Induced Systemic Resistance to Blue Mold: Early Induction and Accumulation of β-1,3-Glucanases, Chitinases, and Other Pathogenesis-Related Proteins (b-Proteins) in Immunized Tobacco

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ABSTRACT

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Tobacco plants (burley Ky 14) were immunized against blue mold by stem injections with sporangiospores of the blue mold pathogen, *Peronospora tabacina*. Enzyme activity assays and western blot analyses indicated that β-1,3-glucanases increased in immunized but not in control plants up to 21 days after stem injection. β-1,3-Glucanases continued to increase in the immunized plants 2 and 6 days after challenge, but in controls comparable levels were detected only 6 days after challenge, when the disease was already extensive. Electrophoretic analyses indicated increases in amounts of several b-proteins in immunized plants prior to challenge. Some b-proteins increased further 2 and 6 days after challenge. Increases in b-proteins were detected in controls only 6 days after challenge. A basal level of chitinases was always detected, but increases in chitinases above this level in immunized plants followed a profile similar to that of the β-1,3-glucanases and other b-proteins. It is likely that β-1,3-glucanases, chitinases, and other b-proteins are coordinately regulated in tobacco. The increases in these proteins coincided with the onset of immunization in plants injected with *P. tabacina*, and the levels were maintained during the period after challenge, when the development of *P. tabacina* was restricted.

The blue mold disease of tobacco is caused by the oomycete *Peronospora tabacina*. Stem injections with spores of the pathogen induce systemic resistance to blue mold (immunization) under both greenhouse (10,48,49) and field conditions (51). Protection, over 95% in immunized plants, compared with water-injected controls, is elicited by a single injection and persists throughout the growing season (48,49). Regenerants of immunized plants obtained from tissue culture are also protected (50), and the factor (signal) or factors responsible for protection are graft-transmissible (49). Biochemical changes associated with immunization have been studied. Levels of β-ionone (39), glucose, and fructose (38) and activities of peroxidase and polyphenol oxidase (S. D. Salt, *personal communications*) are elevated in immunized plants; however, the relationship of these factors to the mechanism or mechanisms of immunization is not yet clear.

The accumulation of soluble proteins in plants infected by viruses (2,36), fungi (14), bacteria (13,28), and viroids (17) has been reported. These proteins, called b-proteins (14,16) or PR-proteins (3), are host-encoded. They are also induced by treatment with chemicals such as polyacrylic acid (15), acetylsalicylic acid (58), and plant hormones (53,55). Electrophoretic analyses of tobacco proteins labeled with ^{14}C-amino acids indicated that at least 10 b-proteins are associated with the hypersensitive response to TMV (54,56). Genes for the tobacco (20,36) and parsley (45) b-proteins have recently been cloned. Subsequently, de novo synthesis of mRNA of b-proteins in tobacco infected by TMV was demonstrated in two cultivars of tobacco (11,20) and in elicitor-treated parsley cells (45). Furthermore, probing of tobacco genomic DNA with cDNA clones specific to b-proteins indicated that b-proteins are coded by a family of genes (37). It has been suggested that these proteins play a role in induced resistance to subsequent viral (15,35,36) as well as fungal (14) infection in hypersensitive tobacco. A positive correlation between b-proteins and resistance to virus has also been reported for interspecific hybrids of *Nicotiana glutinosa* and *N. debneyii* (2). These hybrids produce b-proteins constitutively without induction and develop resistance to challenge inoculation with TMV. However, the accumulation of such proteins with aging, mechanical injury, and osmotic stress, factors not known to induce resistance, suggests that the accumulation of b-proteins alone may not be sufficient to explain disease resistance (12,34). Biological functions of b-proteins have recently been studied (22,27,41). Four of the b-proteins in tobacco were identified as chitinases (27) and four as β-1,3-glucanases (22). In potato, six b-proteins were identified as chitinases and two as β-1,3-glucanases (23). The objective of the present study was to examine tobacco plants that had been stem-injected with *P. tabacina* or water, to investigate the induction and accumulation of β-1,3-glucanases, chitinases, and other b-proteins prior to and subsequent to challenge with the blue mold pathogen. A preliminary report of this research has been presented (52).

