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Functional Implications of the Subcellular Localization of Ethylene-Induced Chitinase and β-1,3-Glucanase in Bean Leaves

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Plants respond to an attack by potentially pathogenic organisms and to the plant stress hormone ethylene with an increased synthesis of hydrolases such as chitinase and β-1,3-glucanase. We have studied the subcellular localization of these two enzymes in ethylene-treated bean leaves by immunogold cytochemistry and by biochemical fractionation techniques. Our micrographs indicate that chitinase and β-1,3-glucanase accumulate in the vacuole of ethylene-treated leaf cells. Within the vacuole label was found predominantly over ethylene-induced electron dense protein aggregates. A second, minor site of accumulation of β-1,3-glucanase was the cell wall, where label was present nearly exclusively over the middle lamella surrounding intercellular air spaces. Both kinds of antibodies labeled Golgi cisternae of ethylene-treated tissue, suggesting that the newly synthesized chitinase and β-1,3-glucanase are processed in the Golgi apparatus. Biochemical fractionation studies confirmed the accumulation in high concentrations of both chitinase and β-1,3-glucanase in isolated vacuoles, and demonstrated that only β-1,3-glucanase, but not chitinase, was present in intercellular washing fluids collected from ethylene-treated leaves. Based on these results and earlier studies, we propose a model in which the vacuole-localized chitinase and β-1,3-glucanase are used as a last line of defense to be released when the attacked host cells lyse. The cell wall-localized β-1,3-glucanase, on the other hand, would be involved in recognition processes, releasing defense activating signaling molecules from the walls of invading pathogens.

INTRODUCTION

Chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39) are present in many higher plants. Both enzymes have been implicated in defense reactions of plants against potential pathogens (Abeles et al., 1971; Boller et al., 1983). In many plants, chitinase and β-1,3-glucanase rapidly accumulate following pathogen attack, after elicitor treatment, and in response to the plant stress hormone ethylene (for review, see Boller, 1985). The substrates of chitinase and β-1,3-glucanase, chitin and β-1,3-glucan, respectively, are major components of the cell walls of many fungi (Wessels and Sietsma, 1981). It has been shown that chitinase and β-1,3-glucanase can degrade isolated fungal (Mauch et al., 1988b) and, due to the lysozyme activity of chitinase, bacterial cell walls (Boller et al., 1983). In addition, physiological concentrations of chitinase and β-1,3-glucanase effectively inhibit growth of many potentially pathogenic fungi (Schlumbaum et al., 1986; Mauch et al., 1988b). Thus, chitinase and β-1,3-glucanase appear to be part of the inducible defense response of higher plants. However, definite proof of an involvement of chitinase and β-1,3-glucanase in resistance mechanisms is still lacking.

To learn more about the possible roles of chitinase and β-1,3-glucanase, it is important to find out where these enzymes accumulate in stressed tissue. To date, only cell fractionation methods have been employed to investigate the subcellular localization of chitinase and β-1,3-glucanase in stressed plant tissue. With one exception (Boller and Vögeli, 1984), chitinase and β-1,3-glucanase have been reported to accumulate extracellularly based on their occurrence in intercellular washing fluids collected from infected leaves (Kauffman et al., 1987; Legrand et al., 1987; Kombrink et al., 1988). These results should be viewed with caution since none of these studies specifically determined whether chitinase and β-1,3-glucanase were actively secreted or whether they were released into the intercellular space because of cell death occurring in the infected tissue.

In this paper we address the question of the subcellular localization of chitinase and β-1,3-glucanase using a combination of immunocytochemical and biochemical fractionation methods. Since chitinase and β-1,3-glucanase are

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also induced by the stress hormone ethylene (Abeles et al., 1971; Boller et al., 1983), we decided to use ethylene-treated bean leaves as an initial model system for our localization studies. Our experimental goals were greatly facilitated by finding two bean varieties that did not show any signs of ethylene-induced leaf yellowing, tissue damage, or leaf abscission, but still reacted to ethylene treatment with a strongly increased synthesis of chitinase and β-1,3-glucanase. Using these bean varieties we show that chitinase and β-1,3-glucanase accumulate mainly in the vacuoles of stressed tissue and that only a small amount of β-1,3-glucanase, but no chitinase, is secreted into the cell wall space. The results of our immunocytochemical studies were fully supported by our cell fractionation experiments. Based on these findings we postulate a dual role for the stress-induced chitinase and β-1,3-glucanase in defense responses.

RESULTS

Induction of Chitinase and β-1,3-Glucanase by Ethylene

We tested 10 bean varieties for the best combination of preservation of ultrastructural integrity and induction of chitinase and β-1,3-glucanase synthesis following exposure of plants to 100 ppm ethylene for 2 days. Surprisingly, two of the varieties tested, cv "Greensleeves" and cv "Kentucky Wonder," did not show any sign of chlorophyll loss, tissue damage, or leaf abscission, even after 5 days in a 100-ppm ethylene atmosphere. The only morphological response observed was a swelling of the epicotyl. However, chitinase and β-1,3-glucanase activities were strongly induced by ethylene. After 48 hr of ethylene treatment, chitinase activity was 30 to 40 times higher and β-1,3-glucanase activity 40 to 50 times higher than in untreated control leaves. The increase in both activities was linear over the first 72 hr of ethylene treatment (data not shown).

