Differential Expression and Induction of Chitinases and β-1,3-Glucanases in Response to Fungal Infection During Germination of Maize Seeds

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Received 21 July 1993. Accepted 5 October 1993.

Chitinases and β-1,3-glucanases are believed to be important in defending plants against pathogens. Here, we examined the expression of β-1,3-glucanases and chitinases in response to infection by the fungus *Fusarium moniliforme* during germination of maize seeds. Accumulation of both hydrolases in embryo and vegetative tissues of maize seedlings were analyzed. Immunoblotting and immunoprecipitation of total *in vitro* translated proteins using antibodies against the maize pathogenesis-related proteins characterized as β-1,3-glucanases and chitinases were performed. We found a coordinated induction of the expression of one β-1,3-glucanase and three chitinase isoforms in maize seedlings in response to infection by the fungus *F. moniliforme*. The observed induction of the expression of the β-1,3-glucanase isoform correlates with an increase in the level of translatable mRNA for this particular isoform. In contrast, a second β-1,3-glucanase isoform was also expressed in embryo and radicle tissues, but its level of accumulation was not increased upon fungal infection. These findings suggest that β-1,3-glucanases, in addition to their possible role in the defense response against pathogens, may have a developmentally regulated expression and a role in the normal process of seed germination. The pattern of accumulation of the various β-1,3-glucanase and chitinase isoforms and their responses to fungal infection in radicles and coleoptiles are distinct from those seen in embryo tissues of maize seedlings. These data indicate that in addition to tissue-specific expression, there is a differential pathogen regulation of the expression of the different β-1,3-glucanases and chitinases during germination of maize seeds.

Plants develop a complex variety of defense responses when infected by pathogens. The synthesis of new proteins that can have direct or indirect action on the course of pathogenesis is a ubiquitous response of monocot and dicot plants to pathogen attack (Lamb *et al.* 1989; Bowles 1990). These induced proteins include cell-wall proteins, enzymes involved in phenylpropanoid and flavonoid metabolism, toxic proteins (thionins), proteins with antimicrobial properties (enzyme inhibitors), lytic enzymes, and a heterogeneous group of proteins known collectively as pathogenesis-related (PR) proteins.

The expression of PR proteins can be induced by different infectious agents, such as viruses, viroids, fungi, and bacteria (van Loon 1985; Bol *et al.* 1990; White and Antoniw 1991; Linthorst 1991). It has been shown that PR proteins are also produced after treatment with specific chemicals (van Loon 1983; Granell *et al.* 1987; Oshima *et al.* 1990) and during certain stages of normal development of uninfected plants, such as during flowering in tobacco (Lotan *et al.* 1989; Neale *et al.* 1990; Ori *et al.* 1990) or during leaf senescence in tomato plants (Vera *et al.* 1988). Accumulation of PR proteins has been studied in great detail in dicotyledonous plants, mainly tobacco and tomato, where pure PR proteins have been obtained. Thus, tobacco PR proteins have been grouped into five groups, and two hydrolytic functions have been described for two of them. One group has β-1,3-glucanase activity, and the other group has chitinase activity (Kauffmann *et al.* 1987; Legrand *et al.* 1987).

In maize, several PR proteins have been found to accumulate in leaves after mercuric chloride treatment or brome mosaic virus (BMV) infection (Nasser *et al.* 1988). Among these PR proteins, several β-1,3-glucanases and chitinases have been identified and purified (Nasser *et al.* 1990).

Concerning the function of β-1,3-glucanases and chitinases, a role for these hydrolytic enzymes as part of the plant defense response has been suggested on the basis of their inhibitory effect on the *in vitro* growth of pathogenic fungi (Mauch *et al.* 1988; Sela-Buurlage *et al.* 1993). Moreover, it has been reported that transgenic tobacco seedlings constitutively expressing a bean chitinase gene show an enhanced resistance to the fungal pathogen *Rhizoctonia solani* (Broglie *et al.* 1991). β-1,3-Glucanases may also be involved in the stimulation of the plant defense reaction by releasing elicitors from fungal cell walls that can stimulate phytoalexin accumulation in the host plant (Takeuchi *et al.* 1990; Ham *et al.* 1991).

Despite the extensive information available on the accumulation of PR proteins (particularly on PR chitinases and β-1,3-glucanases) in leaves, the expression of these groups of PR proteins in other tissues or developmental stages has been less studied. In related research, we cloned and characterized cDNA and genomic sequences corresponding to another PR protein, the PRms protein. The expression of the PRms protein is specifically induced during germination in seed tissues

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MPMI Vol. 7, No. 1, 1994, pp. 23-31
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in response to fungal infection (Casacuberta *et al.* 1991, 1992).

We biochemically characterized the chitinase and β-1,3-glucanase isoforms expressed in maize seedlings and the changes in expression of these isoforms in response to infection with the fungus *Fusarium moniliforme*. Antibodies raised against maize PR proteins that had already been characterized as β-1,3-glucanases and chitinases (Nasser *et al.* 1990) were used for immunoblotting in the present work. Analysis of the total *in vitro* translated proteins was done by immunoprecipitation.

RESULTS

Separation of crude protein extracts by ion-exchange chromatography.

In maize, brome mosaic virus (BMV) infection or mercuric chloride treatment induces the expression and accumulation in leaves of several β-1,3-glucanases and chitinases. Basic and acidic isoforms of both types of hydrolases are found. The acidic β-1,3-glucanases and chitinases can be separated from their respective basic isoforms by ion-exchange chromatography at pH 5.2 (Nasser *et al.* 1988, 1990). In order to obtain a more detailed characterization of the different chitinase and β-1,3-glucanase isoforms detected in maize embryos, this chromatographic procedure was applied to the separation of proteins present in extracts from germinating embryos.

PR proteins are known to be selectively extracted with acidic buffers. Accordingly, a crude protein extract obtained with acidic buffer was prepared from 3-day-old germinated embryos infected with *F. moniliforme*, dialyzed against 20 mM sodium acetate, pH 5.2, at 4° C, and applied to a CM-Sephadex column equilibrated with the same buffer (Fig. 1). The washed-through and bound (eluted with 0.5 M NaCl) protein fractions were collected and subjected to analysis by immunoblotting with antisera raised against maize β-1,3-glucanases and chitinases. Approximately 75% of the amount of total protein applied to the column was found in the acidic protein fraction (Fig. 1, fraction A).