MATERIALS AND METHODS

Biological materials and experimental design. Burley tobacco plants (*N. tabacum* L. 'Ky 14') were grown in the greenhouse (20–26 C in fall and winter and 20–33 C in spring and summer, with daylight supplemented with 16 hr of fluorescent and incandescent light) in 2-L pots containing Pro-Mix Bx (Premier Peat Corp. Marketing, New York, NY). The pots were watered twice a week to saturation with a 0.1% 15:16:17 (N:P:K) fertilizer solution (Peters Fertilizer, W. R. Grace and Co., Fogelsville, PA). Five plants were used per time point in each experiment. The midrib was cut out of the third fully expanded leaf from the top of each plant, and the center portion of each half-lamina of these leaves was obtained. The leaf samples were either processed immediately or frozen in liquid N_2 and stored at −80 C. The samples from plants with identical treatments were pooled for analysis. Each experiment was repeated at least three times. Representative results are presented.

Isolate 82 of *P. tabacina* was used for stem inoculations. The

isolate was collected at Spindletop Farm, Lexington, Kentucky, in 1982. The pathogen was maintained on 6- to 9-wk-old Ky 14 plants grown in growth chambers at 20 C, and leaves with sporulating lesions were stored in a freezer until used to prepare sporangiospore suspensions for stem injections. Isolate 79 of *P. tabacina* was used for challenge inoculations. The isolate was collected from a field near Georgetown, Kentucky, in 1979 and was maintained as described above. Sporangia of both isolates elicit systemic resistance, and both are effective for challenge inoculations (48). Sporangial suspensions of *P. tabacina* were prepared, and approximately 1 ml of the suspensions (5×10^5 sporangiospores per milliliter) was injected into the stems of 8- to 10-wk-old tobacco plants as described (48). Control plants were injected with distilled water. The plants were challenged, 21 days after stem inoculation, with a sporangial suspension of *P. tabacina* (approximately 50 ml per plant, with 10^5 sporangiospores per milliliter) in growth chambers, where they were kept until the termination of the experiment as described (48). Leaf symptoms and sporulation were determined as previously described (48).

Extraction of b-proteins. Pooled leaf samples were collected 15, 21, 23, and 27 days after stem injection and frozen in liquid N_2. A portion (1–3 g) of each mixed sample was ground to a fine powder in a cold mortar, and the resulting powder was extracted with one volume of buffer (pH 2.8) containing 84 mM citric acid, 32 mM Na_2HPO_4, 14 mM β-mercaptoethanol, and 6 mM ascorbic acid. The extracts were centrifuged at 10,000*g* for 30 min, and the pellet was discarded. Protein was determined as described by Bradford (9) (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (Sigma Chemical Co., St Louis, MO) as standard. The samples at 21 days after immunization were taken before the challenge of the leaves with *P. tabacina*.

Enzyme assays. Leaf samples were taken as described above, frozen in liquid N_2, and then extracted in two volumes of 0.2 M Tris HCl buffer (pH 7.8) containing 14 mM β-mercaptoethanol. The enzyme assays appeared more reliable in this procedure than in the procedure for total b-protein extraction described above. Chitinase activity was determined by radiochemical assay with [^{3}H]chitin (50,000 cpm/μmol) as substrate (8). Different dilutions of leaf homogenates were tested to determine the appropriate dilutions for the assay. Dilutions giving a linear relationship of protein to activity were used for enzyme assays. The reaction mixture consisted of 10 μl of diluted homogenate, 0.8 mg of [^{3}H]chitin, and 5 μmol of sodium phosphate buffer (pH 6.4) in a final volume of 0.25 ml. The reaction was stopped after a 45-min incubation at 37 C by the addition of 0.25 ml of 10% aqueous trichloroacetic acid (TCA). After centrifugation, the radioactivity of 0.2 ml of the supernatant was determined.

