MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 3, pp. 234-238, 1991

Induction of β-1,3-Glucanase in Barley in Response to Infection by Fungal Pathogens

W. Jutidamrongphan,[1] J. B. Andersen,[2] G. Mackinnon,[1] J. M. Manners,[3] R. S. Simpson,[1] and K. J. Scott[1]

[1]Department of Biochemistry, The University of Queensland, 4072, Australia; [2]Department of Plant Pathology, Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark; [3]Commonwealth Scientific and Industrial Research Organization, Division of Tropical Crops and Pastures-UQ Plant Pathology Unit, Department of Botany, The University of Queensland, 4072, Australia.
Received 2 July 1990. Revised 21 November 1990. Accepted 7 December 1990.

The sequence of a partial cDNA clone corresponding to an mRNA induced in leaves of barley (*Hordeum vulgare*) by infection with fungal pathogens matched almost perfectly with that of a cDNA clone coding for β-1,3-glucanase isolated from the scutellum of barley. Western blot analysis of intercellular proteins from near-isogenic barley lines inoculated with the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*) showed a strong induction of glucanase in all inoculated lines but was most pronounced in two resistant lines. These data were confirmed by β-1,3-glucanase assays. The barley cDNA was used as a hybridization probe to detect mRNAs in barley, wheat (*Triticum aestivum*), rice (*Oryza sativus*), and sorghum (*Sorghum bicolor*), which are induced by infection with the necrotrophic pathogen *Bipolaris sorokiniana*. These results demonstrate that activation of β-1,3-glucanase genes may be a general response of cereals to infection by fungal pathogens.

Additional keyword: disease resistance.

Infection of barley by the powdery mildew fungus results in increased steady-state levels of a number of host mRNAs (Davidson *et al.* 1987). These barley mRNAs have been termed infection-related (IR) mRNAs, and cDNA clones for six of these mRNAs have been isolated (Davidson *et al.* 1987, 1988). A comparison of the timing and extent of induction of the IR mRNAs in resistant and susceptible near-isogenic barley during infection by the powdery mildew fungus has suggested a role for these mRNAs in resistance (Davidson *et al.* 1988).

The induction of mRNAs in barley during fungal infection resembles that of dicotyledonous plants where a number of host defense-associated genes are known to be activated (Collinge and Slusarenko 1987; Ebel and Grisebach 1988; Templeton and Lamb 1988). In dicots, the identity of many of the induced host mRNAs is established and these mRNAs code for proteins that are either potentially antifungal, e. g., chitinases and β-1,3-glucanase, or that are involved in well-characterized cellular defense processes, e.g., phytoalexin synthesis and lignification. In barley, the function of the proteins encoded by the IR mRNAs has not been identified; however, a comparison of sequences of the barley IR mRNAs with dicot defense genes may permit an identification. By this means we have now identified one of the cloned IR mRNAs of barley as encoding a β-1,3-glucanase. The induction of β-1,3-glucanase activity by fungal infection was confirmed by western blot and enzymatic analysis of intercellular fluids of powdery mildew infected barley. Activation of β-1,3-glucanase activity in response to *Bipolaris sorokiniana* (Sacc.) Shoemaker infection was also shown in barley, wheat, rice, and sorghum.

Nucleotide and/or amino acid sequence data submitted to GenBank, EMBL, and DDBJ as accession number X16274 β-1-3-glucanase.

MATERIALS AND METHODS

Plant and fungal material. Plants of *Hordeum vulgare* (L.) 'Psaknon 4*/ (F14) Man. (R)' of USDA Accession CI 16146 and its near-isogenic susceptible counterpart, CI 16145 (Moseman 1972); *Triticum aestivum* L. 'Zenith'; *Oryza sativa* L. 'Japonicum'; and *Sorghum bicolor* (L.) Moench 'Zulu' were cultivated in controlled growth chambers as described (Manners and Scott 1983). Barley lines used for isolation of intercellular proteins for the western blot experiment were isolines in susceptible Pallas gene background (Kolster *et al.* 1986). Pallas was used with (Pa^+, infection type 4) and without (Pa^-) inoculation. P-23 with the "Laevigatum" resistance gene (M1-[La], infection type 2-3) and P-01 with the "Algerian" resistance gene (M1-a, infection type 0) were used as resistant counterparts. All lines were inoculated with the same powdery mildew isolate (C15).