Crude extracts were also prepared from infected embryos with 0.5 M sodium acetate buffer, pH 5.2, as extraction buffer, and the conditions used for extraction of β-1,3-glucanases and chitinases from maize leaves (Nasser *et al.* 1988, 1990). Protein extracts were subjected to chromatographic separation on CM-Sephadex. Comparison of the elution profiles obtained from crude extracts prepared with the pH 5.2 and pH 2.8 buffers indicated that the pH 2.8 buffer worked more effectively for extracting proteins that elute in the basic fraction (Fig. 1).

Effect of fungal infection on the expression of β-1,3-glucanases in germinating maize embryos.

Immunoblotting of protein extracts obtained from non-infected and infected germinating embryos and immuno-

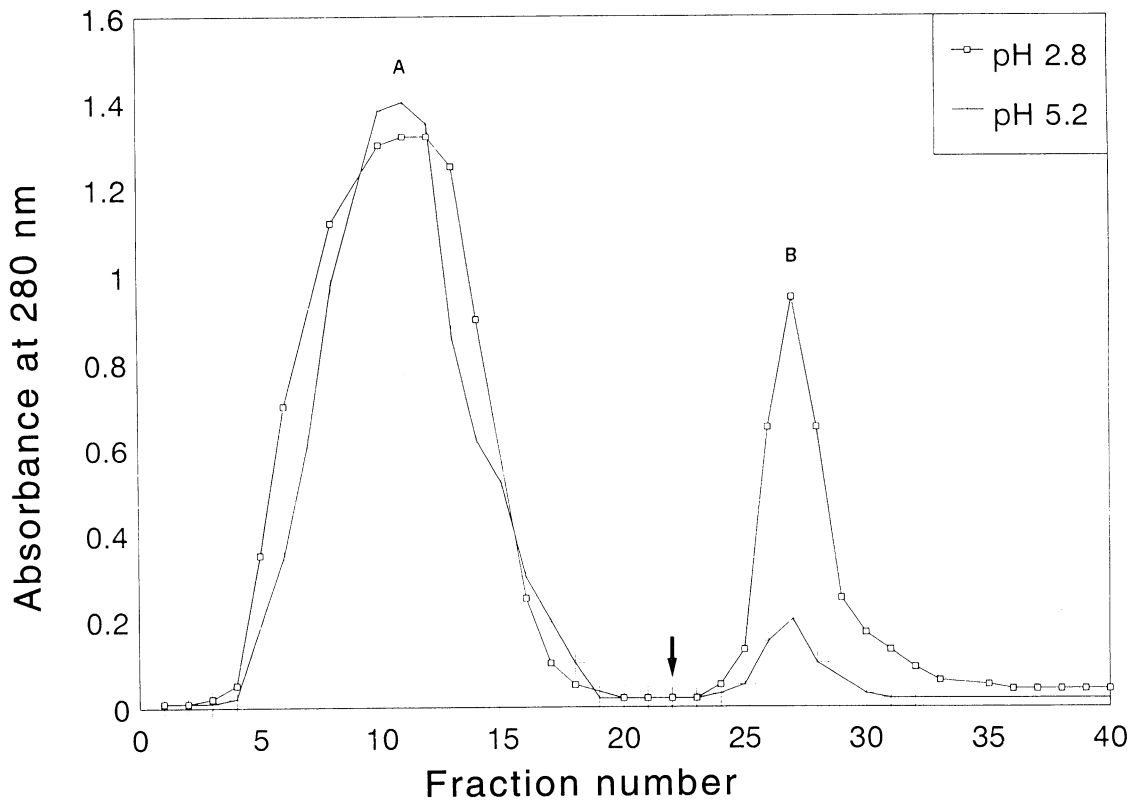

Fig. 1. CM-Sephadex chromatography of proteins from *Fusarium moniliforme*–infected germinating embryos. Two different buffers were used for preparation of protein extracts: phosphate citrate buffer (84 mM citric acid, 30 mM Na_2HPO_4, at pH 2.8, containing 14 mM 2-mercaptoethanol and 6 mM ascorbic acid) (□) and sodium acetate buffer (0.5 M sodium acetate, pH 5.2) (+). The column buffer was 20 mM sodium acetate, pH 5.2. Elution was performed with 0.5 M NaCl (arrow). The protein fraction that passes through the column contains the acidic proteins (A). The fraction eluted with 0.5 M NaCl contains the basic proteins (B).

precipitation of *in vitro* synthesized proteins allowed us to analyze first the ability of germinating embryos to express specific chitinase or β-1,3-glucanase isoforms and, secondly, the effect of fungal infection on the level of accumulation of each specific isoform. In this study, embryos germinated for 20 hr were inoculated with spores of *F. moniliforme* and allowed to continue germination for a total of 2 or 3 days (infected and sterile control embryos).

An antiserum raised against maize PR β-1,3-glucanases, anti-PRm6b antiserum (antiserum provided by G. Burkard, IBMP, Strasbourg, France), was used to detect the presence of β-1,3-glucanases in total protein from crude extracts obtained from germinating embryos. In BMV-infected or chemically treated maize leaves, three β-1,3-glucanase isoforms, two acidic isoforms (the PRm6a and PRm6b proteins of 32 and 30.5 kDa, respectively) and a basic isoform (the PRmBa1 protein, 35 kDa), accumulate. The antiserum raised against pure PRm6b protein reacts with these three β-1,3-glucanase isoforms (Nasser *et al.* 1990).

Results obtained on the expression of β-1,3-glucanases in germinating maize embryos are presented in Figure 2. A β-1,3-glucanase isoform with an apparent molecular weight of approximately 35 kDa accumulates, starting at day 1 of germination. Its level of accumulation increases from day 1 to day 2 and remains at similar levels in protein extracts from both sterile and infected embryos. A second β-1,3-glucanase isoform of approximately 33 kDa, which is not detected in sterile embryos, accumulates only in extracts from infected embryos. Its presence is detectable in 2-day-germinated infected embryos (Fig. 2A, lane d), and its level of accumulation increases drastically from day 2 to day 3 of germination in embryos infected with the fungus *F. moniliforme* (Fig. 2A, lane f).