The activity of β-1,3-glucanase was assayed by measuring the rate of release of reducing sugar from laminarin (Sigma Chemical Co.) as substrate. The assay mixture consisted of 0.4 ml of 0.1 M acetate buffer (pH 4.8) containing 1% laminarin and various volumes of enzyme extract. After a 30-min incubation at 37 C, 1 ml of Somogyi reagent (44) was added, and the solution mixed thoroughly. After the addition of 0.6 ml of distilled water, the mixture was mixed, heated in boiling water for 15 min, and cooled to room temperature. Then 0.5 ml of Nelson's reagent (33) was added, and the mixture was vortexed and incubated 15 min at room temperature. The absorbance was measured at 660 nm (4). Standards of glucose and enzyme, substrate, or buffer blanks were included. Differences in enzyme activities between the treatments were determined by using analysis of the covariance to compare rates of change in enzyme activity over the course of the experiments.

Polyacrylamide gel electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 or 12.5% (w/v) slab gels containing sodium dodecyl sulfate (SDS) (26). The gels were cross-linked with 0.3% (w/v) *N,N'*-methylenebisacrylamide at pH 8.3, and stacking gels were made with 5% (w/v) polyacrylamide at pH 6.8. Samples containing 100 μg of protein were denatured by heating at 100 C for 2 min in 1% SDS containing 100 mM β-mercaptoethanol and Tris-glycine buffer at pH 6.8. The gels were double-stained for protein; they were first stained with Coomassie Brilliant Blue R-250, destained by immersion in a solution containing 10% acetic acid and 50% methanol, and then silver-stained with the Bio-Rad Silver Stain kit (Bio-Rad Laboratories). The procedure of Towbin et al (47) was used for immunodetection with the modifications described by Johnson et al (21).

RESULTS

Induction of β-1,3-glucanases. Tobacco plants stem-injected with *P. tabacina* were protected against disease after subsequent challenge with the fungus. Twenty-one days after stem injection, protection was greater than 95% in immunized plants, compared with controls, as determined by leaf area with lesions and by sporulation (data not shown). β-1,3-Glucanase activity increased in immunized plants prior to challenge, 15 and 21 days after stem injection with *P. tabacina* (Fig. 1B). These immunized plants were challenged by leaf inoculation 21 days following stem injection. Enzyme activity continued to increase in immunized tobacco 2 and 6 days after challenge. In contrast, β-1,3-glucanase activity was not detected in extracts of water-injected control plants prior to challenge (Fig. 1A). Very low activity was sometimes observed in control plants 2 days following challenge. The activity increased by 6 days after challenge (Fig. 1A). At this time disease symptoms were already apparent.

The changes in the levels of proteins cross-reacting with antibodies against bean β-1,3-glucanases in western blots (Fig. 1C and D) showed some correspondence to the observed changes