B. sorokiniana was provided by J. Alcorn, Queensland Department of Primary Industries, and was maintained on agar containing autoclaved wheat straw. For inoculation with *B. sorokiniana*, a spore suspension of approximately 10^5 per milliliter was prepared and sprayed onto the leaves. The plants were then maintained under plastic bags for 24 hr. This fungus was pathogenic on leaves of barley, wheat, rice, and sorghum and produced necrotic lesions on these leaves within 48 hr of inoculation.

Sequence analysis. A cDNA library was prepared from poly(A)$^+$ RNA isolated from barley leaves at 48 hr after inoculation with the powdery mildew fungus by the method of Rutledge *et al.* (1988), except the ligation of the first-strand cDNAs was omitted. The library was probed with

the insert from pRP2, a cDNA clone for an IR mRNA (Davidson *et al.* 1987), and a number of positive clones were isolated. One of these, designated pRP7-25, which contained a 225-base pair (bp) insert, was selected for sequence analysis. DNA sequencing was carried out by the dideoxy method of Sanger *et al.* (1977) with the modifications of Chen and Seeburg (1985). Sequences were determined by analysis of both DNA strands and of overlapping subclones.

Hybridization analysis. Total RNA and poly(A)$^+$ RNA were isolated by standard methods (Manners and Scott 1985). Northern blot analysis was carried out as described (Higgins *et al.* 1985), except that the last washes of the blots were carried out at a lower temperature (42° C).

Intercellular proteins. The leaf material (~5 g) was cut into 5-cm pieces, washed three times with an excess of sterile water, vacuum infiltrated (with water) for 10 min, blotted dry on paper towels, and loaded either on top of 3–4 cm of marbles and centrifuged (15 min, 700 × *g*, 10° C) or onto a nylon mesh filter and centrifuged (5 min, 1,500 × *g*, 10° C). The liquid in the bottom of the tube was collected, quick frozen, freeze-dried, and reconstituted in water or an appropriate buffer. Protein concentrations were determined by the method of Smith *et al.* (1985). Samples were collected daily from 1 to 8 days after inoculation.

β-1,3-Glucanase assays. Proteins were separated on polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose; a semidry blotting system (semidry blotter, JKA-Biotech, Denmark) was used. Barley β-1,3-glucanase antiserum provided by G. Murray Ballance (University of Manitoba) was used diluted 1:500. Blots were developed with alkaline phosphatase-conjugated swine anti-rabbit serum (Dakopatts D306, Glostrup, Denmark).

β-1,3-Glucanase activity was measured essentially as described by Keen and Yoshikawa (1983). The standard assay contained 10 mg of laminarin, 50 mM potassium-acetate, pH 5.3, and 40 μl of enzyme extract in a volume of 1 ml. The mixture was incubated at 30° C, 0.4-ml aliquots were withdrawn at 0 and 15 min, and the enzyme reaction was terminated by heating the aliquots in boiling water for 3 min. The amount of reducing sugar was measured quantitatively with 3,5-dinitrosalicylate by a modification of the method of Bernfeld (1955).

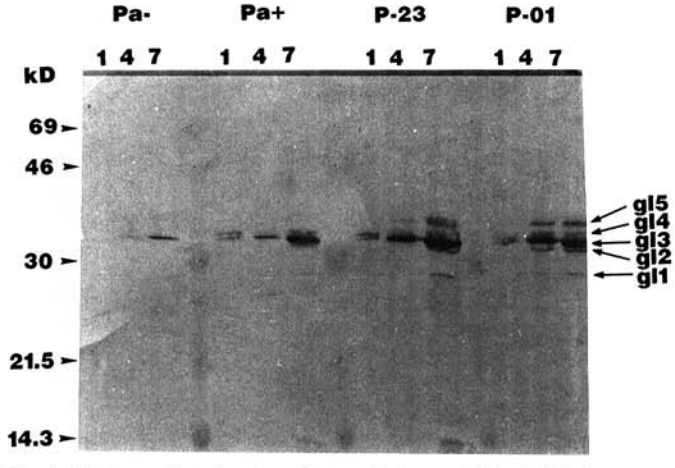

Fig. 1. Western blot showing changes in intercellular β-1,3-glucanases of barley after inoculation with barley powdery mildew. Headings 1, 4, and 7 indicate days after inoculation. Pa$^-$ = Pallas uninoculated, Pa$^+$ = Pallas inoculated, P-23 = M1-(La) resistance, inoculated, P-01 = M1-a resistance, inoculated. Lanes were loaded with equal amounts of protein and incubation with primary antibody was for 1 hr with an antibody generated to the GII protein encoded by pRP2.