β-1,3-Glucanase activities were also determined in crude protein extracts obtained from sterile and *F. moniliforme*–infected germinating embryos as described by Kombrink *et al.* (1988). However, enzyme activity values obtained with extracts from infected embryos were not significantly higher than those obtained with extracts prepared from sterile embryos (10–15% higher in extracts from infected embryos). β-1,3-Glucanase activity measurements, however, confirmed the results obtained on the immunoblot analysis of the protein fractions recovered from the chromatography on CM-Sephadex; that is, activity was found only in the bound protein fraction (basic proteins).

In order to determine the biochemical nature of the immunologically detected embryo β-1,3-glucanases, we separated proteins from crude extracts obtained from 3-day-germinated infected embryos by ion-exchange chromatography on a CM-Sephadex column (as shown in Fig. 1) under conditions in which the basic isoforms are known to bind to the resin. The acidic and basic fractions were screened separately with anti-PRm6b antiserum. After chromatography, the induced isoform was found in the basic protein fraction (Fig. 2B). We were not able to detect the 35-kDa β-1,3-glucanase isoform after chromatographic separation of crude protein extracts in either the acidic or basic protein fractions. Degradation of this protein as a consequence of the separation of a possible protease-inhibitor complex during chromatography may explain its absence. A similar negative result was observed when basic and acidic proteins were screened with anti-PRm7 antiserum.

It can be concluded from the immunoblot analysis results that the two β-1,3-glucanase isozymes are present in protein extracts obtained from *F. moniliforme*–infected embryos. Expression of one of these, a basic β-1,3-glucanase isoform, is induced after fungal infection. This basic isoform may correspond to the basic β-1,3-glucanase isoform that is induced in maize leaves, the PRmBa1 protein. However, in our electrophoretic system, the embryo isoform shows an apparent molecular mass of approximately 33 kDa instead of 35 kDa as described for the PRmBa1 isoform from leaves (Nasser *et al.* 1990). In germinating embryos, we did not observe any immunological reaction with the two other acidic β-1,3-glucanase isoforms, the PRm6b (30.5 kDa) and the PRm6a (32 kDa) proteins that accumulate in BMV-infected or chemically

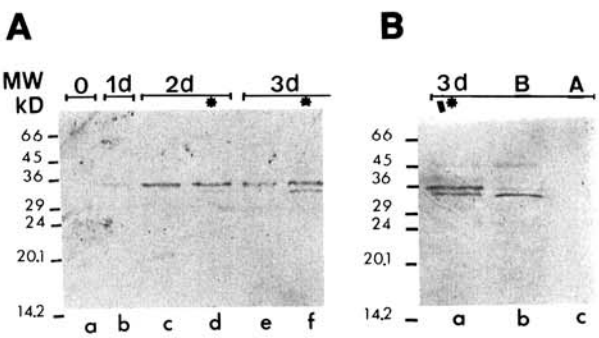

Fig. 2. Detection of embryo β-1,3-glucanases by immunological reaction of embryo protein extracts (100 µg of protein each) with anti-PRm6b antiserum. Five different germination experiments and two preparations of protein extracts were carried out for each germination. **A,** Embryos were dissected from dry sterilized seeds (lane a). After 20 hr of germination (lane b), embryos were inoculated with spores of *Fusarium moniliforme*. Infected and sterile control embryos were allowed to continue germination for a total of 2 days of germination (lanes c and d) or 3 days of germination (lanes e and f). Protein extracts prepared from *F. moniliforme*–infected embryos are marked by an asterisk (lanes d and f). **B,** Protein extract from *F. moniliforme*–infected embryos germinated 3 days (lane a). Protein fractions from chromatography on CM-Sephadex: bound, basic proteins (lane b) and not-bound, acidic proteins (lane c).

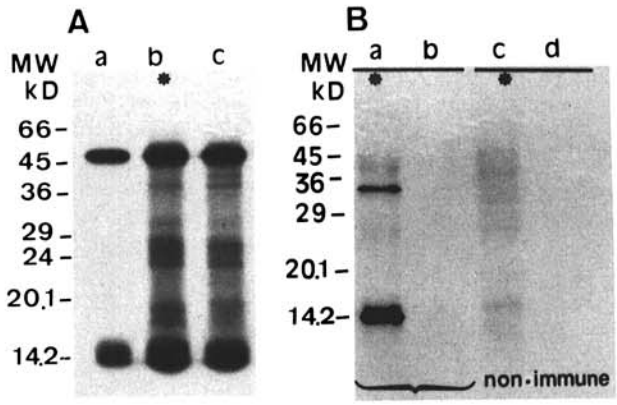

Fig. 3. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of radiolabeled total *in vitro* translation products from poly(A) RNA from *Fusarium moniliforme*–infected (lane b) and sterile (lane c) 2-day germinated maize embryos. Lane a, control without added poly(A) RNA. **B,** *In vitro* translated proteins were subjected to immunoprecipitation with anti-PRm6b antiserum (lane a, infected embryos; lane b, sterile embryos) and with nonimmune serum (lane c, infected embryos; lane d, sterile embryos). Asterisks denote results obtained from infected embryos.

treated maize leaves. Concerning the expression of the 35-kDa β-1,3-glucanase isoform detected in germinating embryos, its level of accumulation increased during germination of maize embryos, but it was not affected by fungal infection. Therefore, we postulate a developmental regulation of the expression of this isoform during embryo germination.

Confirmation that fungal infection induces the expression of the specific β-1,3-glucanase isoform detected by immunoblotting was obtained by analyzing the level of translatable β-1,3-glucanase mRNAs in sterile and *F. moniliforme*–infected germinating embryos. For this purpose, poly(A) RNA isolated from sterile and *F. moniliforme*–infected maize embryos germinated for 2 days was used to direct the synthesis of radiolabeled proteins (Fig. 3A). *In vitro* translations of the β-1,3-glucanase mRNAs that were present among these poly(A) RNAs at this stage of the embryo germination were identified by immunoprecipitation with anti-PRm6b antiserum. As shown in Figure 3B, a single β-1,3-glucanase isozyme with a molecular mass of 33 kDa can be *in vitro* translated and immunoprecipitated from poly(A) RNA obtained from *F. moniliforme*–infected embryos (Fig. 3B, lane a). A low molecular weight immunoprecipitable product was also observed (but was not detected on the immunoblot analysis of protein extracts from infected tissues, Fig. 2). No precipitate was obtained from the translation products synthesized from RNA of noninfected embryos (Fig. 3B, lane b). Controls with nonimmune serum were also performed (Fig. 3B, lanes c and d). The induction here observed on the level of β-1,3-glucanase mRNA for this specific β-1,3-glucanase isoform in response to fungal infection is in agreement with results obtained on the immunoblot analysis of protein extracts from sterile and infected maize embryos (Fig. 2). The 35-kDa isoform was not observed after immunoprecipitation of the *in vitro* translated proteins. This finding suggests that this particular isoform was previously translated and remains present in embryo tissues. The induction observed on the accumulation of the 33-kDa β-1,3-glucanase isoform correlates with an increase in its level of translatable mRNA in response to infection.