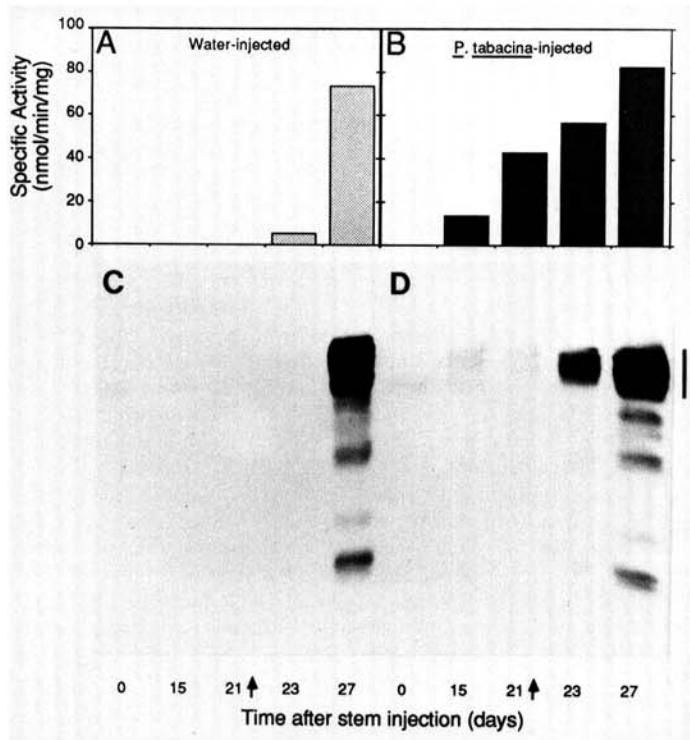

Fig 1. β-1,3-Glucanase activity (**A** and **B**) and western blot analysis of proteins (**C** and **D**) in extracts of tobacco plants stem-injected with water (**A** and **C**) or *Peronospora tabacina* (**B** and **D**). The plants were challenged by sprays of sporangiospore suspensions of *P. tabacina*. The arrows at 21 days indicate the time of challenge. β-1,3-Glucanase activity is expressed as nanomoles of glucose equivalents liberated per minute for each milligram of protein. The data are representative of three separate experiments. Statistical analysis of data obtained from all experiments indicated that the linear response over days is significantly different between different treatments ($p \geq 0.01$). The known locations of bean β-1,3-glucanases (57) are indicated by the bar at the right side of panel D. The identities of the smaller cross-reacting proteins that accumulate at 27 days after stem injection are not known.

in the enzyme activity. This may reflect differences between the extraction buffer used for the enzyme assays and that used to extract the b-proteins for subsequent western blot analysis. Alternatively, some of the samples may contained significant levels of inactive enzyme that, nevertheless, were detected by the antibodies. Western blot analysis should not be taken as a strict quantitative equivalent of enzyme activity assays.

Induction of chitinases. Constitutive levels of chitinase activity were detected in extracts of both control and immunized plants prior to challenge (Fig. 2A and B). Enzyme activity increased in extracts of immunized plants, but not in those of control plants, 15 and 21 days after stem injection with *P. tabacina*. Increased activity was evident in both immunized and control plants 2 and 6 days after challenge; however, higher enzyme activity was detected in immunized plants than in control plants 2 days after challenge (Fig. 2A and B).

Western blot analyses with antibodies against tobacco chitinases indicated the presence of cross-reacting proteins (Fig. 2C and D). To confirm the identities of the proteins, separate gels were sliced, and the proteins were eluted and precipitated by acetone. The proteins were then renatured by dissolving in 6 M guanidine hydrochloride (18), and their activity was determined. Chitinase activity was associated only with the region of the gels corresponding to the major antigens observed in the western blot, as indicated by the bar at the right side of panel D in Figure 2. As was the case for β-1,3-glucanase (see above), changes in the levels of these proteins roughly correlated with changes in activity, but the correspondence was not absolute.

Induction of b-proteins. Several b-proteins appeared to be increased in tobacco plants upon infection with *P. tabacina* (Fig. 3). The most pertinent of these changes (indicated by arrows in Fig. 3) occurred in immunized plants 21 days after stem injection, before challenge. b-Proteins continued to increase in immunized plants 2 and 6 days after challenge, whereas increased amounts were detected in controls only 6 days after challenge. Increased levels of two proteins (approximate mol wt 58,000 and 65,000) were consistently associated with immunization (Fig. 3). The smaller protein was present prior to and after challenge, whereas the larger protein appeared only 6 days after challenge, and only in the immunized plants (Fig. 3).

