

Induction of Chitinases in Tobacco Plants Systemically Protected Against Blue Mold by *Peronospora tabacina* or Tobacco Mosaic Virus

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ABSTRACT

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Direct detection of chitinases after polyacrylamide gel electrophoresis (PAGE) run with an anodic buffer system revealed eight chitinase isozymes in tobacco, designated as C1–C8. Inoculation of three to four lower leaves of tobacco plants with tobacco mosaic virus (TMV) or stem injection of tobacco with sporangiospores of *Peronospora tabacina* systemically protected plants against blue mold caused by *P. tabacina* and systemically elevated the activities of C5 and C6 in the protected plants to about seven times higher than those in controls. On subsequent challenge with *P. tabacina*, the activities of C5 and C6 in the systemically protected plants were further enhanced and were about 10 times higher than those in the controls at 2 days after challenge. The activities of C5 and C6 were markedly elevated in the control plants when symptoms started to appear four days after challenge. However, the activities of C1–C4, C7, and C8 were constant and similar in both the protected and control plants before and after challenge with *P. tabacina*. Experiments with protoplasts and intercellular washing fluid indicated that C1–C3, C5, and

C6 were predominantly located in intercellular spaces, C4 was present in both intracellular and intercellular spaces, and C7 and C8 were uniformly distributed inside and outside of tobacco cells. The data support the suggestion that the induction of chitinases may be considered as one of the general metabolic responses participating in resistance, each of which is nonselectively induced and effective against many but not all pathogens. The coordinate expression of many different resistance mechanisms can restrict development of diverse pathogens and appears to be responsible for both induced resistance of susceptible plants and resistance of noninduced resistant plants. Both kinds of resistance are determined by the regulation of the rapidity and magnitude of gene expression for resistance mechanisms. Although susceptible plants lack gene(s) responsible for the rapid recognition of specific pathogens, they have genes for resistance mechanisms and gene(s) for their regulation. Thus resistance can be induced in susceptible plants by affecting the gene(s) regulating the genes for resistance mechanisms.

Additional keywords: isozyme detection, *Nicotiana tabacum*, systemic induced resistance.

Chitinase (EC 3.2.1.14) and β -1,3-glucanase (EC 3.2.1.39) activities are enhanced in various plants upon infection (4,5,16,23). Because chitin and β -1,3-glucan are major cell wall components of many pathogenic fungi (3,32), these two enzymes have been suggested to play a role in plant defense against pathogens (1,4,5,23). Chitinase and β -1,3-glucanase increase concurrently in plant tissues in response to ethylene (1,16), infection (5,16), and treatment with elicitors (10,16). The induction of the two enzymes is coordinately regulated in bean leaves (31). These two enzymes act synergistically in the partial degradation of isolated fungal cell walls (36), and combinations of the two enzymes strongly inhibit growth of most fungi tested, including those that can not be inhibited by chitinase or β -1,3-glucanase alone (17). These data suggest that a parallel increase in activities of the two enzymes may be important for their optimal function in plant defense.

Stem injection with sporangiospores of *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus (TMV) induces systemic protection of tobacco against blue mold (*P. tabacina*) and disease caused by the virus (6,15,26,34). We have reported that chitinase and β -1,3-glucanase activities were increased systemically in leaves of protected plants (20,29,35), and that the increased activities of two β -1,3-glucanase isozymes (G1 and G2) were positively correlated with the onset and extent of systemic protection (22). However, *P. tabacina* belongs to the Oomycetes, which do not contain chitin in their cell walls (3). This suggests that chitinase should not be effective against *P. tabacina*. Therefore, it is of interest to determine whether any chitinase isozyme, like β -1,3-glucanase, is correlated with the induction of systemic protection. Techniques for detecting chitinase and β -1,3-glucanase isozymes have been developed by Trudel and Asselin (25) and Pan et al (18), respectively. Recently, we developed a technique to detect chitinase, β -1,3-glucanase, and protein pattern after a single separation using polyacrylamide gel electrophoresis (PAGE) (21). This enables us to study the

association of chitinase isozymes with induction of systemic protection and their coordinate induction with β -1,3-glucanase. A preliminary report described aspects of this study (20).