Effect of fungal infection on the expression of chitinases in germinating maize embryos.

The expression of chitinases in germinated embryos was studied by using two different antisera: anti-PRm7 and anti-PRm3. Nasser *et al.* (1990) reported that the antiserum prepared against the acidic chitinase PRm7 protein (34.5 kDa) also reacts with two other chitinase isoforms serologically related to the PRm7 protein, an acidic chitinase, the PRm5 protein (29 kDa), and a basic chitinase, the PRmBa2 protein (30 kDa). In addition to this, two more chitinases have been found to accumulate in BMV-infected or chemically treated maize leaves, the PRm3 and PRm4 proteins (both 25 kDa and acidic). These two isoforms react with antiserum prepared against the PRm3 protein. There is no serological relationship between the two groups of maize chitinases—the group serologically related to the PRm7 protein (PRm7, PRm5, and PRmBa2 proteins), and the PRm3 and PRm4 group of chitinases.

Results obtained on the expression of chitinases in germinating embryos are presented in Figure 4. Figure 4A shows the results obtained with anti-PRm3 antiserum. In crude protein extracts prepared from germinating embryos, an immunological reaction occurs with two proteins, both of approximately 25 kDa. These chitinase isoforms are detected at very low levels in extracts from sterile embryos, and they accumulate at significantly higher levels in extracts from *F. moniliforme*–infected embryos. In 2-day-germinated embryos (1 day postinfection) both isoforms are induced to the same level. However, in 3-day germinated embryos (2 days postinfection), the higher molecular weight isoform seems to be more strongly induced. When the acidic and basic protein fractions were chromatographically separated and probed with anti-PRm3 antiserum, the two chitinase isoforms were eluted in the acidic fraction (Fig. 4A, lane g). Finally, the two acidic 25-kDa chitinase isoforms detected in germinating embryos and whose expression is induced in response to fungal infection most probably coincide with the PRm3 and PRm4 chitinase isoforms (25 kDa and acidic) that accumulate in maize leaves after BMV infection or mercuric chloride treatment.

Figure 4B shows the results obtained from the expression of the second group of maize chitinases with anti-PRm7 antiserum. In extracts from *F. moniliforme*–infected embryos, but not from sterile embryos, a single immunological reaction occurs with a protein of approximately 34 kDa. The chitinase isoform here detected may correspond to the PRm7 isoform (34.4 kDa, acidic) that accumulates in maize leaves. We did not detect either the expression or the induction of the two other chitinase isoforms that are serologically related to the PRm7 protein and are induced in leaves after BMV infection and mercuric chloride treatment.

Results presented in Figures 2 and 4 were obtained with an acidic buffer (phosphate-citrate buffer, pH 2.8) for preparing

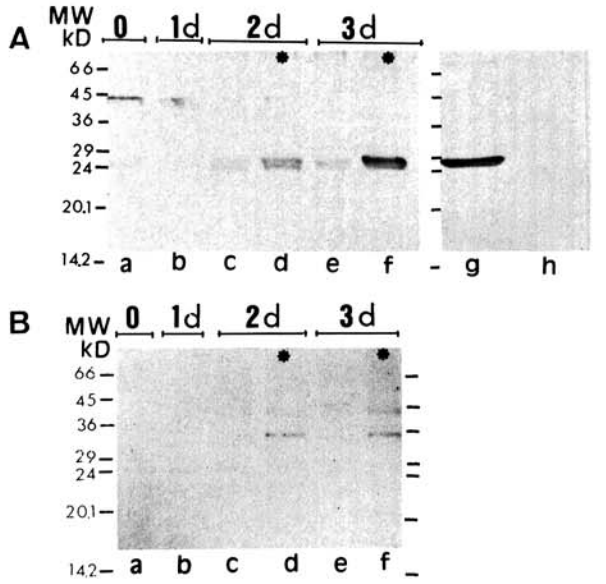

Fig. 4. Detection of embryo chitinases by immunological reaction of embryo protein extracts (100 μg of protein each) with anti-PRm3 antiserum (**A**) and anti-PRm7 antiserum (**B**). Three different germination experiments and two separate preparations of protein extracts for each germination were carried out. Extracts were prepared from sterile and *Fusarium moniliforme*–infected germinated embryos as indicated in Figure 2A (lanes a–f). Protein fractions from chromatography on CM-Sephadex: lane g, acidic proteins (not retained); lane h, basic proteins (bound). Asterisks denote results obtained from infected embryos.

protein extracts from maize embryos. The same proteins were detected on immunoblots when extracts were prepared with 0.5 M sodium acetate buffer, pH 5.2, containing 15 mM β-mercaptoethanol as extraction buffer (for conditions used to extract β-1,3-glucanases from BMV or chemically treated maize leaves, see Nasser *et al.* [1990]). However, higher signal intensities were observed on immunoblots when the pH 2.8 buffer was used as extraction buffer.

Effect of infection by *F. moniliforme* on the germination capability of maize seeds.

We examined the response of maize seeds to increasing doses of *F. moniliforme*. After 24 hr of germination, maize seeds were infected with spores of *F. moniliforme* prepared at various concentrations. Sterile and infected seeds were then germinated for 6 days more. As shown in Figure 5A, the germination capability of maize seeds decreased as the spore content of the inoculum increased. The seedlings that are able to germinate in the presence of *F. moniliforme* develop clear symptoms of infection, mainly in root tissues; this can be seen in Figure 5B. The *F. moniliforme*–infected seedlings were harvested and used for the analysis of expression of β-1,3-glucanases and chitinases in vegetative tissues, radicles, and coleoptiles.

