MPMI Vol. 8, No. 3, 1995, pp. 473–475.

Research Note

Evidence for Transcriptional Regulation of β-1,3-Glucanase as it Relates to Induced Systemic Resistance of Tobacco to Blue Mold

M. Lusso and J. Kuc

Department of Plant Pathology, University of Kentucky, Lexington, KY, 40546, U.S.A.
Received 6 September 1994. Accepted 3 January 1994.

The hybridization signal for β-1,3-glucanase (PR-N) mRNA was detected in the lower inoculated leaves and upper uninfected leaves of N-gene tobacco plants 3 and 12 days, respectively, after inoculation of lower leaves with tobacco mosaic virus (TMV). The signal markedly increased 1 to 2 days after challenge of upper leaves with *Peronospora tabacina*, whereas only a slight increase was apparent 3 to 4 days after mock challenge. The signal was not apparent in mock challenged upper leaves, but it was detected 4 days after challenge in upper leaves of plants mock inoculated with TMV. Plants inoculated with TMV were highly protected against *P. tabacina*. These results indicate a positive association of PR-N mRNA with induced resistance to *P. tabacina* and the apparent sensitization of the induced plants to rapidly produce PR-N mRNA after challenge with *P. tabacina*.

Inoculation of the lower leaves of N-gene tobacco with tobacco mosaic virus (TMV) results in the induction of systemic resistance against TMV and other pathogens (Ross 1961; McIntyre et al. 1981; Ye et al. 1990; Pan et al. 1991) and accumulation of pathogenesis-related proteins (PR proteins) (van Loon 1975; Ye et al. 1990). PR proteins have been suggested to function in plant defense (Carr and Klessing 1990). Four of the tobacco PR-proteins have been identified as β-1,3-glucanases and another four as chitinases (Kauffmann et al. 1987; Legrand et al. 1987). The association of PR proteins with systemic induced resistance (Ye et al. 1990; Pan et al. 1991) and the observation that some purified β-1,3-glucanases and chitinases have inhibitory activities against some fungi in vitro (Mauch et al. 1988) support a possible role for these proteins as plant defense compounds against pathogens. The regulation of expression of some of the tobacco PR proteins, has been reported to occur at the transcriptional level in leaves inoculated with TMV and the unchallenged leaves above (Ward et al. 1991).

In this study, we investigated the association of the transcription of the β-1,3-glucanase (PR-N) gene with systemic induced resistance.

Tobacco (*Nicotiana tabacum* L. cv. Ky 14) plants containing the N-gene for resistance to TMV were used in the experiments (Pan et al. 1991). Isolate 79 of *Peronospora tabacina* Adam (Tuzun and Kuc 1985) was used for challenge inoculations (Pan et al. 1991). Purified TMV was provided by J. Shaw, Department of Plant Pathology, University of Kentucky. The plants were induced with TMV and challenged 12 days after induction with *P. tabacina* as described by Ye et al. 1989.

Leaf tissues were sampled by excising small areas of the lamina with scissors, avoiding large veins. The samples were collected at 0, 3, 6, 9, and 12 days after inoculation or mock inoculation with TMV (three to four lower leaves) and at 0, 1, 2, 3, 4, and 5 days after challenge with *P. tabacina* or mock challenge from the leaves above those inoculated or mock inoculated with TMV (leaf position 3 to 5 from the apex). Samples were weighed and 2 g of tissue was used for RNA isolation as described by Schuler and Zielinski (1989).