MATERIALS AND METHODS

Plants and pathogens. Tobacco (*Nicotiana tabacum* L.) cultivars Ky 14 and Samsun NN were grown in a greenhouse with a photoperiod of 14 h with daylight supplemented by sodium light at a temperature regime of 25–33 C. Seeds were planted in Pro-Mix BX (Premier Peat Corp. Marketing, New York, NY) in small trays and seedlings were watered with a 0.005% 15:16:17 (N:P:K) fertilizer solution (Peters Fertilizer, W. R. Grace and Co., Fogelsville, PA). After 4 wk, seedlings were transplanted to pots (6.5-in pots for small plants and 10-in pots for plants taller than 80 cm) containing Pro-gro 300 (Pro-gro Product Inc., Elizabeth City, NC) and fertilized three times a week with 0.00625% 15:16:17 (N:P:K) Peters fertilizer. Two isolates of *P. tabacina* obtained in Kentucky in 1979 and 1982 (29), designated as isolates 79 and 82, were maintained by weekly transfers of sporangiospores on young Ky 14 tobacco plants in a growth room with a 16-h photoperiod under fluorescent and incandescent light at 20–25 C. Purified TMV in sterile water was provided by Dr. J. Shaw, Department of Plant Pathology, University of Kentucky.

Induction and challenge. The plants were induced by stem injection of 5×10^5 sporangiospores ml^{-1} of *P. tabacina* isolate 82 in the greenhouse (26) or by inoculation with $25 \mu\text{g ml}^{-1}$ of TMV on three to four lower leaves of plants at the 9–10 leaf stage in a growth room with a 14-h photoperiod under white fluorescent and incandescent light at 23 C (34). Control plants were stem-injected with water or mock inoculated. Unless indicated otherwise, leaves above the sites of injection with *P. tabacina* or inoculation with TMV were challenged with 5×10^4 spores ml^{-1} of *P. tabacina* isolate 79 in the growth room, 21 days after stem injection with *P. tabacina* or 12 days after leaf inoculation with TMV (26,34). The isolates of *P. tabacina* used for induction and challenge were chosen based on the availability of inoculum. Both isolates are equally effective when used for induction and challenge.

Preparation of enzyme extract. Leaf lamina tissue was collected at various time intervals after induction and challenge and immediately frozen at -80 C. The samples were extracted with 0.05 M sodium acetate (pH 5.0) buffer by grinding with sea sand at 4 C with a mortar and pestle. The extracts were centrifuged at 10,000 g for 10 min, and the supernatants were dialyzed at 4 C against two changes of H_2O and then two changes of 0.01 M sodium acetate (pH 5.0) overnight (MW cutoff: 3,500). After centrifugation at 10,000 g for 10 min, the supernatants were used as crude enzyme extracts.

Native polyacrylamide gel electrophoresis (PAGE) and detection of chitinase isozymes. Electrophoresis under native conditions was performed on slab gels by the method of Davis (7) with a 3.75% stacking gel and a 15% separating gel. A 15% PAGE gel or 7.5% polyacrylamide isoelectrofocusing (IEF) gel was used to separate and detect chitinase isozymes as described previously (21). Glycol chitin was used as the substrate. To quantify activities of chitinase isozymes, the photographs having the chitinase bands were then scanned with an LKB Ultrosan XL Laser Densitometer (LKB, Bromma, Sweden) (21). Signal areas were used to express the relative chitinase activities.

Isolation of protoplasts and collection of intercellular washing fluid (IWF). Twelve days after leaf inoculation with TMV, protoplasts were isolated from leaves above the sites of induction (2). The leaves above the sites of induction were also infiltrated with water under reduced pressure (24). Infiltrated leaf tissues were blotted dry between paper towels and IWF was collected after centrifugation at 1,000 g for 10 min. The leaves above the sites of induction were extracted, as described earlier, before and after IWF collection, respectively.

Determination of glucose-6-phosphate dehydrogenase activity. Glucose-6-phosphate (G-6-P) dehydrogenase activity was used as

a marker for cytoplasmic contents (24) and determined according to Kornberg and Horecker (11) except that the enzyme extracts were prepared in 0.05 M sodium acetate (pH 5.0) buffer as described above. A unit of enzyme activity was defined as the amount that causes an initial change in absorbance of 1.000 per min at 340 nm (11).