Effects of fungal infection on the expression of β-1,3-glucanases and chitinases in vegetative tissues of maize seedlings.

Acidic protein extracts were prepared from vegetative tissues of sterile and *F. moniliforme*–infected maize seedlings. Immunoblotting was carried out with the antisera against β-1,3-glucanases (anti-PRm6b) and chitinases (anti-PRm3 and anti-PRm7). Results are presented in Figure 6.

The expression of β-1,3-glucanases in radicles of seedlings is reminiscent of the results obtained in germinating embryos. Thus, the non-fungal-induced 35-kDa β-1,3-glucanase isoform is also expressed in radicles (Fig. 6A), and it is observed both in sterile and infected tissues. The 33-kDa β-1,3-glucanase isoform is strongly induced by fungal infection (Fig. 6A, lane b). On the other hand, when protein extracts prepared from coleoptiles are probed with anti-PRm6b antiserum, only the 33-kDa fungal-induced isoform is detected in infected tissues (Fig. 6A, lanes c and d). In summary, the 35-kDa β-1,3-glucanase isoform, which is not fungus-induced, is expressed both in germinating embryos and in radicles but not in coleoptiles.

Concerning the expression of chitinases in radicles of seedlings, the two isoforms that react with anti-PRm3 antiserum in protein extracts from germinating embryos are also detected in protein extracts prepared from radicles. Both isoforms are induced in response to fungal infection in radicles (Fig. 6B, lanes a and b). On the other hand, in extracts prepared from coleoptiles, only one of these chitinase isoforms is detected, and its level of accumulation is notably increased in response to infection (Fig. 6B, lanes c and d).

Finally, anti-PRm7 antiserum was also used to study the expression of chitinases in radicles and coleoptiles of maize seedlings. There does not appear to be any detectable protein band in radicles from noninfected tissues, whereas a weak level of accumulation can be seen in radicles from infected tissues (Fig. 6C, lanes a and b). In coleoptiles, a low level of accumulation of this chitinase isoform, which increases in response to fungal infection, is observed (Fig. 6C, lanes c and d). The same molecular weight chitinase isoform is detected with anti-PRm7 antiserum in both vegetative tissues and embryo tissues.

Results concerning the expression of β-1,3-glucanases and chitinases in vegetative tissues (radicle and coleoptile) of maize seedlings indicate that there is a tissue-specific regulation for the expression of the different isoforms for both types of hydrolases in vegetative tissues of maize seedlings. Thus, the two chitinase isoforms that react with anti-PRm3 anti-

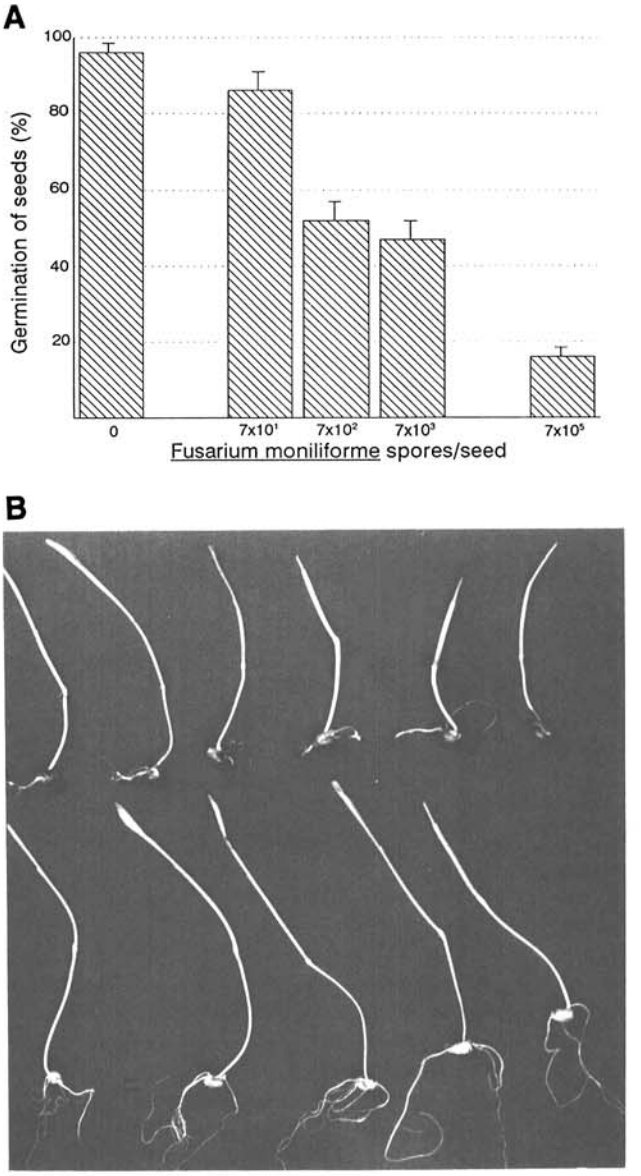

Fig. 5. Effect of infection by *Fusarium moniliforme* on germination of maize seeds. **A,** Sterile and *F. moniliforme*–infected maize seeds were germinated as indicated in Materials and Methods. Seeds were infected with spores of *F. moniliforme* prepared at various concentrations. Results are the average of four different germination experiments. Bars indicate maximum values obtained. **B,** Development of maize seeds that had been germinated for 7 days in the presence (top row) or absence (bottom row) of *F. moniliforme*. Inoculum containing the maximum concentration of spores was used for infection (7×10^5 spores per seed).

serum are expressed and induced in radicles. The third chitinase, the PRm7-related isoform, is induced in both radicle and coleoptile tissues in response to fungal infection, but the overall expression level is higher in the coleoptile. One of the two chitinase isoforms that reacts with the PRm3 antiserum is expressed, and fungus-induced, in radicles but not in coleoptiles. Finally, the constitutively expressed β-1,3-glucanase isoform is detected in radicles but not in coleoptiles. Thus, the patterns of expression of the different β-1,3-glucanase and chitinase isoforms in response to fungal infection indicate that, in addition to a tissue-specific expression, there is a differential pathogen regulation of the expression of these hydrolases.