RESULTS

Sequence comparisons. Comparison of the 225-base nucleotide sequence of pRP7-25 with that of positions 819 to 1043 of a full-length cDNA clone for a specific β-1,3-glucanase in grain of barley (Hoj *et al.* 1989) revealed almost perfect homology (data not shown). The only difference between our sequence and that of the barley grain β-1,3-glucanase cDNA clone occurred at position 1031, where a C was substituted for a G. Comparison of the amino acid coding sequence of clone pRP7-25, which covered the entire insert sequence, revealed it to be identical with that of a part of the amino acid sequence of a β-1,3-glucanase isoenzyme G2 purified from barley grain (Ballance and Svendsen 1988). Comparisons with the sequences of a β-1,3-glucanase of tobacco (Shinshi *et al.* 1988) and a β-1,3/1,4-glucanase of barley (Fincher *et al.* 1986) revealed highly significant homologies of 67 and 78%, respectively. The sequence of pRP7-25 and that of the insert of pRP2 (Davidson *et al.* 1988) were identical, except that the pRP2 insert had a large region (138 bp) of apparently anomalous sequence duplication that most probably occurred during the synthesis and cloning of the cDNA (Davidson *et al.* 1988). These results demonstrated conclusively that clone pRP7-25, and, thus, the original barley clone pRP2 (Davidson *et al.* 1987), coded for a β-1,3-glucanase.

β-1-Glucanase in the intercellular fluid. Antiserum to barley β-1,3-glucanase isolated from malted barley (Ballance and Svendsen 1988) gave a specific reaction to five distinct β-1,3-glucanase isoenzymes (gl1-5) in the leaf intercellular fluid (Fig. 1). Two bands (gl3 [33 kDa] and gl5 [36 kDa] were observed in the Pallas control (Pa$^-$), and band gl3 appeared to increase in amount with time. Band gl5, however, was only visible in the Pa$^-$ control at 7 days and at a very low level compared with gl3. Bands gl2, gl3, and gl4 were induced after infection of the susceptible Pallas cultivar (Pa$^+$). By contrast, all five bands were much more strongly induced after inoculation of the resistant lines (P-23 and P-01). Protein gl3 appears stimulated as early as 1 day after inoculation, and gl1 (28 kDa), gl2 (32.5 kDa), gl4 (34 kDa), and gl5 (36 kDa) appeared 4 days after inoculation (Fig. 1). The new band, gl1, can only be seen in the resistant isolines, and the induction of gl2 and gl4 in Pa$^+$ was slower than in the near-isogenic resistant lines.

Figure 2 shows the activity of β-1,3-glucanase in the intercellular fluid. The activity in the intercellular fluid from uninoculated leaves of both susceptible (CI 16145) and resistant (CI 16146) Psaknon near-isogenic lines was low and did not change markedly with time. The β-1,3-glucanase activity in susceptible inoculated leaves increased at 1 day after inoculation and by the third day after inocu-

lation had increased to 2.8 times the activity of the corresponding control leaves. The enzyme activity in intercellular fluid from resistant inoculated leaves also increased at 1 day after inoculation and continued to increase to 12.9 times the activity in the corresponding control leaves at 3 days after inoculation. When the β-1,3-glucanase activity was expressed as micromoles of glucose equivalents released per minute per milligram of intercellular fluid protein, the level of activity in Psaknon resistant leaves was only slightly higher than that from susceptible leaves (data not shown). This resulted from a 100% increase in the protein content of intercellular fluid from resistant leaves compared with that in susceptible leaves. At least some of this increase in protein content was due to the increase in β-1,3-glucanase. These data on β-1,3-glucanase activity were consistent with our previous observations that induction of mRNA corresponding to pRP2 increased to a significantly higher level in the resistant Psaknon isoline compared with susceptible leaves (Davidson *et al.* 1988). The data were also consistent with the appearance of new isoenzymes as detected by antiserum to barley β-1,3-glucanase. Assays of β-1,3-glucanase activity were also performed on the near-isogenic lines of Pallas. The results indicated 3.5-fold greater activity in the susceptible Pallas isoline at 4 days after inoculation when compared with the uninoculated control (J. B. Andersen, data not shown). The enzyme activity in intercellular fluid from inoculated leaves of the resistant isolines P-23 and P-01 increased to 4.6 and 10.4 times, respectively, the activity in the corresponding control leaves at 4 days after inoculation (J. B. Andersen, data not shown).