RESULTS

Chitinase isozyme pattern and its association with induction of systemic protection. Twelve days after inoculating lower leaves of Ky 14 plants with TMV, the leaves above were challenged with *P. tabacina*. The chitinase isozymes in the challenged leaves were detected on 7.5% IEF gels as described previously (21). The dominant chitinase isozymes detected in the challenged leaves were reported to be acidic proteins (21). Therefore, acidic chitinase isozymes in the challenged leaves were investigated at various times after challenge by using 15% PAGE gels run with an anodic buffer system (21). Eight dominant chitinase isozymes, designated as C1–C8, were detected in the tobacco plants (Fig. 1). Among the eight isozymes, six isozymes, C1–C4, C7, and C8, had constant enzyme activities during the period of induction and challenge in both the protected and control plants. The activities of these six isozymes in the protected plants were similar to those in the control plants during the period of induction and challenge. However, the activities of the other two isozymes, C5 and C6, were correlated with the induction of systemic protection. Twelve days after induction (i.e., at the time of challenge), a significant level of activity of C5 was present in the protected plants, but the activity of C5 was not detectable in the control plants, and the activity of C6 was higher in the protected plants than that in the control plants (Fig. 1A,B). At 2 days after challenge, the activities of C5 and C6 were further elevated in the protected plants, but the activity of C5 was still not detectable in the control plants, and the activity of C6 in the control increased but was still lower relative to the protected plants (Fig. 1C,D). At 4 days after challenge, disease symptoms started to appear in the control plants. At that time, the activity of C5 became detectable and the activity of C6 increased further in the control plants, but the activities of both C5 and C6 were still lower in controls as compared to those in protected plants (Fig. 1E,F). At 6 days

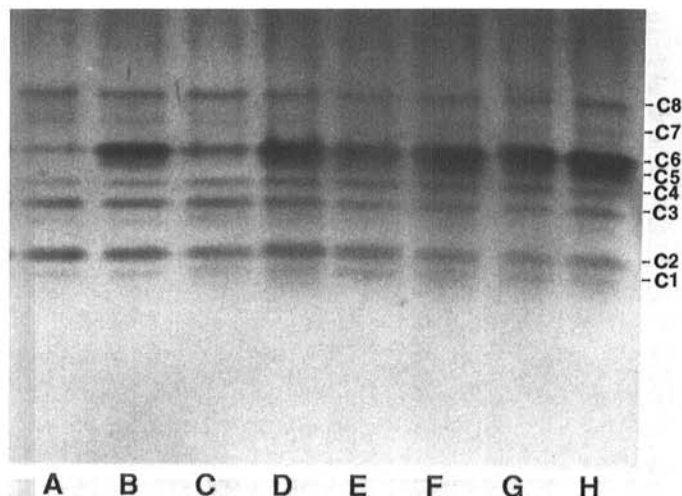


Fig. 1. The effect of inoculating three to four lower leaves of Ky 14 tobacco plants with tobacco mosaic virus (TMV) on the isozyme pattern of chitinase in systemically protected leaves challenged with *Peronospora tabacina*. The noninfected upper leaves of control plants (A, C, E, and G) and TMV-induced plants (B, D, F, and H) were challenged with *P. tabacina*. Crude enzyme extracts were prepared from the challenged leaves at 0 time (A, B), 2 (C, D), 4 (E, F), and 6 (G, H) days after challenge. Crude enzyme extracts (150 μg of total protein) were loaded on 15% PAGE gels and stained for chitinase isozymes. The eight isozymes detected on the gel are designated as C1–C8, respectively.

after challenge, severe symptoms occurred in the control plants, and the activities of C5 and C6 in the control plants were similar to those in the protected plants (Fig. 1G,H).

Because C5 and C6 could not be separated for densitometer scans, the quantitative data are the sum of C5 and C6. Densitometry indicated that 12 days after induction with TMV, the activities of C5 and C6 in the protected plants were about seven times higher than those in controls. On subsequent challenge with *P. tabacina*, the activities of C5 and C6 in the systemically protected plants were further enhanced and were about 10 times higher than those in the controls at 2 days after challenge (the signal area for the protected was 2.3 ± 1.0 ; the area for the control was 22.7 ± 3.5).