DISCUSSION

There is increasing evidence that chitinases and β-1,3-glucanases are important in restricting pathogen infections by virtue of their ability to degrade fungal cell walls (Mauch *et al.* 1988; Boller 1993; Collinge *et al.* 1993). Plant chitinases, either alone or in combination with a β-1,3-glucanase (Mauch *et al.* 1988) or a ribosome-inactivating protein (Leah *et al.* 1991), are known to inhibit mycelial growth of a wide range of fungi.

In contrast to the extensive data concerning the induction of chitinases and β-1,3-glucanases in relation to pathogen attack in leaves of adult plants, expression of these hydrolases in other tissues or developmental stages of the life cycle of a plant have received little attention. This study was undertaken to obtain a better insight into the capability of maize seedlings to synthesize these particular PR proteins, β-1,3-glucanases and chitinases, in response to fungal infection. Protection of the germinating seed is vitally important for the survival of the species.

F. moniliforme is reported to be one of the most widespread pathogens of maize. The fungus causes important damage in crops through seed decay, damping-off, and seedling blight (Nelson *et al.* 1981). This is the fungus most frequently isolated from maize kernels, the inoculum source consequently being seed-transmitted. Moreover, *Fusarium* is a soil-inhabiting fungus that is able to infect plants through the roots. Induction of plant defense genes such as chitinase and β-1,3-glucanase genes in seed tissues and in the roots of germinating seedlings may be one factor in reducing colonization of *F. moniliforme*.

Analysis of the expression of β-1,3-glucanases and chitinases and factors affecting their expression is complicated by the finding that several isoforms, both acidic and basic, have been found for each of these hydrolases. The contribution and possible function of the specific isoforms, both in healthy plants and in response to infection by pathogens, remains unsolved. A differential regulation of β-1,3-glucanase mRNAs in response to pathogen infection has been reported in leaves of tobacco plants (Ward *et al.* 1991). Here, messages for the acidic glucanases are induced similarly to the mRNAs for other PR proteins, while a basic glucanase shows a different response. Similarly, a differential induction of basic and acidic tobacco chitinases has been demonstrated in response to various forms of stress (Memelink *et al.* 1990; Brederode *et al.* 1991).

In maize, it has been demonstrated that infection by brome mosaic virus or treatment with mercuric chloride induces accumulation of several β-1,3-glucanases and chitinases in leaves (Nasser *et al.* 1988, 1990). Results presented here show a coordinated induction on the level of accumulation of a basic β-1,3-glucanase (Figs. 2 and 3) and two acidic chitinase isozymes (Fig. 4A) in germinating maize embryos in response to infection by the fungus *F. moniliforme*. Accumulation of a third chitinase isoform is also observed (Fig. 4B), but its biochemical nature has not been determined. As is the case for the PR protein group, induction of the expression of the maize β-1,3-glucanases and chitinases appears to be a nonspecific response of the host to pathogens, since they accumulate in response to infection both by BMV in leaves and by *F. moniliforme* in seedling tissues.

When patterns of expression of β-1,3-glucanases and chitinases in germinating embryos and in vegetative tissues of maize seedlings are compared, differences are found among the various isoforms of each type of hydrolase in response to fungal infection. Similarly, different patterns are observed among the vegetative tissues (i.e., radicles and coleoptiles) from maize seedlings, indicating that in addition to a pathogen-regulated expression, an important tissue-specificity is also controlling the expression of these hydrolases.

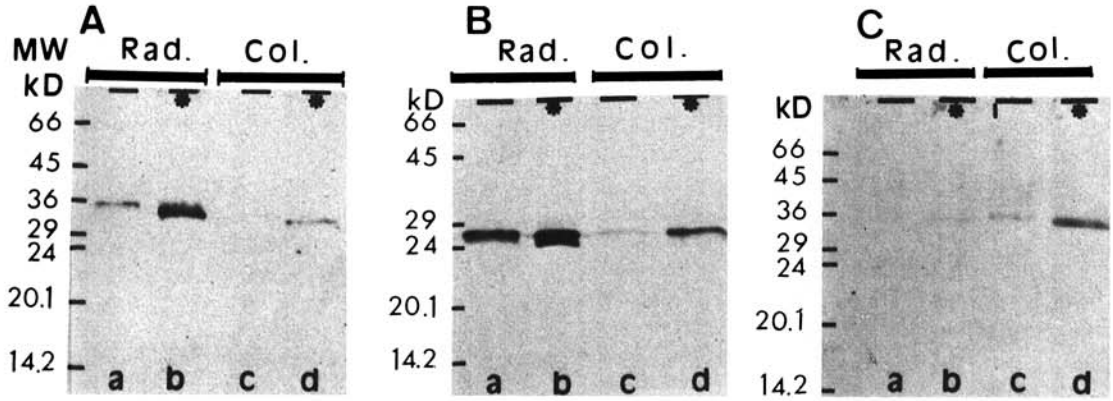

Fig. 6. Detection of β-1,3-glucanases and chitinases in vegetative tissues of maize seedlings. **A,** β-1,3-Glucanase isoforms that react with anti-PRm6b antiserum. **B,** Chitinases detected with anti-PRm3 antiserum. **C,** Chitinases detected with anti-PRm7 antiserum. Radicles and coleoptiles were obtained from seedlings that had been germinated for 7 days without (lanes a and c) or with (lanes b and d) infection by *Fusarium moniliforme* (infection at 24 hr of germination as indicated in Materials and Methods); 100 μg of each extract was analyzed. Asterisks denote results obtained from infected embryos.

Concerning the induction of expression of the basic β-1,3-glucanase, our results showed that accumulation of this particular β-1,3-glucanase isoform results from an increase in the level of its translatable mRNA. In opposition to this, a second serologically related β-1,3-glucanase isoform (35 kDa) is expressed in germinating embryos and in radicles from seedlings, but its expression is not affected by fungal infection. These results point to a diversity of functions for these hydrolytic enzymes during germination of maize seeds. A role for the 35-kDa non-fungus-induced β-1,3-glucanase isoform during natural processes in germination can be postulated. The expression of this particular β-1,3-glucanase isoform would consistently be developmentally regulated during germination. In relation to this, β-1,3-glucanases are known to participate in processes such as pollen formation, removal of wound callus, senescence, and flowering. Results obtained by several authors indicate that there is a correlation between accumulation of specific β-1,3-glucanases and chitinases with tissues in which major cell wall disruption takes place (i.e., flower reproductive organs) (Ori 1990; Neale 1990; Campillo and Lewis 1992). With regard to the possible role of the 35-kDa β-1,3-glucanase isoform during germination of a cereal seed, it may well be involved in the degradation of storage polymers or in the removal of cell walls, which are major processes during seed germination. β-1,3-Glucanases may participate in the cell wall degradation by hydrolyzing regions of contiguous β-1,3-linked glucosyl residues in wall β-1,3-1,4-glucan.

