benthamiana* (30), canola (*Brassica napus*) (6,8), tomato (*Lycopersicon esculentum*) (43), and rice (*Oryza sativa*) (22). However, subsequent evaluations of these transgenic plants for response to filamentous fungal pathogens

have not always been conclusive. For example, inoculation of transgenic *N. sylvestris* (29) and *N. benthamiana* (30) with *Cercospora nicotianae* revealed no differences when compared with nontransgenic controls. In contrast, increased chitinase levels in transgenic tobacco (7,8,17,44) and canola (8) were reported to significantly reduce susceptibility to *Rhizoctonia solani*.

Cucumber (*Cucumis sativus* L.) and carrot (*Daucus carota* L.) are affected by a number of fungal pathogens, some of which are difficult to control with conventional disease management approaches. To evaluate whether chitinases could play a role in enhancing disease tolerance, pickling cucumber and fresh market carrot cultivars were genetically engineered using *Agrobacterium tumefaciens* to overexpress different chitinase genes from various plant species (14,36). The evidence for stable integration and expression of these genes has been presented elsewhere (14,36). The objectives of this study were (i) to evaluate three transgenic lines of cucumber, each transformed with a chitinase gene originating from petunia (acidic), tobacco (basic), or bean (basic), for response to inoculation with the fungal pathogens *Alternaria cucumerina* (leaf spot), *Botrytis cinerea* (gray mold), *Colletotrichum lagenarium* (anthracnose), and *Rhizoctonia solani* (stem blight), and (ii) to evaluate two transgenic lines of carrot, transformed with the petunia and tobacco chitinase genes, for response to inoculation with *Alternaria radicina* (black rot), *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotium rolfsii* (southern blight), and *Thielaviopsis basicola* (black root rot).

MATERIALS AND METHODS

Plant materials: Cucumber. Selected transgenic lines of cucumber cv. Endeavor, each expressing an acidic chitinase gene cloned from petunia, a basic chitinase gene from tobacco (24), or a basic chitinase gene from bean (9), designated 105/196, 105/198, and 105/492, respectively, were evaluated. Each chimeric gene was under the control of the constitutive cauliflower mosaic 35S promoter. The evidence for integration and expression of the genes in the transgenic tissues has been previously described (36). The same plants of each transgenic line as described (36) were regenerated from embryogenic calli following *Agrobacterium tumefaciens*-mediated

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transformation and selection for resistance to kanamycin (36). The plants were multiplied for assay in this study by means of a cell suspension culture system as previously described (37). Nontransgenic plants that were included as controls were obtained as in-vitro-grown seedlings propagated in tissue culture media (33). All regenerated plantlets and seedlings at the two-true-leaf stage were subsequently transferred into plastic pots (12.5 × 12 cm) containing previously-sterilized potting mix (Sunshine Mix 1, Fisons Hort. Inc., Vancouver, BC) and placed in a Conviron growth chamber maintained at 24°C, 96% relative humidity, and a 16-h photoperiod. Plants were evaluated for response to fungal inoculation 2 to 6 weeks after transplanting.

Carrot. Transgenic lines of carrot cvs. Golden State and Nanco, containing one of the above chitinase genes, were evaluated. The transgenic plants were regenerated from transformed calli following *A. tumefaciens*-mediated transformation and multiplied by means of a cell suspension culture system as previously described (14). Evidence for the stable integration and expression of the genes has been presented elsewhere (14). Nontransgenic controls, which consisted of either seedlings grown in tissue culture or plantlets derived from embryogenic calli, were obtained by means of the same tissue culture procedures as for transformation (14). All seedlings and plantlets were transferred to sterile potting mix after 4 to 6 weeks of growth in vitro and maintained at 22 to 26°C under a 16-h photoperiod provided by Gro-Lux lamps at an intensity of 140 $\mu\text{E s}^{-1} \text{m}^{-2}$. Plants were

evaluated at various times during their growth, ranging from 4 to 12 months after transplanting.

Fungal pathogens. The following cucumber pathogens were used in this study: (i) *Colletotrichum lagenarium* race 1 (provided by J. Kuc, University of Kentucky), used in the evaluation of all three transgenic lines; (ii) *Alternaria cucumerina* (provided by R. X. Latin, Purdue University), used in the evaluation of transgenic lines expressing tobacco and bean vacuolar chitinases; (iii) *Botrytis cinerea* (isolated from carrot leaves), used in the evaluation of the transgenic line expressing bean chitinase, and (iv) *Rhizoctonia solani* AG-4 (provided by T. C. Wehner, North Carolina State University), used in evaluation of the transgenic line expressing bean chitinase. All fungal cultures were maintained on potato dextrose agar and incubated in the dark at an ambient temperature of 23 to 25°C. Colonies of *C. lagenarium* were subcultured to fresh medium every 4 to 5 weeks; colonies of the other fungi were subcultured every 2 weeks.