Comparison of chitinase isozymes in tobacco plants. Crude enzyme extracts from different sources were loaded on the same 15% PAGE gel to determine whether the same isozymes are correlated with the induction of systemic protection in different tobacco cultivars or in tobacco plants induced by different agents. As shown in Figure 2, the same mobilities were found for the isozymes from TMV-infected Samsun NN leaves (Fig. 2B), and for the isozymes from challenged, systemically protected leaves of Samsun NN plants induced by TMV (Fig. 2D) and Ky 14 plants induced by leaf inoculation with TMV (Fig. 2F) or by stem injection with *P. tabacina* (Fig. 2H). Two days after challenge with *P. tabacina*, the activities of C5 and C6 in the protected leaves were higher, relative to the controls, not only in the Ky 14 plants induced by TMV inoculation (Fig. 2E,F), but also in the Ky 14 plants induced by stem injection with *P. tabacina* (Fig. 2G,H) and Samsun NN plants induced by TMV inoculation (Fig. 2C,D). However, the activities of C1–C4, C7, and C8 were virtually constant in extracts from the different sources.

Localization of chitinase isozymes. Protoplasts were isolated from the unchallenged systemically protected leaves of TMV-induced plants and the crude enzyme extract was prepared as described for leaves. Crude enzyme extracts were also prepared from the unchallenged systemically protected leaves of Ky 14 plants induced by TMV inoculation, from the systemically protected leaves after collection of IWF by filtration and centrifugation and from the IWF obtained from the systemically protected leaves. G-6-P dehydrogenase activity was assayed in these enzyme

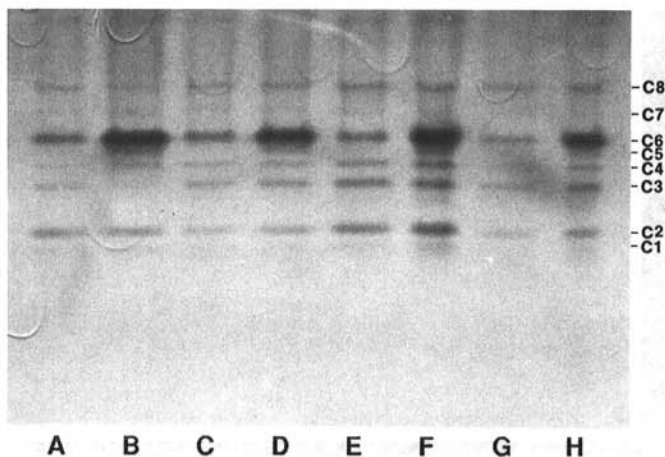


Fig. 2. Comparison of chitinase isozymes from Samsun NN and Ky 14 tobacco plants induced with tobacco mosaic virus (TMV) or *Peronospora tabacina* on 15% PAGE gels. **A**, mock-inoculated tobacco Samsun NN leaves (6 days after treatment); **B**, TMV-inoculated Samsun NN leaves (6 days after inoculation); **C**, challenged, not mock-inoculated upper leaves from mock-inoculated Samsun NN plants (2 days after challenge); **D**, challenged systemically protected leaves from Samsun NN plants induced with TMV (2 days after challenge); **E**, challenged, not mock-inoculated upper leaves from mock-inoculated Ky 14 plants (2 days after challenge); **F**, challenged systemically protected leaves from Ky 14 plants induced with TMV (2 days after challenge); **G**, challenged leaves of Ky 14 plants stem-injected with water (2 days after challenge); **H**, challenged systemically protected leaves of Ky 14 plants stem-injected with *P. tabacina* (2 days after challenge).

extracts and used as a marker for cytoplasmic contents, because this enzyme is largely confined to the cytoplasm in plants (9). The specific activity of G-6-P dehydrogenase in the protoplasts was higher than that in the systemically protected leaves from which the protoplasts were isolated (Fig. 3A,B), suggesting that the protoplasts were intact and cytoplasmic proteins were still compartmentalized inside the cells. Collection of IWF did not significantly change the specific activity in the leaves (Fig. 3B,C), indicating that there was little leakage of cytoplasmic contents into the IWF. This is further supported by the fact that the specific activity in the IWF was lower than that in the leaves (Fig. 3D). Therefore, the activities of chitinase isozymes were assayed in these enzyme preparations to investigate their locations in the systemically protected leaves. Because the same amount of total protein (200 μ g) from each preparation was loaded on the same PAGE gel, the activity of each isozyme may be considered as specific activity. Lower specific activities of C1–C3, C5, and C6 were detected in the protoplasts that had intact cytoplasmic contents inside than in the leaves from which the protoplasts were isolated (Fig. 3A,B). Furthermore, the specific activities of C1–C3, C5, and C6 in the leaves were reduced after a single collection of IWF by filtration and centrifugation (Fig. 3B,C), and their