Induction of β-1,3-glucanase mRNA in barley and other cereals. The insert from the barley β-1,3-glucanase clone pRP2 was used as a hybridization probe to determine whether induction of mRNAs for a β-1,3-glucanase could be demonstrated in barley and other cereals after infection by the pathogenic fungus *B. sorokiniana*, which has a very wide host range among cereals. Similar hybridization to the β-1,3-glucanase probe was detected in barley, wheat, rice, and sorghum (Fig. 3), showing that the mRNA increased in amount in all four cereals from 0 to 48 hr after inoculation by *B. sorokiniana*. Uninoculated controls for all four plant species showed no induction of the mRNA, whereas mock-inoculated (H_2O treated) controls were performed for wheat and barley only and similarly showed no induction.

DISCUSSION

A comparison of the nucleotide and deduced amino acid sequences of the cDNA clones pRP7-25 and pRP2 to those corresponding to the well-characterized β-1,3-glucanase of barley grain (Ballance and Svendsen 1988; Hoj *et al.* 1989) has permitted us to positively identify the function of one of the previously cloned infection-related mRNAs of barley (Davidson *et al.* 1987). Estimates of the amount of this mRNA in near-isogenic resistant and susceptible barley during infection by the powdery mildew fungus have shown that its early induction correlates with resistance conditioned by the *Mla, Mlp*, and *Mlk* genes (Davidson *et al.* 1988). Thus, regulation of genes coding for β-1,3-glucanase may be important in determining resistance to the powdery mildew fungus. In addition, recent studies with other fungal pathogens have also implicated the β-

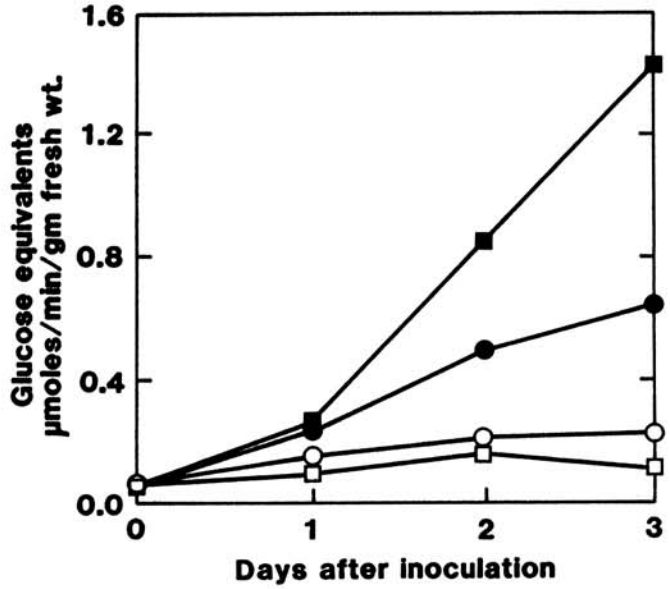

Fig. 2. Activity of β-1,3-glucanase in intercellular extracts of barley during infection by the powdery mildew fungus. β-1,3-Glucanase activity was measured in leaf intercellular fluid from control (○ □) and inoculated (● ■) near-isogenic susceptible (○ ●) and resistant (□ ■) lines of barley differing for the *Mlp* gene. Standard deviation on quadruplicate experiments ranged from 0.006 to 0.044 μmoles $min^{-1}g^{-1}$ and differences between resistant and susceptible lines were significant (>95% *P*) at 2 and 3 days after inoculation.