The following carrot pathogens were used in this study: (i) *Alternaria radicina* (provided by B. Pryor, University of California, Davis); (ii) *Botrytis cinerea* (isolated from carrot leaves); (iii) *Rhizoctonia solani* (provided by T. C. Wehner); (iv) *Sclerotium rolfsii* (isolated from carrot roots); and (v) *Thielaviopsis basicola* (isolated from carrot roots). All cultures were maintained on V8 agar (34) and incubated at 22 to 28°C on the laboratory bench. Colonies were subcultured prior to use in the inoculation experiments.

Inoculation procedure for cucumber: Whole plant assay. Conidia of *C. lagenarium* were obtained by flooding colonies, 14 to 20 days after the last subculture, with sterile distilled water and collecting the spore suspension with a Pasteur pipette. The spore density was adjusted to 10^7 per ml with the aid of a hemacytometer. Expanded leaves, one leaf per plant, of transgenic and nontransgenic (control) plants were inoculated by applying 16 or 30 droplets of the conidial suspension (5 μl each) to the adaxial surface. In each experiment a group of 10 to 12 plants representing the transgenic line and a comparable group of control plants were used. To obtain plants of comparable size, transgenic plants were 24 to 34 days old while control plants were 14 to 18 days old, after transfer to soil. The lines were evaluated in separate experiments and each experiment was repeated at least once with similar numbers of plants. Transgenic lines 105/198 and 105/492 were also inoculated with *A. cucumerina*. A 12-day-old culture was used as the source of hyphal inoculum. Plants were inoculated by placing two mycelial plugs (4-mm-diameter) on the adaxial surface of each leaf that was basipetally next to the leaf inoculated with *C. lagenarium*. Both inoculations with *C. lagenarium* and *A. cucumerina* were carried out at the same day. The experiment was repeated once.

Transgenic line 105/492 was inoculated with *B. cinerea* and *R. solani* simultaneously. Nine-day-old cultures of both pathogens were used as the source of hyphal inoculum. Inoculation with *B. cinerea* was conducted by placing two agar plugs (4-mm-diameter) on the adaxial surface of each of the youngest fully expanded leaves of the plants, one plug on each side of the leaf. Each leaf that was basipetally next to those inoculated with *B. cinerea* was inoculated with *R. solani*, also by placing two agar plugs of hyphal inoculum. Inoculations with both fungi were carried out at the same day, and the experiment was repeated once.

Following all inoculations, plants were maintained under the same conditions that preceded inoculation, except that during the first 24 h, the relative humidity within the growth chamber was increased to 99%. The development of disease symptoms was recorded at times ranging from 3 to 12 days after inoculation. The number of lesions formed and the diameter of lesions were recorded. The data for each pathogen were averaged over each experiment, and the standard error of the means for all replicate plants was calculated.

Detached leaf assay. A detached leaf assay was also used to evaluate responses to *B. cinerea* and *R. solani*. Each leaf was placed, adaxial surface up, in a petri dish (100 × 20 mm) containing moistened filter paper. One group of 5 to 8 leaves of 105/492 and one from control plants were