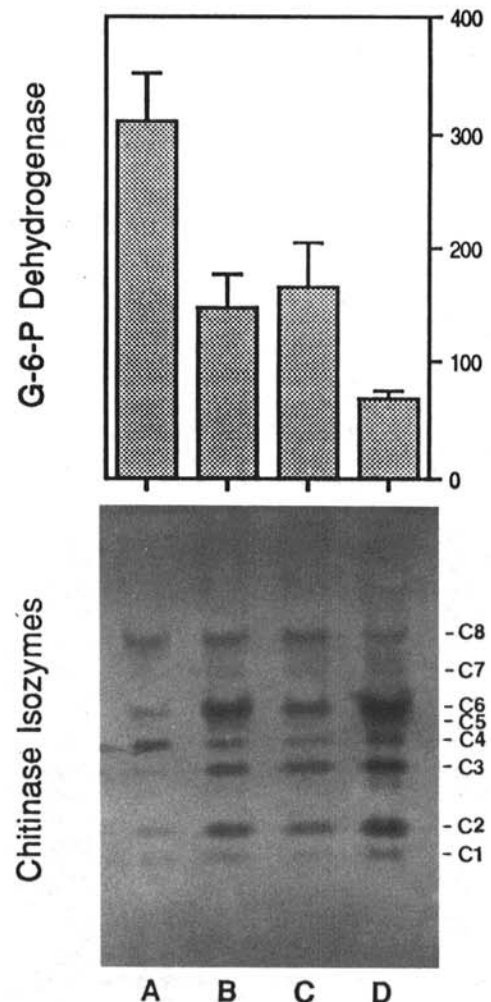


Fig. 3. Localization of chitinase isozymes. The activities of chitinase isozymes and glucose-6-phosphate (G-6-P) dehydrogenase were assayed in protoplasts from **A**, unchallenged systemically protected leaves from Ky 14 plants induced with tobacco mosaic virus (TMV); **B**, crude enzyme extract from the same leaves as above; **C**, from the same leaves as above after collection of intercellular washing fluid (IWF); and **D**, IWF from the same leaves as above. Two-hundred micrograms of total protein was applied per lane on a 15% anodic PAGE gel to assay chitinase isozymes. G-6-P dehydrogenase activity was expressed as milliunits per milligram of protein and used as marker for cytoplasmic contents. The error bars are the standard deviations of three replications.

specific activities were higher in IWF than in the crude extract of the leaves although there was a lower amount of cytoplasmic contents in IWF (Fig. 3B,D). This suggested that C1-C3, C5, and C6 are at least predominantly located in intercellular spaces. Higher specific activity of C4 was detected in the protoplasts than in the leaves (Fig. 3A,B), indicating that a significant amount of C4 was within the cells. After collection of IWF, the specific activity of C4 was reduced in the leaves (Fig. 3B,C), and the specific activity was higher in IWF than in the leaves (Fig. 3B,D), indicating that a substantial amount of C4 was also freely located in intercellular spaces. The specific activities of C7 and C8 were similar in the protoplasts (Fig. 3A), in the crude extract of leaves before (Fig. 3B) and after (Fig. 3C) collection of IWF, and in the IWF (Fig. 3D). This suggests that C7 and C8 were uniformly distributed inside and outside of tobacco cells.

DISCUSSION

Susceptible tobacco plants can be systemically protected against blue mold (*P. tabacina*) by stem injection with sporangiospores of *P. tabacina* (6,26) or by inoculating three to four lower leaves with TMV (34). Twelve days after leaf inoculation with TMV, systemic induced resistance reached a maximum (34), at this time the activities of acidic chitinases C5 and C6 were increased in the systemically protected leaves (Fig. 1). Similarly, 21 days after stem injection with *P. tabacina*, tobacco plants were highly protected (27), and the activities of the two acidic chitinases were also increased in the protected leaves (data not shown). Upon challenge with *P. tabacina*, the activities of these two chitinases were further elevated in the protected plants (Fig. 1). The activities in the control plants were also increased upon challenge, but they were lower than those in the protected plants at the early stage, 0-4 days after challenge. By 4 days after challenge, disease symptoms started to appear in the control plants, and at that time the chitinase activities began to markedly increase in the control. Optical and electron microscopy showed that blue mold development in the protected plants was restricted 2 days after challenge (33). The crucial difference in the enzyme activities between the protected and control plants at the early stage of challenge (2 days after challenge) was observed not only in Ky 14 plants induced by leaf inoculation with TMV but also in Ky 14 plants induced by stem injection with *P. tabacina* and Samsun NN plants induced by leaf inoculation with TMV (Fig. 2). Assays for chitinase isozymes in extracts of isolated protoplasts and in IWF indicated that chitinases C5 and C6 were primarily located in intercellular spaces, where fungal pathogens are likely to encounter them after penetration.