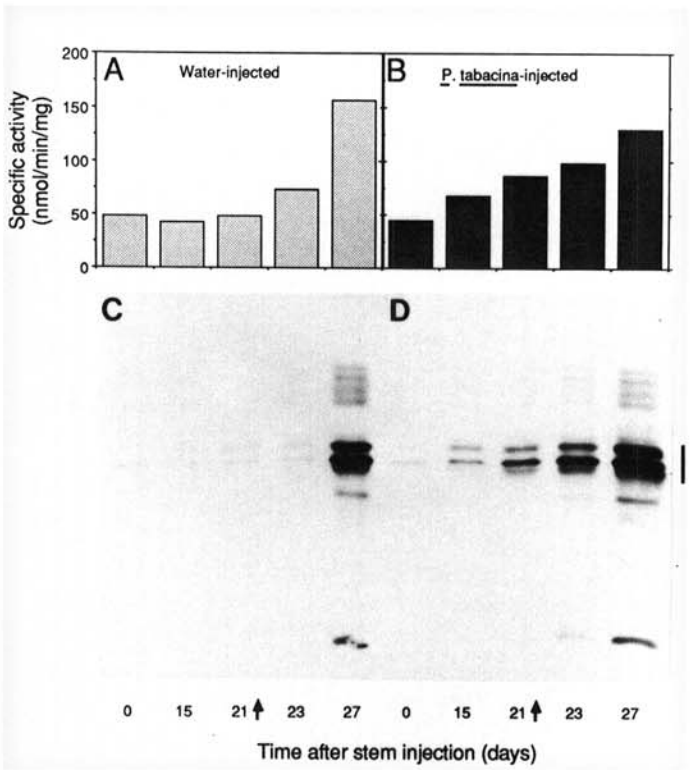

Fig. 2. Chitinase activity (**A** and **B**) and western blot analysis of proteins (**C** and **D**) in extracts of tobacco stem-injected with water (**A** and **C**) or *Peronospora tabacina* (**B** and **D**). The plants were challenged by sprays of sporangiospore suspensions of *P. tabacina*. The arrows at 21 days indicate the time of challenge. Chitinase activity is expressed as nanomoles of *N*-acetylglucosamine equivalents liberated per minute for each milligram of protein. The data are representative of four separate experiments. Statistical analyses of data obtained from all experiments indicated that the linear response over days is significantly different between different treatments ($p \geq 0.01$). The known locations of active chitinases (19) are indicated by the bar at the right side of panel D. The identities of other cross-reacting proteins are not known.

DISCUSSION

The early accumulation of β-1,3-glucanases, chitinases, and several other b-proteins in tobacco plants is associated with induced systemic resistance against blue mold. These observations suggest the b-proteins, especially β-1,3-glucanases, may have a role in the immunization of tobacco. β-1,3-Glucan and chitin are major cell wall components of many fungi (5), and β-1,3-glucanases and chitinases can hydrolyze cell walls of some fungal pathogens and nonpathogens in vitro (40,42). On the basis of these observations, these enzymes have been proposed as