Fifteen micrograms of the RNA samples was electrophoresed in a 1% agarose gel containing formaldehyde and then blotted onto a nylon membrane by capillary elution as described in Sambrook et al. (1989). The probe used was the full-length cDNA of ß-1,3-glucanase (PR-N) containing 1,053 bp, labeled with digoxigenin-dUTP by using the "Genius 2 DNA Labeling Kit" (Boehringer Mannheim Corp.) as per the instruction of the supplier. The probe was obtained from X. S. Ye, Department of Plant Pathology, University of Kentucky. The membrane was prehybridized for 24 h at 42°C in 50% formamide, 0.5% SDS, 6× SSC (0.9 M NaCl, 0.9 M sodium citrate), 5× Denhardt's reagent, 100 μg ml^{-1} denatured herring sperm DNA. The probe was denatured by incubation at 100°C for 5 min then added into the prehybridization solution to give a 20 ng ml^{-1} final concentration. Hybridization proceeded for 24 h at 42°C. The membrane was washed twice in 2× SSC, 0.1% SDS for 10 min at room temperature, then three times at 42°C in 0.5× SSC, 0.1% SDS for 15 min. High-stringency conditions were used to avoid cross-hybridization on Northern blots. The hybridized probe was immunodetected with an alkaline phosphatase-conjugated antidigoxigenin antibody and then visualized with the chemiluminescent substrate Lumi-Phos 530 (Schaap et al. 1989) (Boehringer Mannheim Corp.) as per the instruction of the supplier.

Corresponding author: J. Kuc

Journal Series Paper 94-11-152 of the Kentucky Agricultural Experiment Station, Lexington 40546

Induced systemic resistance in tobacco has been shown to increase the total β-1,3-glucanase activity and distinct isoforms of the enzyme (Pan et al. 1991). Ward et al. (1991) showed that the mRNA levels of the β-1,3-glucanases PR-2, PR-Q´ and a basic glucanase increase locally and systemically in response to TMV inoculation of lower leaves, indicating that these isoforms are regulated at the transcriptional level. However, the regulation of the isoform PR-N was not reported in leaves inoculated with TMV and the leaves above, and data were not presented for induced and noninduced plants after challenge to demonstrate the sensitization phenomenon at the transcriptional level. Northern blot analyses of total RNA isolated from leaves at various times after inoculation with TMV or mock inoculation, indicated an increase in the hybridization signal for PR-N in TMV-inoculated leaves (Fig. 1). The mRNA encoding the β-1,3-glucanase (PR-N) was detected in inoculated leaves at 3 days after inoculation, and the hybridization signal continued to increase until at least 12 days after inoculation. On mock-inoculated plants treated with water, the hybridization signal was not detected (results not shown).

The PR-N mRNA was first detected 4 days after challenge in the upper leaves of noninduced plants (Fig. 2A), and was not detected in unchallenged upper leaves of noninduced plants (results not shown).

With leaves above those inoculated with TMV, the β-1,3-glucanase isoform PR-N increased markedly 2 days after challenge with *P. tabacina* (Pan et al. 1991). In our study the mRNA for the enzyme was evident at the time of challenge (12 days after induction) in upper leaves of induced plants (Fig. 2B) and it markedly increased 1 to 2 days after challenge and continued to increase until 4 to 5 days after challenge. A slight increase in β-1,3-glucanase mRNA was first detected 3 days after mock challenge of induced plants (Fig. 2C).

Previous reports have demonstrated that resistance induced to *P. tabacina* by TMV was first clearly evident 3 to 6 days after induction (Ye et al. 1989; Pan et al. 1991). The protection data obtained from the plants from which the samples for RNA where collected showed that the plants inoculated with TMV were much more resistant to *P. tabacina* than the noninduced plants (88% protection based on the area of lesions).