The association of chitinases C5 and C6 with induction of systemic protection was similar in pattern to the association of two acidic β -1,3-glucanases, G1 and G2 (19,20,22). The data support a coordinate induction of chitinases and β -1,3-glucanases in systemically protected plants. This is in agreement with the report that chitinases and β -1,3-glucanases are coordinately induced by ethylene in bean leaves (31). The use of a recent technique for detection of chitinase, β -1,3-glucanase and protein patterns after a single separation using PAGE facilitates studies of the coordinate induction of chitinases and β -1,3-glucanases relevant to systemic induced resistance (21).

Chitinase isozymes C1-C4, C7, and C8, however, did not appear to be associated with the induction of systemic protection, because the activity of these six isozymes was virtually constant and similar in both induced and control plants before and after challenge with *P. tabacina* (Fig. 1). This phenomenon was observed not only in Ky 14 plants induced by leaf inoculation with TMV but also in Ky 14 plants induced by stem injection with *P. tabacina* and Samsun NN plants induced by leaf inoculation with TMV (Fig. 2).

The parallel induction of chitinases C5 and C6 and β -1,3-glucanases G1 and G2 in the systemically protected plants is consistent with the previous reports that chitinase and β -1,3-glucanase act synergistically in the partial degradation of isolated fungal cell wall (36), and combinations of the two enzymes inhibit growth

of fungi that can not be inhibited by chitinase or β -1,3-glucanase alone (17). However, *P. tabacina* belongs to the Oomycetes, and they do not contain chitin in their cell walls (3).

Therefore, it seems likely that the induction of chitinases C5 and C6 may be considered one of the general metabolic responses, associated with plant disease resistance, which include accumulation of chitinases, β -1,3-glucanases, peroxidases, PR-proteins, phytoalexins, lignin, callose, and hydroxyproline-rich glycoproteins (4,5,12,13,30). Such responses occur concurrently in various plants after infection by various pathogens (4,5,12,13,30), and they can nonspecifically inhibit the growth and/or development of various pathogens (4,5,13,17). Furthermore, some of these changes occur upon infection even though an obvious role for the change in disease resistance is not evident (e.g., PR-proteins are markedly elevated in resistant tobacco plants upon infection by TMV [30], although PR-proteins apparently do not directly affect disease caused by TMV) (8,14,35). It appears improbable to consider any one of these metabolic responses as a resistance mechanism solely responsible for restricting development of a specific pathogen. Although these responses have been proposed as resistance mechanisms (4,5,12,13,30), the genes encoding the mechanisms should be called genes for resistance mechanisms or defense genes and not resistance genes. Kuć and co-workers have presented evidence that susceptibility or resistance is determined not by the presence or absence of genes for resistance mechanisms but by the rapidity and magnitude with which the genetic information is expressed (12,13,28). This evidence is further supported by numerous reports that resistance can be induced systemically in susceptible plants by using various biotic and abiotic agents (12,13,28). Systemic induced resistance is non-specific and effective against various pathogens (12,13,28). The general metabolic responses associated with natural resistance of noninduced resistant plants are similar, if not identical, to those associated with systemic induced resistance (12,13,19,20,29,33,34,35). Both kinds of resistance are determined not by the presence or absence of genes for resistance mechanisms but by the rapidity and magnitude of gene expression. Although each individual resistance mechanism is nonspecifically effective against many, but not all, pathogens, the coordinated expression of many different resistance mechanisms, if not all, can inhibit various pathogens and appears to be responsible for both induced resistance of susceptible plants and resistance of noninduced resistant plants. The genes for resistance mechanisms and genes for their regulation are probably present in all plants and insure the survival of plant species. The regulation of such genes by biotic or abiotic agents may find use for the effective control of plant disease.

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