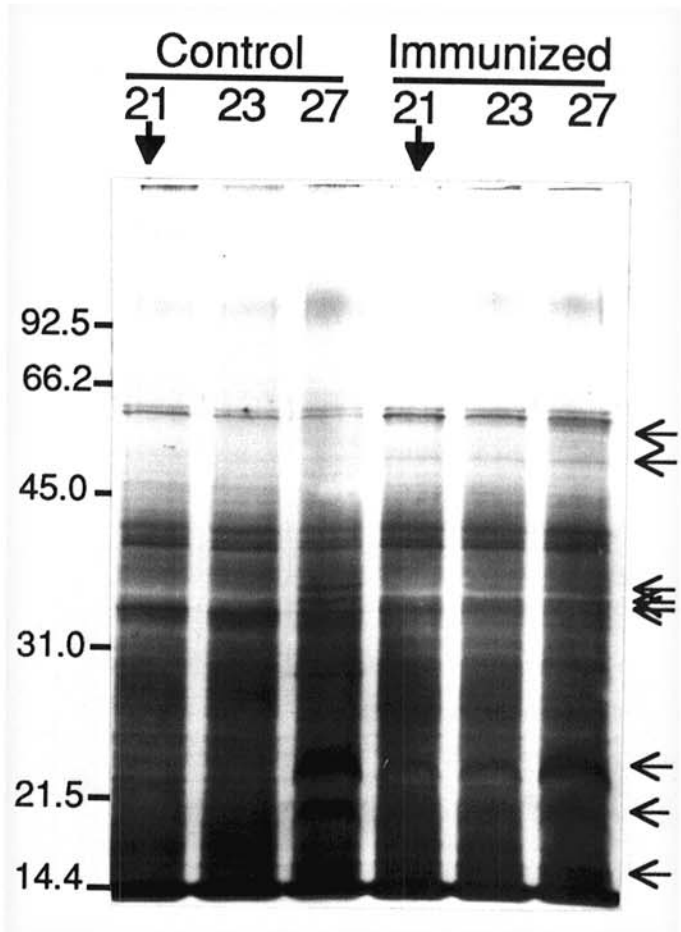

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of b-proteins in tobacco plants stem-injected with water (control) or *Peronospora tabacina* (immunized) before and after challenge with the same fungus. The plants were challenged 21 days after stem injection, as indicated by arrows at the top of the figure. The numbers at the top of the figure indicate the time (in days) after stem injection. The most pertinent changes in b-proteins are indicated by arrows at the right side of the figure. SDS-PAGE was performed in a polyacrylamide gel (10%, w/v) double-stained with Coomassie Brilliant Blue R-250 prior to silver staining, as described in the text. The following markers were used as molecular weight standards: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase B (92,500).

important in the disease resistance of plants (1,7,8,61). *P. tabacina*, an oomycete, does not contain chitin as a cell wall component; however, β-1,3-linked glucans are present in the wall (5). β-1,3-Glucanases may, therefore, be a component of the plant's defense against *P. tabacina* by restricting the development of the pathogen in leaf tissue.

Low levels of protection (45–60%) are consistently obtained 2 wk following stem injection with *P. tabacina* (48,49), and in this study a low level of β-1,3-glucanase activity was detected 15 days after stem injection with *P. tabacina*, but not after stem injection with water. The rapid increase in the level of β-1,3-glucanase observed 21 days after stem injection may account in part for the increase in the effectiveness of immunization, which reaches a maximum (>95% in this study) 21 days after stem injection (48,49). β-1,3-Glucanase is also high in the 1- to 3-day period during which the development of *P. tabacina* is restricted after challenge (46) in immunized plants. Enzyme activity was barely detectable in one experiment (not detected by western blots) in controls 2 days after challenge. Six days after challenge, β-1,3-glucanase levels in the controls equaled those in the immunized plants. However, this late response would not have provided resistance, because the development of the pathogen was already extensive, and sporulation was evident.

This paper is, to our knowledge, the first report indicating a positive correlation between the induction of resistance to *P. tabacina* and the accumulation of β-1,3-glucanases, chitinases, and other b-proteins. In light of these results, it is interesting to note that previous studies have demonstrated resistance to blue mold induced by the inoculation of TMV-resistant tobacco with the virus (30,59). Other studies have indicated that resistance to TMV can be induced in tobacco by stem injections with *P. tabacina* (29,59). Hypersensitive resistance to TMV is also associated with the accumulation of these proteins (35,56,60). On the basis of the results presented in this paper, β-1,3-glucanase and possibly other b-proteins may participate in the observed resistance to blue mold. However, it seems unlikely that the enzymes directly protect against viruses or bacteria.

An increase in chitinase activity is positively correlated with systemic induced resistance to viral, bacterial, and fungal pathogens in cucumber (31,32). In the immunization of tobacco described here, there was also a positive correlation between enhanced chitinase and induced immunity, although the degree of the increase (approximately twofold at 21 days) was much less than the increase in β-1,3-glucanase. It is evident that the induction of chitinase in plants does not require a pathogen with chitin. Chitinase is one of a series of b-proteins, including β-1,3-glucanase, that are likely to be coordinately regulated.

The role of other b-proteins in defense reactions to microorganisms is not known. Early accumulation may indicate a general response to pathogens in resistant plants, which is consistent with induced resistance to a broad spectrum of pathogens (24). The rapid increase of b-proteins 2 days after challenge, in immunized but not in control plants, suggests further that immunized plants are "sensitized" to respond more rapidly after challenge than control plants. The results are analogous to phaseolin accumulation in immunized green beans challenged with *Colletotrichum lindemuthianum* (24,25) and the rapid syntheses of phenylalanine ammonia lyase, chalcone synthetase, and chalcone isomerase in green beans inoculated with incompatible races of the fungus (6,43). In contrast, the induction of these enzymes is much later in the compatible interaction.

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