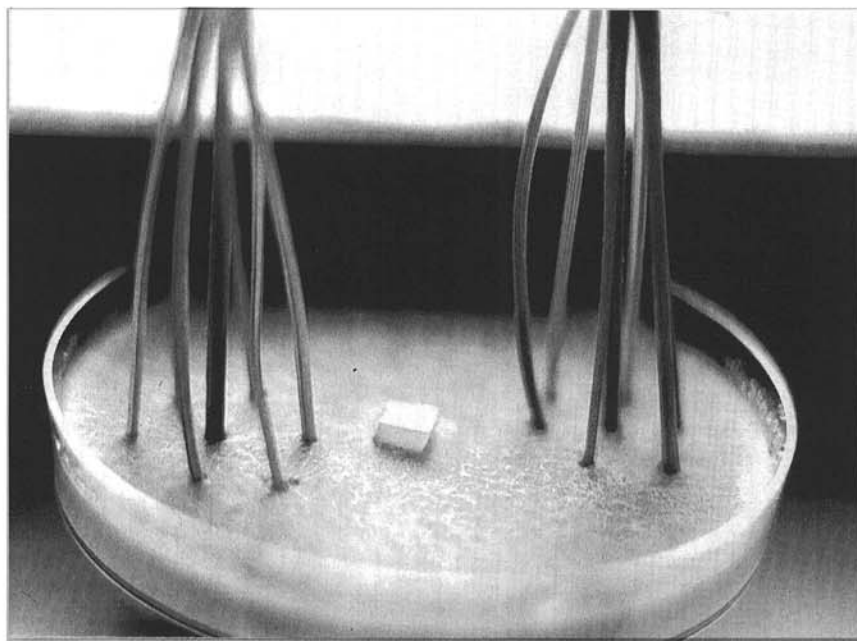


Fig. 1. Detached petiole assay used to evaluate the response of transgenic and nontransgenic carrots to fungal inoculation. Petioles were placed upright into a colony of *Rhizoctonia solani*; left group of petioles are from transgenic plants, the right group from nontransgenic. Photograph was taken 1 day after the experiment was set up.

inoculated with the pathogens by placing two mycelial plugs (4 mm diameter) on each leaf, one on each side of the leaf. A different set of leaves was used for *B. cinerea* and *R. solani*. Two control leaves were inoculated with potato dextrose agar plugs as a negative control. The extent of lesion development was determined after 7 to 9 days of incubation at 25°C.

Inoculation procedure for carrot. Two transgenic lines (105/196 and 105/198) were evaluated with all of the pathogens by means of a detached petiole assay. Petioles from transgenic and control plants of each cultivar were selected from similar-aged plants. The petioles were excised at the crown, the leaves were trimmed off, and 10- to 16-cm-long sections were washed under running tap water for 3 to 5 min. Petioles of similar diameter were grouped and cut into 8-cm-long sections; six segments were combined and assayed together as one sample. Petioles from transgenic and control plants were inserted upright into actively growing colonies (in 100 × 15 mm petri dishes) of the fungal pathogens; the distal ends of the petioles were grouped and held together with tape (Fig. 1). Where possible, samples of six petiole segments from transgenic and control plants were placed on the same fungal colony, at opposite ends. The colony ages for the respective fungal pathogens were as follows: *A. radicans* (14 days), *B. cinerea* (4 or 7 days), *R. solani* (4 or 7 days), *S. rolfii* (4 or 7 days), and *T. basicola* (14 days). The petri

dishes with the petioles were sealed in plastic bags lined with moistened paper towels. The extent of lesion development from the base of the petiole upward was measured after 2, 4, and 7 days of incubation at 22 to 24°C. Lesion length was measured to the nearest millimeter on each petiole, and the mean lesion length for each colony was determined. In each experiment, two to three cultures of each inoculum age were used to assess each cultivar/transgenic line. The experiment was conducted five times, each with two to three replicate colonies of each pathogen, using petioles from the same transgenic plants collected at different times during growth. Data from all of the experiments were subjected to analysis of variance. Untransformed data were analyzed by the general linear modeling (GLM) program in the SAS statistical software package (SAS Institute, Cary, NC). Pairwise comparisons of treatment means (lesion size) were made for transgenic versus control tissues for both cultivars at each of the three assessment dates and for the two inoculum types. Significant differences are indicated at $P = 0.01$ according to the least significant difference test.

RESULTS

Response of cucumber to fungal pathogens: *C. lagenarium* race 1. Leaves inoculated with *C. lagenarium* developed lesions typical of those caused by this

pathogen. Disease development was rated as the percentage of inoculum droplets that resulted in lesion formation and the average diameter of developing lesions. There was no significant difference in the percentage of lesion formation (4 and 12 days postinoculation) (Fig. 2A), or in the diameter of lesions (12 days postinoculation) (Fig. 2B), between transgenic 105/196 and control plants. Similarly, evaluations of transgenic lines 105/198 and 105/492 showed that the percentage of lesion formation on transgenic and control plants at all evaluation days (3, 6, and 9 days postinoculation) was not significantly different (Figs. 3 and 4). However, for the two latest evaluation dates with lines 105/198 and 105/492, lesions on transgenic plants were slightly larger than those on the control plants (Figs. 3 and 4). All of the results were consistent between leaves on different plants and between experiments.

***A. cucumerina*.** Following inoculation of transgenic line 105/198 with *A. cucumerina*, hyphae of the fungus grew from inoculum plugs onto the leaf. Lesions were visible 9 days after inoculation, and the extent of lesion formation was similar on both transgenic and control leaves (data not shown).