As shown in Figure 1A, the induction of chitinase and β-1,3-glucanase by ethylene was also monitored by SDS-PAGE. Compared to a control extract (lane 1), extracts from ethylene-treated leaves showed several additional protein bands (lane 2). Two of them were identified as chitinase and β-1,3-glucanase, respectively, by affinity adsorption to the respective enzyme substrates. Addition of pachyman, a water-insoluble β-1,3-glucan, quantitatively removed the ethylene-induced 36-kD protein from the extract (gluc, lane 3). After removal of the 36-kD protein, the extract showed no more glucanase activity, indicating that the 36-kD protein is indeed a β-1,3-glucanase. Addition of chitin quantitatively removed a broad ethylene-induced band of about 33-kD from the extract (lane 6). With the removal of the 33-kD protein, the extract lost its chitinase activity, indicating that the 33-kD protein is a

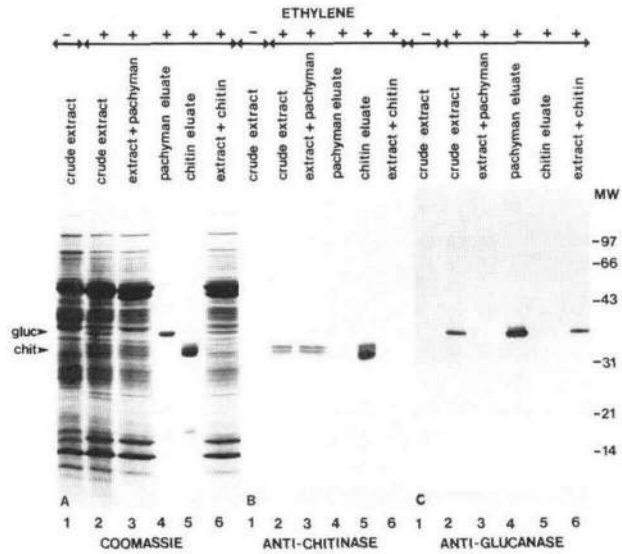


Figure 1. Identification of Ethylene-Induced Chitinase and β-1,3-Glucanase and Specificity of Antibodies.

(A) Coomassie-stained SDS-PAGE gel.
(B) Immunoblots stained with antichitinase antibodies.
(C) Immunoblots stained with anti-β-1,3-glucanase antibodies.
Lanes 1, proteins from control leaves; lanes 2, proteins from ethylene-treated bean leaves; lanes 3, same as lane 2 but proteins with an affinity for β-1,3-glucans were removed by adsorption to pachyman; lanes 4, proteins with an affinity to β-1,3-glucans, e.g., β-1,3-glucanase, removed from the preparation shown in lane 3; lanes 5, proteins with an affinity to chitin, e.g., chitinase, removed from the preparation shown in lane 6; lanes 6, same as lane 2 but proteins with an affinity to chitin were removed by adsorption to chitin. Molecular weight markers are indicated on the right. Chitinase (chit) and β-1,3-glucanase (gluc) are marked on the left.

chitinase. Both adsorbed enzymes were released from their substrates by incubation in SDS sample buffer (lanes 4 and 5).

Specificity of Antibodies

The specificity of the antibodies used for the immunolocalization studies was tested on protein gel blots of SDS-PAGE gels as illustrated in Figure 1. Figure 1B shows that the antibodies raised against chitinase from bean were monospecific. The antibodies reacted with chitinase (lanes 2, 3, and 5) and did not recognize β-1,3-glucanase, even at high concentrations (lane 4), or any other protein present in the extract. Since the crude serum raised against β-1,3-glucanase purified from pea recognized a number of other proteins besides β-1,3-glucanase on immunoblots, we affinity-purified the antibodies using bean glucanase bound to nitrocellulose (see "Methods"). As shown in Figure 1C, this procedure removed most of the cross-contamination. However, some faint background in the higher molecular

weight range could still be observed. The reason for this background smear is not known. It is important to note that none of the background was ethylene-induced. It occurred in control extracts (lane 1) and in extracts of ethylene-treated leaves (lanes 2, 3, and 6). The antiglucanase antibodies did not recognize chitinase (lane 5). Neither antisera recognized the two lower molecular weight proteins which were present in the pachyman eluates and chitin eluates, respectively, shown in Figure 1A.

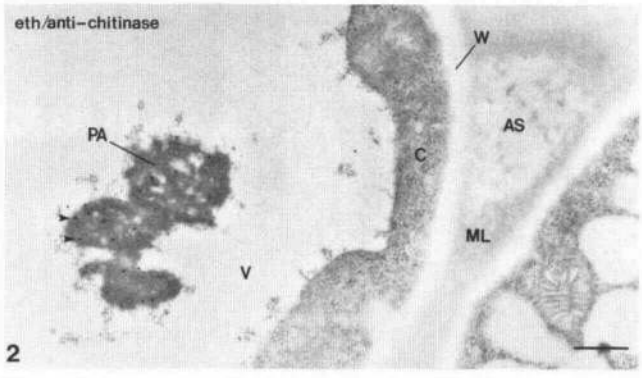
Immunogold Localization of Chitinase and β-1,3-Glucanase

The ethylene-induced chitinase and β-1,3-glucanase could be readily localized by immunocytochemical methods. Bean leaf tissue fixed with glutaraldehyde followed by OsO_4 and embedded in LR White acrylic resin retained a high degree of protein antigenicity. Postfixation with OsO_4 only slightly reduced the density of the immunolabeling but strongly improved ultrastructural preservation. Figure 2 shows that antibodies specific for chitinase labeled large electron dense protein aggregates present in the vacuoles of ethylene-treated bean leaves. No label was found over the cytoplasm and over the cell wall. An example of the labeling pattern obtained with antibodies specific for β-1,3-glucanase is presented in Figure 3. Similar to the antichitinase antiserum, the antiglucanase antibodies heavily labeled the ethylene-induced vacuolar protein aggregates. In addition, glucanase label was also found over the expanded middle lamella region of the cell wall, indicating that some of the glucanase is present extracellularly.