The mechanisms involved in the induction of systemic resistance are still not known. Some reports support a role for the PR proteins, in plant resistance, including the hydrolytic enzymes glucanases and chitinases (Mauch et al. 1988; Carr and Klessing 1990; Ye et al. 1990; Pan et al. 1991). The isoform pattern of β-1,3-glucanase in age-dependent resistance (Wyatt et al. 1991) and in induced tobacco plants (Pan et al. 1991; Ye et al. 1992) indicate a association of the β-1,3-glucanase proteins, PR-N and PR-O, with resistance to *P. tabacina*, a fungus containing β-1,3-glucans but not chitin in its cell wall. Our results indicate an increase in the PR-N mRNA in the TMV-inoculated leaves and in uninfected leaves of induced plants. After challenge, PR-N mRNA increases sooner in challenged leaves of induced as compared to noninduced plants. These data provide evidence that the induction of PR-N isoform in leaves inoculated with TMV and leaves distant from those inoculated with TMV is regulated at the transcriptional level as was observed before with other glucanase isoforms (Ward et al. 1991). It also supports other results from our laboratory, with Western blot and enzyme activity analyses, that leaves of induced plants are sensitized to react rapidly after inoculation with *P. tabacina* (Tuzun et al. 1989; Pan et al. 1991).

The existence of a translocated factor which triggers the activation of resistance mechanisms in plant defense is accepted as one of the initial steps in induced systemic resistance, although the identity of this factor is still unknown. The early systemic activation of the PR-N gene in induced tobacco plants that we observed in our experiments further

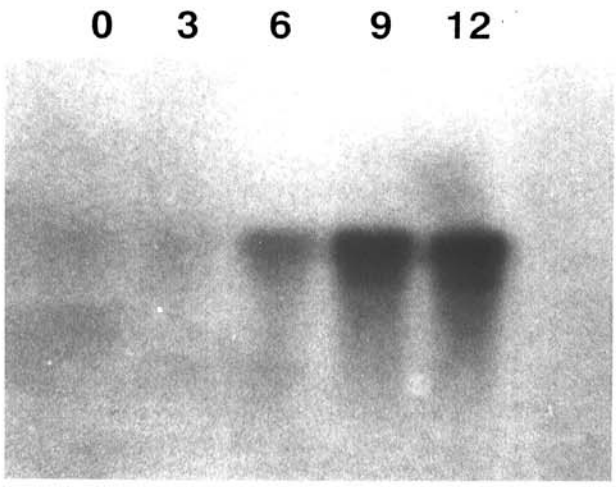

Fig. 1. Effects of tobacco mosaic virus inoculation on transcript levels of the β-1,3-glucanase (PR-N) gene. RNA from inoculated leaves was isolated at 0, 3, 6, 9, and 12 days after inoculation, electrophoresed through denaturing gel, transferred to nylon membranes, and then hybridized with a PR-N cDNA probe labeled with digoxigenin-dUTP. Exposure time was 30 min.

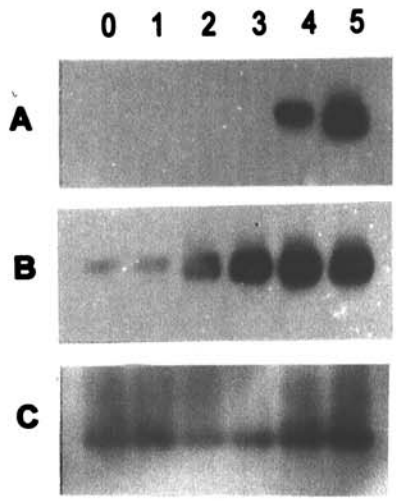

Fig. 2. Effects of induced systemic resistance against *Peronospora tabacina* on transcript levels of the β-1,3-glucanase (PR-N) gene. RNA from leaves above those mock inoculated with tobacco mosaic mosaic virus (A) or inoculated with TMV (B,C), was isolated at 0 to 5 days after challenge (A,B) or mock challenge (C), with *P. tabacina.* Isolated RNA was electrophoresed through denaturing gels, transferred to nylon membranes, and then hybrized with a PR-N cDNA probe labeled with digoxigenin-dUTP. Exposure time for A and B was 60 min and C was 90 min. The transcript for PR-N was not detected in unchallenged leaves of noninduced plants.

supports the hypothesis that a signal for induced systemic resistance moves in the plant and either directly or indirectly activates resistance mechanisms which include increased transcription of the gene for the PR-N protein. The mechanism(s) underlying the apparent sensitization of the induced plant to respond to challenge at the transcriptional level may be a critical factor in induced systemic resistance.