***B. cinerea* and *R. solani*.** Following inoculation of transgenic line 105/492 with *B. cinerea*, the leaves were rapidly infected, as indicated by the formation of visible lesions. Although hyphal growth

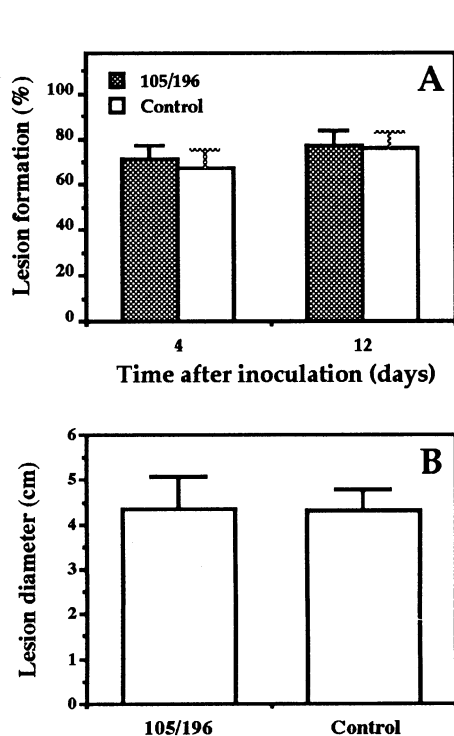


Fig. 2. Lesion development in cucumber cv. Endeavor transgenic (105/196) and control plants following inoculation with *C. lagenarium* race 1. (A) Percentage of lesion formation 4 and 12 days postinoculation. (B) Diameter of lesions 12 days postinoculation. Vertical bars represent standard errors of the mean.

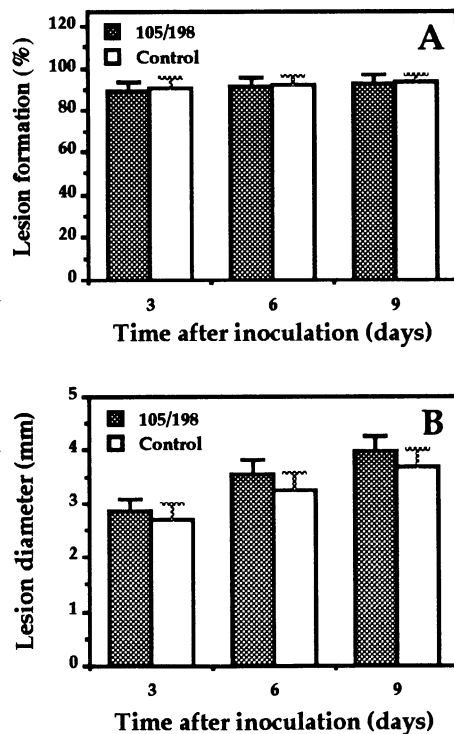


Fig. 3. Lesion development in cucumber cv. Endeavor transgenic (105/198) and control plants following inoculation with *C. lagenarium* race 1 (3, 6, and 12 days postinoculation). (A) Percentage of lesion formation. (B) Diameter of lesions. Vertical bars represent standard errors of the mean.

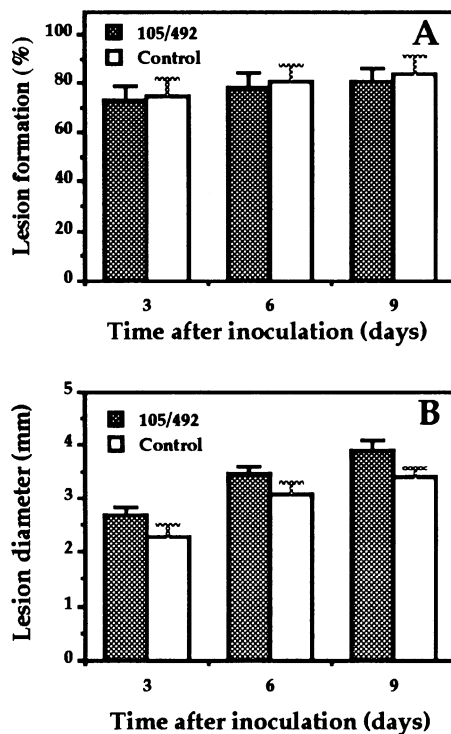


Fig. 4. Lesion development in cucumber cv. Endeavor transgenic (105/492) and control plants following inoculation with *C. lagenarium* race 1 (3, 6, and 12 days postinoculation). (A) Percentage of lesion formation. (B) Diameter of lesion. Vertical bars represent standard errors of the mean.

from inoculum plugs was evident on most of the leaves inoculated with *R. solani*, no lesions or only small irregular lesions were formed; there was no detectable difference in lesion development between transgenic and control plants. With *B. cinerea*, lesion diameters on leaves of transgenic and control plants were 6.7 and 7.0 mm, respectively, on whole plants; lesion sizes were 16.3 and 15.2 mm, respectively, on detached leaves (Fig. 5). In all tests, there were no significant differences between the lesion sizes on leaves of transgenic and control plants.