Both chitinase and glucanase pass through the Golgi apparatus. Figure 4 shows a high magnification view of ethylene-treated leaves labeled with antiglucanase antibodies. Gold label is present over the middle lamella and over a Golgi complex. As exemplified in Figure 5, Golgi labeling was also observed in sections of ethylene-treated leaves labeled with antichitinase antibodies. Occasionally, a light labeling of flocculent material in the vacuole by antichitinase antiserum, shown in Figure 5, and by antiglucanase antibodies (not shown) was observed. Such flocculent material was also observed in the vacuoles of noninduced control cells. However, as shown in Figure 6, in control cells it did not label with antichitinase or antiglucanase antibodies. The large vacuolar protein aggregates that labeled for both chitinase and glucanase were only found in ethylene-treated bean leaves. They were absent from control leaves. Labeling of control cells with antibodies specific for β-1,3-glucanase, shown in Figure 7, revealed a light labeling of the middle lamella region of cell corners. Thus, small amounts of β-1,3-glucanase appear to be present extracellularly in control cells.

Immunocytochemical Controls

Several controls were used to establish the specificity of the immunolabeling pattern. Figure 8 shows that, if immune serum were replaced by nonimmune serum, no gold par-



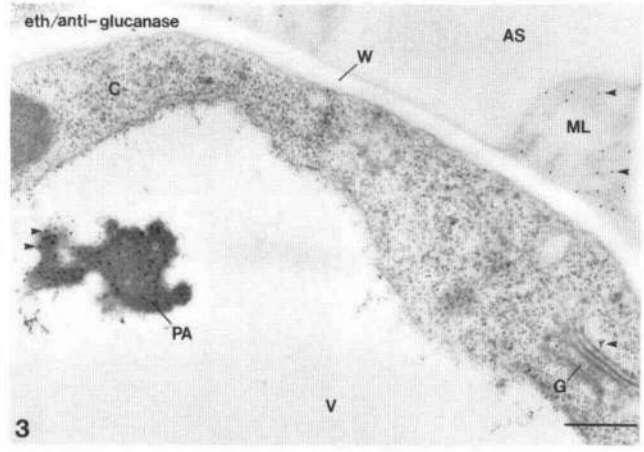


Figure 2. Immunocytochemical Localization of Chitinase.

Section incubated with antichitinase antibodies followed by protein A-gold (17 nm). Gold label (▶) is found exclusively over ethylene-induced protein aggregates in the vacuoles. No label is seen extracellularly. Bar = 0.5 μm. The abbreviations used are: AS, air space; C, cytoplasm; CP, chloroplast; W, cell wall; G, Golgi complex; ML, middle lamella; PA, protein aggregate; V, vacuole.

Figure 3. Immunocytochemical Localization of β-1,3-Glucanase in Ethylene-Treated Bean Leaves.

Section incubated with anti-β-1,3-glucanase antibodies followed by protein A-gold (11 nm). Label (▶) is found over electrondense vacuolar protein aggregates, over a Golgi complex, and over expanded regions of the middle lamella adjacent to air spaces. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

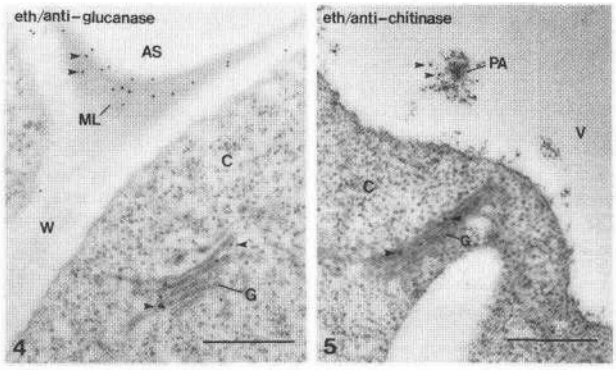


Figure 4. Immunolabeling with Anti-β-1,3-Glucanase Antibodies.

Label (►) is present over a Golgi complex and over the middle lamella region of the cell wall. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

Figure 5. Immunolabeling with Antichitinase Antibodies.

Gold label (►) is found over Golgi complexes and over small protein aggregates in the vacuole. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

ticles were observed over the ethylene-induced vacuolar protein aggregates and over the middle lamella. In addition, no labeling of Golgi complexes was found (not shown). Labeling was not due to the unspecific sticking of protein A-gold or IgGs to the vacuolar protein aggregates or to the middle lamella, since no gold particles were found over these locations if antibodies to the chlorophyll-protein CP II*, a thylakoid membrane complex, or antibodies to the cell wall polysaccharide xyloglucan were used (not shown). Immunolabeling with antibodies against the pectic cell wall polysaccharide rhamnogalacturonan I revealed a cell wall labeling pattern similar to β-1,3-glucanase (not shown). However, the labeling for the peptic polysaccharide was not ethylene-induced and, in contrast to the glucanase, labeling, was prevented by preadsorption to polygalacturonic acid, indicating that the two antisera recognize different antigenic sites. Figure 9 shows that preadsorbtion of the antiglucanase antibodies with an excess of purified β-1,3-glucanase prior to the immunolabeling prevents the binding of gold label to the vacuolar protein aggregates and to the middle lamella. Similarly, if the antichitinase antibodies were preadsorbed with an excess of purified chitinase, labeling of the vacuolar protein aggregates completely disappeared (not shown). Finally, the antichitinase antibodies and the antiglucanase antibodies did not label vacuolar protein deposits of clover root tips, indicating that both antisera have no general affinity for vacuolar protein aggregates (not shown). Together these results indicate that the labeling pattern observed on thin sections of fixed bean leaves incubated with antichitinase or antiglucanase antibodies is due to the specific interaction of the antibodies with their respective antigens.