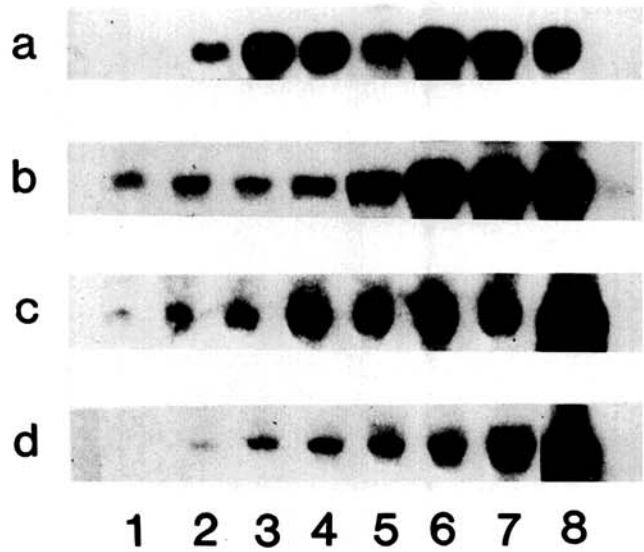

Fig. 3. Induction of mRNAs detected by the β-1,3-glucanase probe pRP2 derived from barley in leaves of barley (a), wheat (b), sorghum (c), and rice (d) during infection by *Bipolaris sorokiniana*. The signal from total RNA obtained at the following times is shown: Lane 1, 0 hr after inoculation, lanes 2–7 at 2, 4, 8, 12, 24, and 48 hr after inoculation. In a, b, c, and d lane 8 represents the signal from a sample of total RNA obtained from leaves of barley at 48 hr after inoculation with an incompatible race of the powdery mildew fungus and was included as a positive control.

1,3-glucanase mRNA in nonhost resistance reactions in barley (Scott *et al.* 1990).

The western blots that used a β-1,3-glucanase antiserum and the enzyme assays reported here showed a strong association of protein production with resistance and confirm the mRNA induction results previously published (Davidson *et al.* 1988). Other authors have found multiple forms of β-1,3-glucanase in the intercellular fluid, and these always appear together with chitinases and other "pathogenesis-related" (PR) proteins (Kauffmann *et al.* 1987; Kombrink *et al.* 1988). This also occurs in barley where chitinase (J.B. Andersen, unpublished data) and a protein serologically related to tobacco PR-1 (L. Wolfe and K. J. Scott, unpublished data) are found in the intercellular fluid. The association of intercellular β-1,3-glucanase with resistance will now require cytoimmunological examination to determine the site of the enhanced enzyme activity and whether it is present at penetration sites or surrounds the invading fungal structures.

There is evidence to implicate β-1,3-glucanase in resistance of plants to some fungi (Kauffmann *et al.* 1987). For example, the enzyme has antifungal activity when combined with plant chitinase, and, in dicots, enzyme activity is induced by fungal pathogens (Mauch *et al.* 1984, 1988). In dicots, β-1,3-glucanase genes are considered to constitute a part of the general array of defense genes induced during pathogenesis. Our results indicate that the induction of β-1,3-glucanase mRNA also occurs during fungal infection of a number of cereals. This identification of a cereal defense gene product as a β-1,3-glucanase indicates that some parts of the general defense response of cereals may closely resemble that previously demonstrated in dicots (Boller 1987; Kauffmann *et al.* 1987; Kombrink *et al.* 1988; Mauch *et al.* 1984). Obviously some pathogens such as *B. sorokiniana* can successfully infect cereals despite their ability to rapidly induce the β-1,3-glucanase mRNA, and it is possible that this fungus is either insensitive to the enzyme or produces an inhibitor as reported for *Colletotrichum lindemuthianum* Sacc. & Magnus (Lams.-Scrib.) (Albersheim and Valent 1974).

Recently, Bohlmann *et al.* (1988) have shown that another class of antifungal proteins, the leaf-specific thionins, are also induced in barley during infection by the powdery mildew fungus. Taken together, the data reported in this paper on induction of β-1,3-glucanase mRNA and the work on leaf thionins indicate that barley has a multicomponent response to fungal infection. Further investigation of the IR mRNAs of barley may reveal the function of other components in this response. Ultimately, investigation of the regulation of these genes should permit the manipulation of expression of these genes to test their roles in resistance to fungal pathogens.

ACKNOWLEDGMENTS

We greatly appreciate the cooperation of G. Fincher (La Trobe University, Melbourne) and associates for making available the barley β-1,3-glucanase cDNA sequence before publication. This work was supported by grants from the Barley Research Council and the Australian Research Council. W. J. was supported by a scholarship from the Australian International Development Program for Colleges and Universities. We are grateful to M. Hobbs for assistance in computer analysis of sequences. We are greatly indebted to G. Murray Ballance (University of Manitoba) for providing a sample of β-1,3-glucanase antiserum. The antiserum was prepared during a postdoctoral stay at Carlsberg Research Centre in Copenhagen.

LITERATURE CITED

Albersheim, P., and Valent, B. 1974. Host-pathogen interactions. VII. Plant pathogens secrete proteins which inhibit enzymes of the host capable of attacking the pathogen. Plant Physiol. 53:684-687.

Ballance, G. M., and Svendsen, I. B. 1988. Purification and amino acid sequence determination of an endo-1,3-β-glucanase from barley. Carlsberg Res. Commun. 53:411-419.

Bernfeld, P. 1955. Amylase, α and β. Methods Enzymol. 1:149-158.