Response of carrot to fungal pathogens. The detached petiole assay was a sensitive and reproducible method for rating the extent of fungal development on transgenic and control tissues. In general, significant differences were not apparent until 4 days after inoculation with most of the pathogens tested. Following extensive fungal colonization, there was collapse of the petioles within 5 to 7 days due to extensive cell maceration. To prevent this, each group of petioles was supported by a central wooden stake, which held them upright until the experiment was completed (Fig. 1).

Transgenic line 105/196 was inoculated with all of the pathogens and lesion development was assessed at 2, 4, and 7 days postinoculation. Statistical analyses of the data did not show a significant difference between transgenic and nontransgenic plants in several experiments (data not shown). Transgenic line 105/198 was also challenged with the pathogens and com-

parisons were made with control plants. With *B. cinerea*, lesion size was significantly ($P = 0.01$) smaller in transgenic plants of both Golden State and Nanco at 4 and 7 days after inoculation (Fig. 6). There was no apparent influence of inoculum age (4 or 7 days) on lesion development. With *R. solani*, lesion development was significantly reduced in the transgenic cultivar Nanco at all evaluation times (2, 4, and 7 days) when a 4-day-old culture was used as inoculum (Fig. 7). The transgenic cultivar Golden State showed a significant reduction only after 7 days. When the inoculum age was increased to 7 days, only Nanco showed a significant reduction in lesion size and only at day 7. Overall lesion sizes with 7-day-old inoculum were lower in both control and transgenic plants compared with 4-day-old inoculum (Fig. 7).

With *S. rolfisii*, both transgenic cultivars showed a significant reduction in lesion size only at day 7 (Fig. 8), with 4-day-old inoculum. With 7-day-old inoculum, overall lesion sizes were smaller and transgenic cultivar Nanco showed significant reductions in disease at all evaluation times (Fig. 8), while Golden State had lower disease only at day 7.

DISCUSSION

The results from this study have shown that (i) chitinase overexpression enhanced tolerance to pathogen infection in carrot but not in cucumber tissues, (ii) plant cultivar and/or gene integration event may alter the disease response, (iii) different chitinase genes (and the corresponding

protein) differ in antifungal activity in planta, and (iv) pathogenic fungi differ in susceptibility of chitinases in vivo.

The lack of significant differences between transgenic and nontransgenic tissues of cucumber to fungal infection could be explained by the relatively small increase in overall chitinase activity in transgenic leaves (36). Previous analyses of chitinase levels in the same plants as those used in this study indicated that expression levels were increased by 1.5-fold to twofold (36). In other studies in which reductions in disease development due to chitinase overexpression were reported, the increases ranged from 14-fold (44) to up to 44-fold (8). Thus, selection of lines with the greatest enzyme levels might increase the likelihood of obtaining a disease tolerant phenotype. Broglie et al. (8) and Lin et al. (22) reported that the level of resistance to *R. solani* in transgenic plants expressing a basic chitinase gene was correlated with the level of chitinase enzyme expression. In carrot tissues, analyses conducted with the same group of plants as those used in this study confirmed the expression of the chitinase gene (14) and preliminary experiments indicated that the magnitude of these increases was greater than that obtained for cucumber (Z. K. Punja and S. H. T. Raharjo, unpublished data).

Plant chitinases are grouped into different classes (6,27) and it was recently shown that, in tobacco, members of one class (class I, basic) have higher activity against fungi than another class (class II, acidic) has (12,40). While the bases for

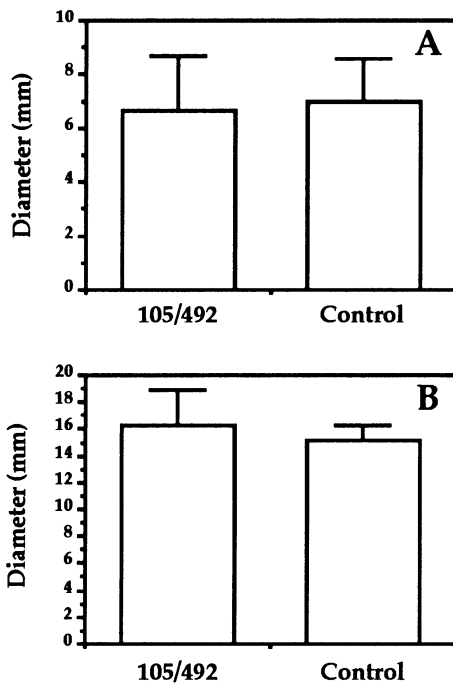


Fig. 5. Diameter of lesions on leaves of cucumber cv. Endeavor transgenic (105/492) and control plants following inoculation with *B. cinerea* (7 days postinoculation). (A) Intact plants. (B) Detached leaves. Vertical bars represent standard errors of the mean.