Intracellular and Extracellular Distribution of Chitinase and β-1,3-Glucanase as Determined by Biochemical Methods

Figure 10A compares the protein pattern of bean leaf extracts and intercellular washing fluids (IWF) collected from ethylene-treated leaf tissue. Chitinase and β-1,3-glucanase are clearly visible in the extract from ethylene-treated leaves (lane 2). Only a few bands are present in the IWF (lane 3). One of them has an apparent molecular weight similar to that of β-1,3-glucanase. No major band corresponds to chitinase. As shown in Figure 10B, immunoblotting with a combination of antichitinase and antiglucanase antibodies confirmed that glucanase but no detectable amounts of chitinase were present in the IWF.

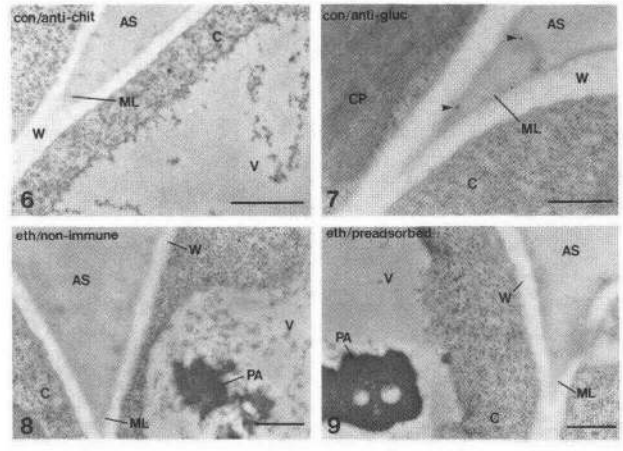


Figure 6. Section of Control Bean Leaf Labeled with Antichitinase Antibodies.

No label is present over the middle lamella and over the flocculent material in the vacuole. Large vacuolar protein aggregates were not found in noninduced control tissue. Background label is very low. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

Figure 7. Section of Control Bean Leaf Labeled with Anti-β-1,3-Glucanase Antibodies.

Gold label (►) is found exclusively over the middle lamella region of the cell wall. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

Figure 8. Section of Ethylene-Treated Bean Leaf Incubated with Nonimmune Sera Followed by Protein A-Gold.

No gold label is found over the vacuolar protein aggregates and over the middle lamella. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

Figure 9. Section of Ethylene-Treated Bean Leaf Incubated with Anti-β-1,3-Glucanase Antibodies Preadsorbed with an Excess of Purified Glucanase.

No label above background is found over the vacuolar protein aggregate and over the middle lamella. Bar = 0.5 μm. Abbreviations are defined in Figure 2.

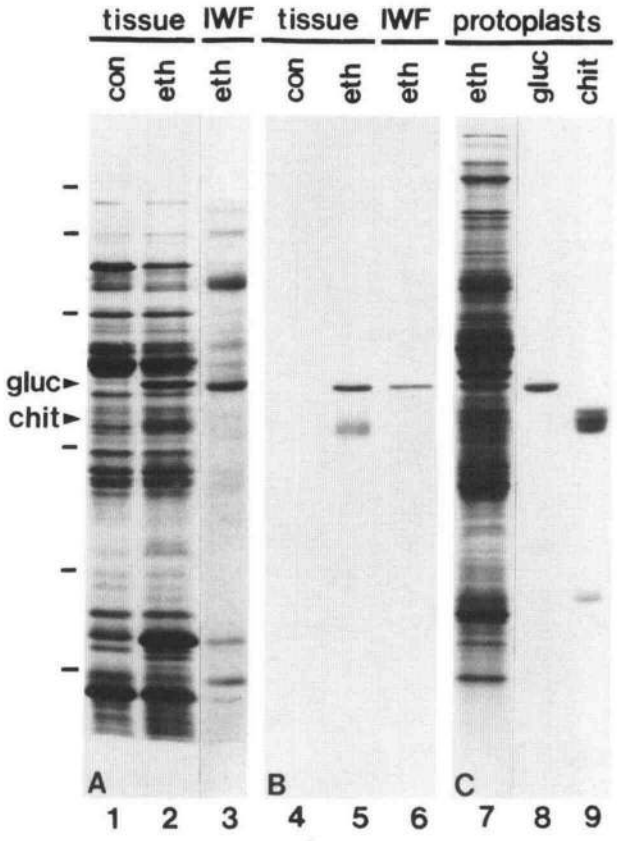


Figure 10. SDS-PAGE of Leaf Tissue, Intercellular Washing Fluids, and Protoplasts.