Bohlmann, H., Calusen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V., and Apel, K. 1988. Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. EMBO J. 7:1559-1565.

Boller, T. 1987. Hydrolytic enzymes in plant disease resistance. Pages 385-413 in: Plant-Microbe Interactions. Molecular and Genetic Perspectives Vol. 2. T. Kosuge and E. W. Nestor, eds. Macmillan Publishing Co., New York.

Chen, E. Y., and Seeburg, P. H. 1985. Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. DNA 4:165-170.

Collinge, D. B., and Slusarenko, A. J. 1987. Plant gene expression in response to pathogens. Plant Mol. Biol. 9:389-410.

Davidson, A. D., Manners, J. M., Simpson, R. S., and Scott, K. J. 1987. cDNA cloning of mRNAs induced in resistant barley during infection by *Erysiphe graminis* f. sp. *hordei*. Plant Mol. Biol. 8:77-85.

Davidson, A. D., Manners, J. M., Simpson, R. S., and Scott, K. J. 1988. Altered host gene expression in near-isogenic barley conditioned by different genes for resistance during infection by *Erysiphe graminis* f. sp. *hordei*. Physiol. Mol. Plant Pathol. 32:127-139.

Ebel, J., and Grisebach, H. 1988. Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: A molecular analysis. Trends Biochem. Sci. 13:23-27.

Fincher, G. B., Lock, P. A., Morgan, M. M., Lingelbach, K., Wettenhall, R. E. H., Mercer, J. F. B., Brandt, A., and Thomas, K. K. 1986. Primary structure of the (1-3, 1-4)-β-D-glucan 4-glucanohydrolase from barley aleurone. Proc. Natl. Acad. Sci. USA 83:2081-2085.

Higgins, C. M., Manners, J. M., and Scott, K. J. 1985. Decrease in three messenger RNA species coding for chloroplast proteins in leaves of barley infected with *Erysiphe graminis* f. sp. *hordei*. Plant Physiol. 78:891-894.

Hoj, P. B., Hartman, D. J., Morrice, N. A., Doan, D. N. P., and Fincher, G. B. 1989. Purification of (1-3)-β-glucan endohydrolase isoenzyme II from germinating barley and determination of its primary structure from a cDNA clone. Plant Mol. Biol. 13:31-42.

Keen, N. T., and Yoshikawa, M. 1983. β-1,3-Endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol. 71:460-465.

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

Kolster, P., Munk, L., Stolen, O., and Lohde, J. 1986. Near-isogenic barley lines with genes for resistance to powdery mildew. Crop Sci. 26:903-907.

Kombrink, E., Schroder, M., and Hahlbrock, K. 1988. Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc. Natl. Acad. Sci. USA 85:782-786.

Manners, J. M., and Scott, K. J. 1983. Translational activity of polysomes of barley leaves during infection by *Erysiphe graminis* f. sp. *hordei*. Phytopathology 73:1386-1392.

Manners, J. M., and Scott, K. J. 1985. Reduced translatable messenger RNA activities in leaves of barley infected with *Erysiphe graminis* f. sp. *hordei*. Physiol. Plant Pathol. 26:297-308.

Mauch, F., Hadwiger, L. A., and Boller, T. 1984. Ethylene: Symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. 76:607-611.

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

Moseman, J. G. 1972. Isogenic barley lines for reaction to *Erysiphe graminis* f. sp. *hordei*. Crop Sci. 12:681-682.

Rutledge, R. G., Seligy, B. L., Cote, M.-J., Dimock, K., Lewin, L. L.,

and Tenniswood, M. P. 1988. Rapid synthesis and cloning of complementary DNA from any RNA molecule into plasmid and phage M13 vectors. Gene 68:151-158.
Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
Scott, K. J., Davidson, A. D., Jutidamrongphan, W., Mackinnon, G., and Manners, J. M. 1990. The activation of genes of wheat and barley by fungal phytopathogens. Aust. J. Plant Physiol. 17:229-238.
Shinshi, H., Wenzler, H., Neuhaus, J.-M., Felix, G., Hofsteenge, J., and Meins, F., Jr. 1988. Evidence for N- and C- terminal processing of a plant defense-related enzyme: Primary structure of tobacco prepro-β-1,3-glucanase. Proc. Natl. Acad. Sci. USA 85:5541-5545.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.
Templeton, M. O., and Lamb, C. J. 1988. Elicitors and defense gene activation. Plant Cell Environ. 11:395-401.