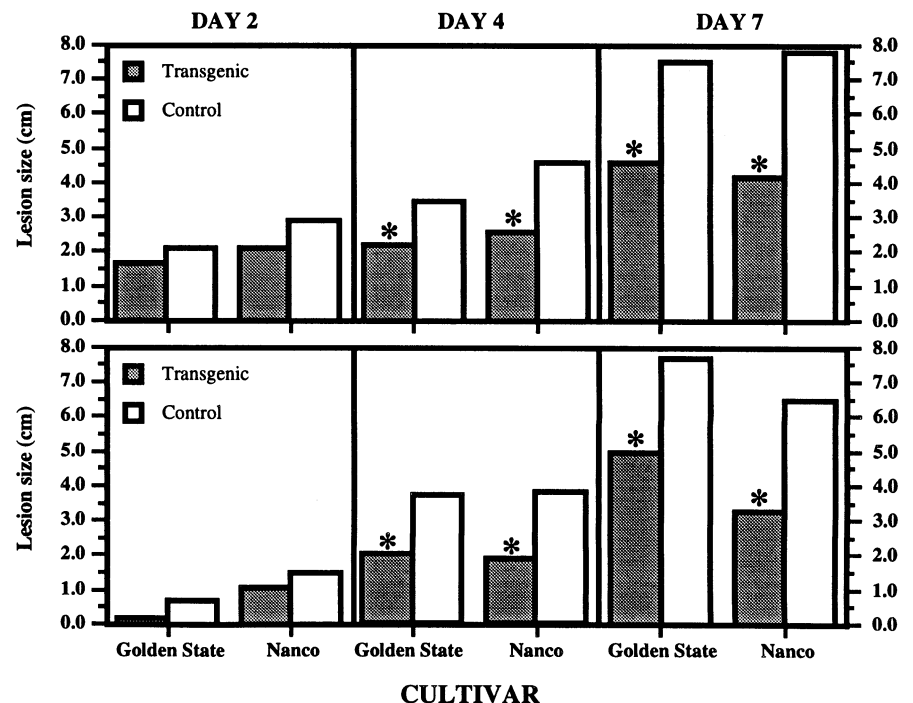


Fig. 6. Lesion development on transgenic (105/198) and control petioles of two carrot cultivars at 2, 4, and 7 days after inoculation with *Botrytis cinerea*. Response to inoculation with (top panel) a 4-day-old culture and (lower panel) a 7-day-old culture. (*) indicate significantly lower disease compared with the control ($P = 0.01$).

these differences have not been determined, the selection of the chitinase for transformation studies can influence the outcome with regard to disease response. When the research leading up to this study was initiated (14,36), little was known about the differences in antifungal activity of various classes of chitinases. The acidic (class II) chitinase from petunia used in this study (105/196) may have lower antifungal activity, accounting for the lack of an effect when expressed in transgenic cucumber and carrot plants. Although the acidic chitinases are postulated to play an important role during the early stages of host-pathogen interactions (26), the inherent differences in antifungal activity may influence the outcome. Modified targeting of a basic (vacuolar) chitinase to the extracellular space has been achieved (28,40,44) and these plants may have a higher level of tolerance to infection provided that the chitinase expression levels are similar.

The response of nontransgenic and transgenic cultivars of carrot, i.e., Nanco and Golden State, to disease development varied depending on the fungal pathogen. Previous work on chitinase gene transformation into plants has generally been conducted with one cultivar, e.g., in tobacco (8,17,40), canola (8), tomato (43), and rice (22). The inclusion of additional cultivars and evaluation against a range of fungal pathogens should provide useful information on the range of variability of the host-pathogen response in these transgenic plants.