(A) Stained with Coomassie Blue.
(B) An immunoblot stained with a combination of antichitinase and antiglucanase antibodies.
(C) Stained with Coomassie Blue.
Lane 1, 50 μg of protein from noninduced control leaves; lane 2, 50 μg of protein from ethylene-treated bean leaves; lane 3, 15 μg of protein of the intercellular washing fluid (IWF) collected from ethylene-treated leaves; lane 4, 20 μg of protein from control leaves; lane 5, 20 μg of protein from ethylene-treated leaves; lane 6, 5 μg of protein from the IWF collected from ethylene-treated leaves; lane 7, 60 μg of protein from protoplasts isolated from ethylene-treated leaves; lane 8, β-1,3-glucanase isolated from the protoplast preparation shown in lane 7 by adsorption to pachyman; lane 8, chitinase isolated from the protoplast preparation shown in lane 7 by adsorption to chitin. Molecular weight markers indicated on the left are as in Figure 1.

Chitinase and glucanase showed similar intracellular localization and solubility characteristics; in particular, both enzymes were readily soluble in the solution used for the isolation of IWFs. Therefore, the absence of chitinase in the IWF indicates that the glucanase present in the IWF was probably not released from cells broken during the collection of the IWF. No glucanase or chitinase was detected in the IWF of control leaves. It should be noted that Figure 10A overestimates the abundance of the cell wall localized β-1,3-glucanase in ethylene-treated leaves, since the IWF protein loaded in lane 3 was isolated from 250 mg of leaf tissue, whereas the protein from whole tissue loaded in lane 2 corresponds to only 20 mg of leaf tissue. After the removal of the extracellular glucanase, the leaf tissue contained, on average, only about 5% less glucanase activity compared with the starting material.

The intracellular accumulation of chitinase and β-1,3-glucanase was established by isolating protoplasts from ethylene-treated bean leaves. Figure 10C shows that both enzymes, identified by affinity adsorption to their substrates, were abundant in isolated protoplasts. No significant difference in the specific chitinase and glucanase activities was observed between extracts from isolated protoplasts and leaf tissue, indicating that the majority of these enzymes accumulate intracellularly. In Figure 10C, chitinase is resolved in three bands, suggesting that bean chitinase exists in three molecular forms, all of which accumulate intracellularly.

Vacuolar Localization of Chitinase and β-1,3-Glucanase

Figure 11A compares the protein pattern of protoplasts and vacuoles isolated from ethylene-treated bean leaves. Two proteins with molecular weights similar to those of chitinase and β-1,3-glucanase were abundant in the vacuolar preparation. Immunoblotting with a combination of antichitinase and antiglucanase antibodies, shown in Figure 11B, revealed that these two proteins were chitinase and β-1,3-glucanase, respectively. Microscopic observation showed that the vacuolar preparations were contaminated with up to 10% of unlysed protoplasts and vacuoles with sticking cytoplasmic caps. However, it appears clear from Figure 11A that the vacuolar preparation was enriched in chitinase and β-1,3-glucanase. The same chitinase activities were loaded in lane 2 and 3. Clearly, lane 3 contains much less protein than lane 2. We estimate that the chitinase and glucanase were at least 6 times enriched in the vacuolar preparation.

DISCUSSION

The goal of this work was to refine our knowledge of the postulated functions of stress-induced chitinase and β-1,3-glucanase by determining the subcellular distribution of these enzymes in leaf tissues.

Possible Role of Chitinase and β-1,3-Glucanase in Tissue Aging Processes

The induction of chitinase and β-1,3-glucanase in bean leaves by ethylene has been described before (Boller et

al., 1983; Vögeli et al., 1988). Our data confirm that the induction of these enzymes by ethylene is a quantitatively important event. In contrast to the saturation kinetics reported earlier, we found a linear increase of chitinase and β-1,3-glucanase activity over a time period of more than 3 days, leading to a twofold higher induction factor for both enzymes than reported earlier. This result can probably be attributed to the fact that the bean varieties used in this study (cv Greensleeves and cv Kentucky Wonder), in contrast to the varieties used before, survived ethylene treatment undamaged. Even after 5 days in an ethylene atmosphere, they showed no signs of ethylene-induced premature aging such as chlorophyll loss, cell damage, or leaf abscission. Thus, there appears to be no direct linkage between the induction of chitinase and β-1,3-glucanase and ethylene-induced aging processes and leaf abscission. It will be interesting to test whether ethylene-induced enzymes, such as proteases and the basic form of cellulase, postulated to play a role in senescence and leaf abscission (Roberts et al., 1985; Sexton et al., 1985), are still induced by ethylene in the bean varieties used for this study.

Subcellular Localization of Stress-Induced Chitinase and β-1,3-Glucanase

The unusual resistance of the bean varieties to ethylene-induced aging phenomena and the fact that ethylene treatment, unlike pathogen attack, does not lead to only locally increased enzyme activities, greatly facilitated our localization studies. It is important to note that pathogen attack, elicitor treatment, and ethylene have been shown to induce the same molecular forms of chitinase and β-1,3-glucanase (Mauch et al., 1988a). Ethylene-treated tissue should therefore provide a valid model system to establish the initial sites of accumulation of these enzymes in stressed tissue in general.