To help determine if PR-N has a role in induced systemic resistance, the β-1,3-glucanase (PR-N) cDNA was introduced in the sense and antisense orientations in tobacco plants, which have been regenerated and are being tested for their resistance to *P. tabacina* using plants induced and not induced with TMV.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the Brazilian Education Agency (FAPESP), and by grants from the R. J. Reynolds Tobacco Co. and Cooperative Agreement 43YK-5-0030 of the United States Department of Agriculture.

LITERATURE CITED

Carr, J. P., and Klessing, D. F. 1990. The pathogenesis-related proteins of plants. Pages 65-109 in: Genetic Engineering: Principles and Methods, Vol. 11. J. K. Setlow, ed. Plenum Press, New York.

Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. 1987. Biological functions of pathogenesis-related proteins: Four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J. 6:3209-3212.

Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. 1987. Biological functions of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc. Natl. Acad. Sci. USA 84:6750-6754.

MacIntyre, J. L., Dods, A., and Hare, J. D. 1981. Effects of localized infections of *Nicotiana tabacum* by tobacco mosaic virus on systemic resistance against diverse pathogens and insects. Phytopathology 71:297-301.

Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolyses in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and beta-1,3-glucanase. Plant Physiol. 88:936-942.

Pan, S. Q., Ye, X. S., and Kuc, J. 1991. Association of β-1,3-glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. Physiol. Mol. Plant Pathol. 39:25-39.

Ross, A. F. 1961. Systemic acquired resistance induced by localized virus infections in plants. Virology 14:340-358.

Sambrook, J., Fritisch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schaap, A. P., Akhavan, H., and Romano, L. J. 1989. Clin. Chem. 35:1863-1864.

Schuler, M. A., and Zielinski, R. E. 1989. Methods in Plant Molecular Biology. Academic Press, San Diego, California.

Tuzun, S., and Kuc, J. 1985. Movement of a factor in tobacco infected with *Peronospora tabacina* Adam which systemically protects against blue mold. Physiol. Plant Pathol. 26:321-330.

Tuzun, S., Rao, N., Vogeli, U., Schardl, C. L., and Kuc, J. 1989. 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. Phytopathology 79:979-983.

Van-Loon, L. C. 1975. Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. Samsun and Samsun NN. IV. Similarity of qualitative changes of specific proteins after infection with different viruses and their relationship to acquired resistance. Virology 67:566-575.

Ward, E. R., Payne, G. B., Moyer, M. B., Williams, S. C., Dincher, S. S., Sharkey, K. C., Beck, J. J., Taylor, H. T., Ahl-Goy, P., Meins, Jr., F., and Ryals, J. A. 1991. Differential regulation of β-1,3-glucanase messenger RNAs in response to pathogen infection. Plant Physiol. 96:390-397.

Wyatt, S. E., Pan, S. Q., and Kuc, J. 1991. β-1,3-Glucanase, chitinase & peroxidase activities in tobacco tissues resistant and susceptible to blue mold as related to flowering, age and sucker development. Physiol. Mol. Plant Pathol. 39:433-440.

Ye, X. S., Pan, S. Q., and Kuc, J. 1989. Pathogenesis-related proteins and systemic resistance to blue mold and tobacco mosaic virus induced by tobacco mosaic virus, *Peronospora tabacina* and aspirin. Physiol. Mol. Plant Pathol. 35:161-175.

Ye, X. S., Pan, S. Q., and Kuc, J. 1990. Association of pathogenesis-related proteins and activity of peroxidase, β-1,3-glucanase and chitinase with systemic induced resistance to blue mold of tobacco but not to systemic tobacco mosaic virus. Physiol. Mol. Plant Pathol. 36:523-531.

Ye, X. S., Pan, S. Q., and Kuc, J. 1992. Specificity of induced systemic resistance as elicited by ethephon and tobacco mosaic virus in tobacco. Plant Sci. 84:1-9.