In summary, regulation of the expression of chitinases and β-1,3-glucanases in response to fungal infection occurs in a coordinated fashion in tissues of maize seedlings. Results previously reported by our laboratory indicate that the induction of these hydrolases is also coordinated with the induction of another PR protein, the PRms protein, during germination of maize seeds) (Casacuberta *et al.* 1991, 1992; Cordero *et al.* 1992). Differential responses to fungal infection and tissue-specific expression are found in the expression of both types of hydrolases. This is interesting in view of the recent observation that only specific chitinases and β-1,3-glucanases exhibit antifungal activity when they are tested, either alone or in combination, for their ability to inhibit *in vitro* growth of the fungus *F. solani* (Sela-Buurlage *et al.* 1993). It will now be interesting to obtain a more profound characterization of the specific fungus-induced chitinase and β-1,3-glucanase isoforms here reported and their ability to inhibit the growth of *F. moniliforme*. Expression of β-1,3-glucanase and chitinase genes induced by fungal infection would be part of the complex of mechanisms that seedlings may use during germination to defend themselves against pathogen attack.

MATERIALS AND METHODS

Plant material.

Maize seeds (*Zea mays*, pure inbred line W64A) were used as the experimental material. Seeds were sterilized as previously described (Casacuberta *et al.* 1991). Dry sterilized seeds were dissected, and the embryo was placed on wet filter paper for 20 hr to allow germination. Embryos were then inoculated with a conidial suspension of the fungus *F. moniliforme* by adding 50 µl (2,000–3,000 spores per milliliter) to each embryo. Inoculated embryos (and sterile control embryos) were allowed to continue germination for the required period of time.

Vegetative tissues (radicle and coleoptile) were also obtained from sterile and *F. moniliforme*–infected maize seedlings. For this, sterile seeds were germinated in sterile medium (Casacuberta *et al.* 1991) for 24 hr and then inoculated with the conidial suspension of *F. moniliforme* as indicated above. Infected and sterile control seeds were allowed to continue germination for another 6 days.

Preparation of protein extracts and immunoblotting.

Sterile and *F. moniliforme*–infected germinating embryos were harvested and ground to a powder with a pestle in a prechilled mortar in liquid nitrogen. Chilled extraction buffer (84 mM citric acid, 30 mM Na_2HPO_4, at pH 2.8, containing 14 mM 2-mercaptoethanol and 6 mM ascorbic acid) was added to the powder (2 ml/g fresh weight tissue). The phosphate-citrate buffer was also used for preparation of protein extracts from vegetative tissues (radicle, coleoptile) from maize seedlings. Extraction was carried out at 4° C for 60 min with continuous slow stirring. The buffer extracts were then centrifuged at 10,000 rpm for 15 min at 4° C, and the supernatant was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Protein content was assayed by the method of Bradford (1976) using the Bio-Rad (Bio-Rad, Richmond, CA) dye reagent and bovine serum albumin as a standard.

Proteins obtained from 3-day-germinated embryos infected with *F. moniliforme* were subjected to ion-exchange chromatography basically as described by Nasser *et al.* (1990). For this, acidic protein extracts were dialyzed against 20 mM sodium acetate buffer, pH 5.2, and subjected to separation on a CM-Sephadex column equilibrated with the same buffer. Characterization of β-1,3-glucanases and chitinases from maize leaves indicated that the protein fraction that passes through the column contains the acidic isoforms, and the protein fraction that is retained (eluted with 0.5 M NaCl in the same sodium acetate buffer) contains the basic isoforms of these hydrolytic enzymes. Proteins present in each fraction were further analyzed by immunoblotting.

Immunoblots were prepared essentially according to the procedure of Towbin *et al.* (1979). SDS-PAGE was performed by the method of Laemmli (1970) using 15% (w/v) polyacrylamide gels. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes, blocked in PBS buffer (1× PBS = 0.05M Na phosphate, pH 7.5, 0.15M NaCl) containing 0.1% Tween and 3% w/v nonfat dry milk, and incubated for 2 hr at room temperature with the appropriate antiserum. Antibodies raised against maize PR proteins described as β-1,3-glucanases and chitinases that accumulate in maize leaves in response to BMV infection or mercuric chloride treatment were obtained by Nasser *et al.* (1988, 1990). These antibodies were used in this work to study the expression of both hydrolases in germinating maize embryos. Thus, an antiserum raised against maize β-1,3-glucanases (anti-PRm6b antiserum) and two different antisera raised against chitinases (anti-PRm3 and anti-PRm7 antisera) were used in this work. These antisera were provided by G. Burkard (IBMP, Strasbourg, France). Swine anti-rabbit immunoglobulin–alkaline phosphatase conjugate (Dako A/S, Copenhagen) was used as second antibody. Serological reac-

tions were detected by the colorimetric method with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

***In vitro* translation and immunoprecipitation.**

Total RNA was prepared from noninfected and *F. moniliforme*–infected embryos germinated for 2 days basically as described by Logeman *et al.* (1987). Poly(A) RNA was selected by oligo(dT) cellulose chromatography and used for *in vitro* translation experiments.

In vitro translation of the poly(A) RNA was performed in a nuclease-treated rabbit reticulocyte lysate (Amersham). Incubation mixtures were supplied with 1 mCi/ml ^{35}S-methionine (1,000 Ci/mmol, Amersham) and 0.05 μg/μl poly(A) RNA. After translation, 25% was directly electrophoresed; the rest was used for immunoprecipitation.

Immunoprecipitation of the translation products with the antiserum against maize β-1,3-glucanases (anti-PRm6b antiserum) using protein A–Sepharose CL 4B (Pharmacia, Uppsala, Sweden) was carried out by the method of Borgese (Borgese and Gaetani 1980). SDS-PAGE was performed by the method of Laemmli (1970) using a 15% acrylamide separating gel. After electrophoresis, the gels were fixed for 20 min in methanol/acetic acid/water (30:10:60, v/v), immersed on Enhance (New England Nuclear, Boston, MA) for 20 min, and dried under vacuum in a gel drier (45 min). Fluorographs were obtained by exposing the dry gels at –70° C for 18 days to preflashed X film.