The cell fractionation techniques employed to date to study the subcellular localization of stress-induced chitinase and β-1,3-glucanase have yielded variable results. The demonstration of these enzymes in the IWFs of tobacco leaves infected with *Phytophthora infestans* (Kombrink et al., 1988) and potato leaves infected with *Tobacco Mosaic Virus* (Kauffman et al., 1987; Legrand et al., 1987), has led to the proposal that chitinase and β-1,3-glucanases produced in response to stress accumulate extracellularly. However, there are problems involved with the collection of intercellular washing fluids from infected or hypersensitively reacting tissues. Many pathogen-induced proteins accumulate to especially high levels around infection sites (Antoniw and White, 1986; Métraux and Boller, 1986). The lysis of such cells during infection is likely to alter the protein pattern of the intercellular washing fluids, making it impossible to determine whether chitinase and β-1,3-glucanase are actively secreted or whether they are released into the extracellular space only because of host cell lysis. In other cell fractionation studies, chitinase has been shown to be vacuolar in bean (Boller and Vögeli, 1984), and extracellular in the uninfected upper leaves of virus-infected cucumber (Boller and Métraux, 1988). This suggests that stress-induced chitinase and glucanase may accumulate at different locations in different plant species.

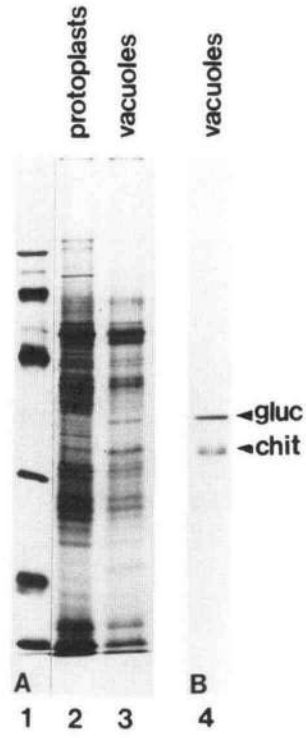


Figure 11. SDS-PAGE of Protoplasts and Vacuoles Isolated from Ethylene-Treated Bean Leaves

(A) Silver-stained gel.
(B) An immunoblot stained with a combination of antichitinase and antiglutanase antibodies.
Lane 1, molecular weight markers as indicated in Figure 1; lane 2, 2 μg of protein from protoplasts isolated from ethylene-treated bean leaves; lanes 3 and 4, protein from vacuoles isolated from ethylene-treated leaves. Equal amounts of chitinase were loaded in lane 2 and lane 3.

Our combined immunocytochemical and cell fractionation experiments demonstrate that, at least in bean leaves, all the chitinase and most (>95%) of the β-1,3-glucanase accumulate in the vacuoles of ethylene-treated leaves. Immunogold cytochemistry located both enzymes to large vacuolar protein aggregates (Figures 2 and 3). In accordance with the ethylene-induced nature of chitinase and β-1,3-glucanase, such aggregates were only found in ethylene-treated leaves but not in untreated control leaves. Whether these aggregates are naturally formed as a result of the high concentration of these enzymes in the vacuoles

or whether they are an artifact of the fixation conditions used is unclear at the present time.

The validity of the immunolabeling results is dependent on the specificity of the antibodies used. The antichitinase antibodies recognized only chitinase on immunoblots of crude leaf extracts (Figure 1B). However, the antiglucanase antibodies, even after affinity purification, showed a slight cross-reactivity in the higher molecular weight range on immunoblots (Figure 1C). This background staining, in contrast to the section labeling, was not ethylene-induced. Therefore, we conclude that the observed section labeling is not caused by the slight unspecificity of the antiglucanase antibodies. Preadsorption of either antiserum with its specific antigen completely abolished section labeling (Figure 9), indicating that the labeling pattern is indeed due to the specific interaction of the antibodies with β-1,3-glucanase and chitinase, respectively.

The immunocytochemical localization of chitinase and glucanase to the vacuoles was confirmed by cell fractionation experiments (Figures 10 and 11). The specific enzyme activities in protoplasts and leaf tissue did not differ significantly, indicating that the majority of the two enzymes accumulates intracellularly. The vacuolar localization of chitinase and β-1,3-glucanase was supported by the finding that both enzymes were present in high concentrations in isolated vacuoles. Although the vacuolar preparations were slightly contaminated with cytoplasm, we consider it unlikely that the sixfold enrichment of chitinase and β-1,3-glucanase in these preparations was due to specific nonvacuolar cytoplasmic contaminants that were exceptionally rich in chitinase and β-1,3-glucanase.

Some glucanase label was found over the middle lamella region of the cell walls in control leaves (Figure 7) and in enhanced amounts in ethylene-treated leaves (Figures 3 and 4), indicating that small quantities of β-1,3-glucanase are secreted during normal development and that secretion of this enzyme increases in stressed tissue. The cell wall-associated glucanase labeling was not due to the release of vacuolar glucanase from cells broken during tissue fixation. If this were the case, one would expect also to find some chitinase in the extracellular space. However, no chitinase was detected extracellularly. The extracellular nature of some of the glucanase in ethylene-treated leaves was confirmed by the presence of glucanase, but not chitinase, in the IWF (Figure 10). No glucanase was detectable in the IWF of control tissue, which already had a very low overall glucanase activity.