The extent to which chitinase causes cell wall degradation and inhibition of fungal growth is influenced by fungal cell wall composition, which differs with the taxonomic grouping of the fungi (15,38,45). In general, the regions most susceptible to chitinase attack are the growing tips (1,3,7,11,31). However, few studies have evaluated the response of a range of pathogenic fungi to different classes of chitinases in vitro (5). Usually, a *Trichoderma* species is included as the candidate fungus since it is sensitive to chitinase (5,39). However, several pathogens, including *R. solani* (1,2,7,18), *S. rolfsii* (18,31), *F. oxysporum* (18) and *F. solani* (40,43), and *T. basicola* (46), are known to be affected by chitinase. Transgenic plants that were more tolerant to infection by *R. solani* (2,7,8,17,44) and *F. oxysporum* (43) have been described, and the efficacy of bacteria that express chitinases for biocontrol of *S. rolfsii*, *R. solani*, and *F. oxysporum* has been demonstrated (18).

In this study, transgenic carrots had reduced lesion size and rate of expansion when inoculated with *R. solani*, *S. rolfsii*, and *B. cinerea* but not when challenged with *A. radicans* and *T. basicola*. The susceptibility of hyphae of the latter two fungi to chitinase is not known. The reduction in disease development in transgenic carrots was less marked when the colonies used as

inoculum were actively growing (4 days old) in *S. rolfsii* compared with 7-day-old cultures, likely due to the more rapid and prolific growth and the availability of nutrients in the former. A comparable inoculum-dose response was observed in transgenic tobacco inoculated with *R. solani*

(8). The detached petiole assay method was developed to ensure uniformity of inoculum and disease response. Although representing an artificial method, the exposure of cut petioles to a mycelial culture is a vigorous test since the inoculum is growing from a nutrient base. Under field

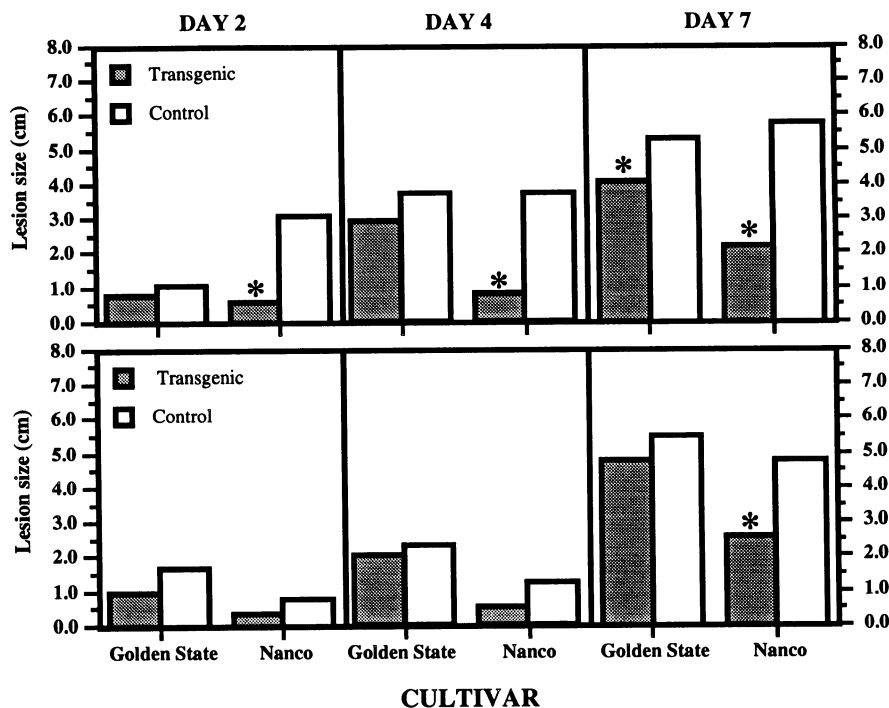


Fig. 7. Lesion development on transgenic (105/198) and control petioles of two carrot cultivars at 2, 4, and 7 days after inoculation with *Rhizoctonia solani*. Response to inoculation with (top panel) a 4-day-old culture and (lower panel) a 7-day-old culture. (*) indicate significantly lower disease compared with the control ($P = 0.01$).