ACKNOWLEDGMENTS

We are grateful to G. Burkard for providing us with the antisera against maize β-1,3-glucanases and chitinases. We thank M. I. Murillo for excellent technical assistance. This work was supported by the Plan Nacional de Investigación Científica y Desarrollo Tecnológico (grant AGR91-0677).

LITERATURE CITED

Bol, J. F., Linthorst, H. J. M., and Cornelissen, B. J. C. 1990. Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. 28:113-138.

Boller, T. 1993. Antimicrobial functions of the plant hydrolases, chitinase and β-1,3-glucanase. Pages 391-397 in: Developments in Plant Pathology. Vol. 2, Mechanisms of Plant Defense Responses B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Borgese, N., and Gaetani, S. 1980. Site of synthesis of rat liver NADH-cytochrome b reductase, an integral membrane protein. FEBS Lett. 112:216-220.

Bowles, D. J. 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. 59:873-907.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye-binding. Anal. Biochem. 72:248-254.

Brederode, F. T., Linthorst, H. J. M., and Bol, J. F. 1991. Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol. Biol. 17:1117-1125.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Diddle, P., Knowlton, S., Jeffry Mauvais, C., and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194-1197.

Campillo, E., and Lewis, L. N. 1992. Occurrence of 9.5 cellulase and other hydrolases in flower reproductive organs undergoing major cell wall disruption. Plant Physiol. 99:1015-1020.

Casacuberta, J. M., Puigdoménech, P., and San Segundo, B. 1991. A gene coding for a basic pathogenesis-related (PR-like) protein from *Zea mays*. Molecular cloning and induction by a fungus (*Fusarium moniliforme*) in germinating maize seeds. Plant Mol. Biol. 16:527-536.

Casacuberta, J. M., Raventós, D., Puigdoménech, P., and San Segundo, B. 1992. Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. Mol. Gen. Genet. 234:97-104.

Collinge, D. B., McKragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmunsen, U., and Vad, K. 1993. Plant chitinases. Plant J. 3:31-40.

Cordero, M. J., Raventós, D., and San Segundo, B. 1992. Induction of PR proteins in germinating maize seeds infected with the fungus *Fusarium moniliforme*. Physiol. Mol. Plant Pathol. 41:189-200.

Granell, A., Bellés, J. M., and Conejero, V. 1987. Induction of pathogenesis-related proteins in tomato by citrus exocortis viroid, silver ion and ethephon. Physiol. Mol. Plant Pathol. 31:83-90.

Ham, K.-S., Kauffmann, S., Albersheim, P., and Darvill, A. G. 1991. Host-pathogen interactions XXXIX. A soybean pathogenesis-related protein with β-1,3-glucanase activity releases phytoalexin elicitor-active heat-stable fragments from fungal walls. Mol. Plant-Microbe Interact. 4:545-552.

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.

Kombrink, E., Schröder, 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.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277:680-685.

Lamb, C. J., Lawton, M. A., Drom, M., and Dixon, R. A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215-224.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266:1564-1573.

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

Linthorst, H. J. M. 1991. Pathogenesis-related proteins of plants. C. R. Plant Sci. 10:123-150.

Logeman, J., Schell, J., and Willmitzert, L. 1987. Improved method for the isolation of RNA from plant tissues. Anal. Biochem. 163:16-20.

Lotan, T., Ori, N., and Fluhr, N. 1989. Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1:881-887.

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.

Memelink, J., Linthorst, H. J. M., Schilperoot, R. A., and Hoge, J. H. C. 1990. Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol. Biol. 14:119-126.

Nasser, W., de Tapia, M., and Burkard, G. 1990. Maize pathogenesis-related proteins: Characterization and cellular distribution of β-1,3-glucanases and chitinases induced by brome mosaic virus infection or mercuric chloride treatment. Physiol. Mol. Plant Pathol. 36:1-14.

Nasser, W., de Tapia, M., Kauffman, S., Montasser-Kouhsari, S., and Burkard, G. 1988. Identification and characterization of maize pathogenesis-related proteins. Four maize PR proteins are chitinases. Plant Mol. Biol. 11:529-538.

Neale, A. D., Wahleithner, J. A., Lund, M., Bonnett, H. T., Kelly, A., Ry Meeks-Wagner, D., Peacock, J., and Dennis, E. S. 1990. Chitinase, β-1,3-glucanase, osmotin and extensin are expressed in tobacco explants during flower formation. Plant Cell 2:673-684.

Nelson, P. E., Toussoun, T. A., and Cook, R. J., eds. 1981. *Fusarium* Diseases: Biology and Taxonomy. Pennsylvania State University Press, University Park.

Ori, N., Sessa, G., Lotan, T., Himmelhoch, S., and Fluhr, R. 1990. A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. EMBO J. 9:3429-3436.

Oshima, M., Itoh, H., Matsuika, M., Murakami, T., and Ohashi, Y. 1990. Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein in transgenic tobacco. Plant Cell 2:95-106.

Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A. B., Melchers, L. S., van den Elxen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases

exhibit antifungal activity. Plant Physiol. 101:857-863.
Takeuchi, Y., Yoshikawa, M., Takeba, G., Tanaka, K., Shibata, D., and Horino, O. 1990. Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor-releasing factor, β-1,3-glucanase, in soybean. Plant Physiol. 93:673-682.
Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.
van Loon, L. C. 1983. The induction of pathogenesis-related proteins by pathogens and specific chemicals. Neth. J. Plant Pathol. 89:265-273.
van Loon, L. C. 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4:111-116.
Vera, P., Hernandez-Yago, J., and Conejero, V. 1988. Immunocytochemical localization of the major "pathogenesis-related" (PR) protein in tomato plants. Plant Sci. 55:223-230.
Ward, E. R., Payne, G. B., Moyer, M. B., Willians, S. C., Dincher, S. S., Sharkey, K. C., Beck, J. J., Taylor, H. T., Ahl-Goy, P., Meins, 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.
White, R. F., and Antoniw, J. F. 1991. Virus induced resistance responses in plants. Crit. Rev. Plant Sci. 9:443-455.