Both chitinase and β-1,3-glucanase have been shown to be synthesized as larger precursors with an N-terminal signal peptide (Broglie et al., 1986; Shinshi et al., 1988; Vögeli et al., 1988), suggesting that they are synthesized on membrane-bound polysomes and cotranslationally transferred into the lumen of the endoplasmic reticulum. Our immunolabeling results show that both enzymes further pass through the Golgi apparatus before they are transported to the vacuole or the extracellular space (Figures 4 and 5). What are the regulatory mechanisms that allow all chitinase and the majority of the β-1,3-glucanase to be delivered to the vacuole while a small amount of β-1,3-glucanase is secreted? It was recently shown that positive targeting information is required for the transport of plant proteins to the vacuole (Tague and Chrispeels, 1988) and that secretion is a default pathway in higher plants (Dorel et al., 1988). Since similar amounts of both chitinase and glucanase were produced and no chitinase was secreted, it seems unlikely that the secretion of some of the β-1,3-glucanase is merely due to the overloading of the Golgi sorting mechanism with the large amounts of the newly synthesized enzymes. At present we have no evidence to suggest any molecular differences between the secreted and the vacuolar glucanase that could explain their different accumulation sites. Antiglucanase antibodies occasionally stained a band with a molecular weight slightly higher than that of β-1,3-glucanase on immunoblots of pachyman eluates (Figure 1C, lane 4). However, the molecular weight of this band did not correspond to the glucanase present in IWFs. Since this band showed an affinity for β-1,3-glucan, it could represent the preprotein form of β-1,3-glucanase. In bean and other plants, chitinase and β-1,3-glucanase are encoded by multiple genes and are synthesized as preproteins (Hedrick et al. 1988; Shinshi et al., 1988). The maturation of a glucanase from tobacco was recently shown to be quite complicated, including N- and C-terminal processing and an intermediary glycosylation step (Shinshi et al., 1988). Hence, the vacuolar and the extracellular forms of β-1,3-glucanase could either arise from different mRNAs or from differential processing of the enzymes leading to its targeting.

Model of the Roles of Chitinase and β-1,3-Glucanase in Plant Defense Responses

Based on our results and those of others we propose a model for the roles of chitinase and β-1,3-glucanase in defense responses as illustrated in Figure 12. When fungal pathogens initially grow in the intercellular space of their host plants, they make contact with the β-1,3-glucanase molecules localized in the middle lamella along the air spaces. Upon contact, the β-1,3-glucanase is postulated to release oligosaccharide fragments from the β-1,3-glucan-containing fungal cell wall. Oligosaccharides released by β-1,3-glucanase from isolated fungal walls have been demonstrated to act as (exo-) elicitors of phytoalexin production (Keen and Yoshikawa, 1983). Many plant genes coding for defense-related products have been shown to be activated by β-1,3-glucans (Ryan, 1987). However, except for preliminary evidence for a β-1,3-glucan elicitor receptor in plasma membrane fractions of soybean cells (Schmidt and Ebel, 1987), knowledge of the actual signal transduction pathways is very rudimentary (Dixon, 1986).

It is interesting to note that the cell wall labeling pattern

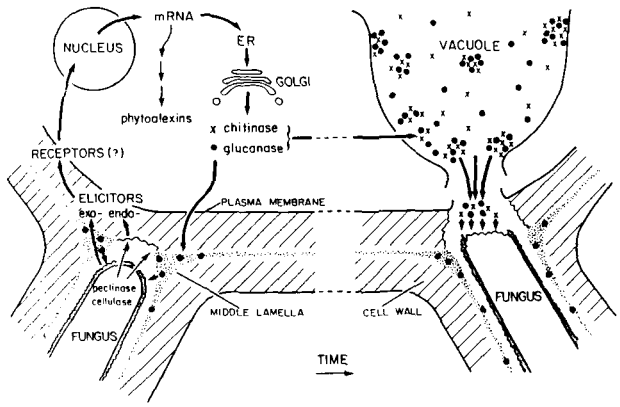


Figure 12. Model Outlining the Roles of Chitinase and β-1,3-Glucanase in a Bean Plant's Defense Against Pathogen Attacks.

observed for β-1,3-glucanase coincides with the distribution of the pectic polysaccharides in plant cell walls (Moore and Staehelin, 1988). This places the β-1,3-glucanase molecules, postulated to release exo-elicitors from fungal cell walls, in the same location as the cell wall molecules that appear to give rise to defense-related endo-elicitors (Nothnagel et al., 1983; Bishop et al., 1984). As shown by Davis et al. (1986), exo- and endo-elicitors appear to act synergistically in the induction of defense responses.

Like many other inducible host defense responses (Dixon, 1986), the induction of chitinase and β-1,3-glucanase by elicitors and by ethylene has been shown to be regulated at the mRNA level (Broglie et al., 1986; Vögeli et al., 1988) and probably results from an increased transcription of these genes (Hedrick et al., 1988). Our results show that some of the newly synthesized β-1,3-glucanase is secreted, possibly to augment the signaling system. However, most of the β-1,3-glucanase and all of the chitinase is deposited into the vacuole. These enzymes become functional only when host cells are lysed during pathogenesis; e.g., when fungal enzymes digest the host cell walls, thereby causing the protoplast to burst, or when the pathogen triggers a hypersensitive response of the surrounding host tissue. Why should enzymes with a postulated defense function accumulate in the vacuole, at a location where they come into contact with the invading pathogen only relatively late or not at all during the infection process? We think the advantage of the vacuolar localization lies in the possibility of suddenly flooding the invading fungus with potentially lethal concentrations of chitinase and β-1,3-glucanase upon lysis of some host cells. Indeed, physiological concentrations of chitinase and β-1,3-glucanase have been shown to strongly inhibit the growth of many potentially pathogenic fungi in in vitro assays (Mauch et al., 1988b). In fungi, wall extension is restricted to the hyphal tips and is thought to represent a delicate balance between synthesis and degradation of the main cell wall components, such as chitin and β-1,3-glucan (Wessels and Sietsma, 1981). Exogenously applied chitinase and β-1,3-glucanase have been demonstrated to interfere with this balance, killing many fungi by lysing their hyphal tips (Mauch et al., 1988b). An extracellular deposition of chitinase and β-1,3-glucanase would have the advantage of allowing an early contact between the antifungal enzymes and the pathogen, but would also have several potential drawbacks. For instance, during early stages of a defense response, the concentration of enzymes may not be adequate to significantly interfere with the growth of the fungus and, therefore, would not only be wasted, but may also give the fungus a chance to adapt to the slowly increasing extracellular hydrolytic activities by adjusting its own cell wall synthetic machinery at the fungal apex. In contrast, by accumulating the enzymes in the vacuole, release can be delayed until effective concentrations have been produced and the sudden release of high concentrations of chitinase and β-1,3-glucanase upon breakage of the vacuoles gives the fungus no time to adapt to the rapidly changing conditions. Further studies will have to prove whether in infected tissue chitinase and β-1,3-glucanase actually fulfill the roles outlined in this report.