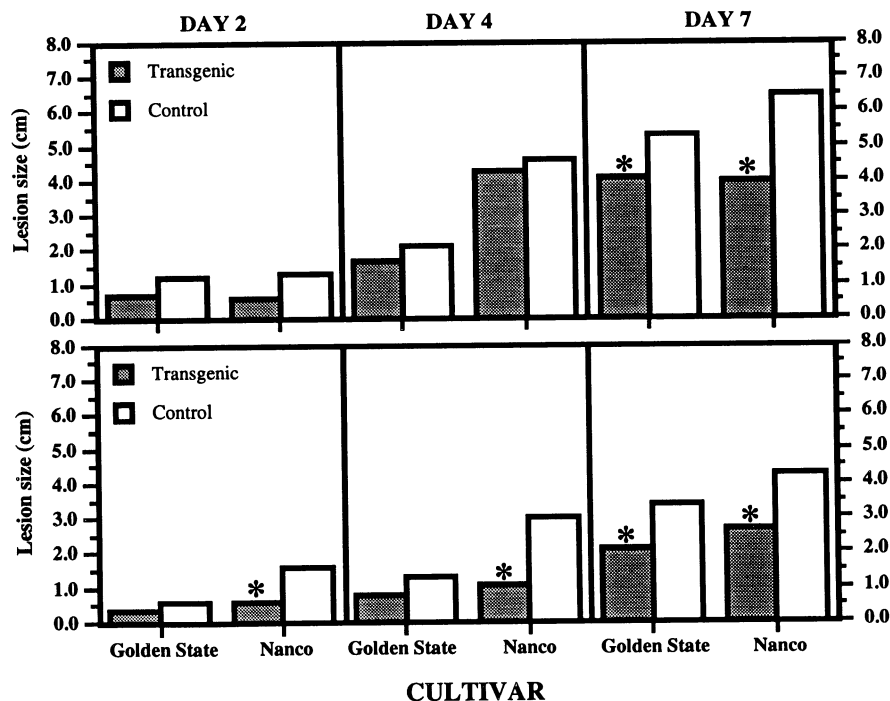


Fig. 8. Lesion development on transgenic (105/198) and control petioles of two carrot cultivars at 2, 4, and 7 days after inoculation with *Sclerotium rolfsii*. Response to inoculation with (top panel) a 4-day-old culture and (lower panel) a 7-day-old culture. (*) indicate significantly lower disease compared with the control ($P = 0.01$).

conditions, mycelia of *B. cinerea*, *R. solani*, and *S. rolfisii* generally infect the petioles at the crown and frequently this occurs at wound sites (10,16,32). Any statistical differences between transgenic and non-transgenic tissues with the laboratory inoculation method used in the study should be indicative of whole plant responses in the field, where inoculum pressure would be lower. The reduction in lesion size was more apparent at later evaluation times (7 days), possibly because the reduction of fungal growth in transgenic tissues was cumulative. In other studies, transgenic plants exhibited a delay in the appearance of disease symptoms and had lower overall disease incidence when compared with nontransgenic plants (7,17,19,22,43,44).

When transgenic plants are infected by different fungal pathogens, the manner in which tissues are colonized by the invading hyphae could influence the disease response. In tobacco, overexpression of a basic (vacuolar) chitinase had no effect on *Cercospora nicotiana* (29,30), a pathogen that grows intercellularly and that would not encounter the intracellular chitinase unless host cells were lysed (12). Similarly, biotrophic pathogens that grow intercellularly would not be expected to be adversely affected by basic chitinases accumulating in the vacuole. Thus, knowledge of pathogen behavior in host tissues should be considered prior to evaluating the transgenic plants. In this study, the fungi that were inhibited by vacuolar chitinase, i.e., *R. solani*, *S. rolfisii*, and *B. cinerea*, are pathogens that invade intracellularly and cause host cell collapse and death (2,10,41), sometimes in advance of the growing mycelium (10,41). The sudden release of a vacuolar chitinase upon cell collapse would be expected to have a pronounced effect on pathogen growth (3). In contrast, both *A. radicans* and *T. basicola* do not cause extensive cell disruption or death (34) and this could be one of the reasons why they were not affected.

A major constituent of fungal cell walls, in addition to chitin, is β -1,3 glucan (3,38, 45), which serves as a protective barrier to the action of chitinases (3,25). The susceptibility of fungi to chitinases in combination with glucanase is greater than to either one alone (12,25,40,43). The expression of both chitinase and glucanase genes in transgenic tobacco (40,47) and tomato (43) has provided significantly higher protection against fungal development. Therefore, chitinase overexpression in combination with glucanase, other PR-proteins (12, 19,20,42), or other enzymes that enhance the levels of antifungal compounds (20, 21), should provide greater and more broad-spectrum activity against fungal pathogens.

NOTE ADDED IN PROOF

A recent article by Grison et al. (Nature Biotech. 14:643-646, May 1996) has demonstrated

that disease development due to three different fungal pathogens on transgenic oilseed rape expressing a tomato chitinase gene was reduced under field conditions.

ACKNOWLEDGMENTS

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