METHODS

Plant Material

Seeds of *Phaseolus vulgaris* L. cv Greensleeves and cv Kentucky Wonder (Burpee Seed Co.) were grown in vermiculite under a 16 hr:8 hr light:dark regime. After 12 to 14 days, when the primary leaves were just fully expanded, the seedlings were placed in an air-tight Plexiglas chamber and treated with 100 ppm ethylene under continuous light. If not mentioned otherwise, plants treated for 48 hr were used for the experiments.

Protein Extraction

Primary leaves of bean were ground in liquid nitrogen with a mortar and pestle. The resulting powder was extracted (4 mL/g) with cold 0.1 M sodium citrate buffer, pH 4.6, containing 5 mM EDTA, 10 mM sodium ascorbate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol. The extract was centrifuged at 20,000*g* for 20 min and the proteins in the supernatant were precipitated by adding ammonium sulfate to 95% saturation. After centrifugation (20 min at 20,000*g*) the sediment was redissolved in 10 mM Tris-HCl, pH 7.5 (enzyme assays and protein determination) or in 20 mM Tris-HCl, pH 8.0, containing 0.5 M sodium chloride (affinity adsorption experiments).

For the extraction of intercellular washing fluids (IWF), leaves were cut in 2 cm-wide strips and washed extensively in water. The leaf strips were then vacuum-infiltrated for 10 min with water containing 5 mM EDTA, 10 mM sodium ascorbate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol. The leaf strips were blotted dry and rolled into 20 ml syringes. The syringes

were placed in centrifuge tubes and centrifuged at 800*g* for 10 min. The IWF at the bottom of the tube was collected. For direct comparison leaves were extracted in the infiltration solution and processed as described above.

Affinity Adsorption of Chitinase and β-1,3-Glucanase

Proteins with an affinity for chitin or β-1,3-glucan, respectively, were selectively removed from bean leaf extracts by the addition of regenerated chitin prepared as described (Boller et al., 1983) or pachyman (Behring Diagnostics). The suspensions were incubated at 0°C for 1 hr on a shaker. The sediment formed after centrifugation was washed twice with 20 mM Tris-HCl, pH 8.0, and twice with 20 mM sodium acetate buffer, pH 5.0. Both wash solutions contained 0.5 M sodium chloride. After two final washes in water, the proteins bound to the insoluble substrates were released by boiling the sediment in SDS sample buffer.

Antibodies

Polyclonal antibodies raised in rabbits against chitinase purified from bean (Vögeli et al., 1988) or β-1,3-glucanase purified from peas (Mauch et al., 1988a) were kind gifts of Dr. U. Vögeli, Lexington, KY, and Dr. L.A. Hadwiger, Pullman, WA, respectively. The anti-CP* antiserum and the anti-cell wall polysaccharide antisera raised against xyloglucan and rhamnogalacturonan I were the respective gifts of Drs. T.G. Dunahay and Dr. P.J. Moore, University of Colorado, Boulder, CO. The IgG fraction was isolated from the immune sera by protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) column chromatography according to the manufacturer's instructions. The anti-pea glucanase IgG fraction was further affinity-purified using bean β-1,3-glucanase bound to nitrocellulose following the method of Olmsted (1981) or with an affinity support made by binding purified β-1,3-glucanase from peas (Mauch et al., 1988a) to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970). Prior to electrophoresis, the proteins were precipitated from crude extracts by the addition of 1.5 volumes of ice-cold methanol containing 1% acetic acid and overnight incubation at −20°C. The protein pellet was washed with 100% ice-cold ethanol followed by 70% ice-cold ethanol, dried in a Speed Vac and dissolved in SDS sample buffer. Silver staining was performed as described (Blum et al., 1987). Proteins were transferred electrophoretically to nitrocellulose sheets (0.45 μm, Bio-Rad) as described (Towbin et al., 1979). To effectively transfer chitinase and β-1,3-glucanase, it was necessary to include 0.1% SDS in the transfer buffer. For immunodetection the nitrocellulose sheets were blocked in PBS (20 mM sodium phosphate/150 mM sodium chloride, pH 7.2) containing 10% (w/v) nonfat milk powder and then incubated for 2 hr in primary antibody diluted in PBS containing 3% nonfat milk. The antigens were visualized by the indirect immunoperoxidase technique as described in the Bio-Rad technical sheet.

Electron Microscopy

Primary leaves of bean were cut in small pieces (~1 mm^2) and fixed in 2% (v/v) glutaraldehyde/0.1 M sodium phosphate buffer, pH 7.0, for 2 hr at room temperature. After three buffer washes, the material was postfixed in 1% (w/v) OsO_4/0.1 M sodium phosphate buffer, pH 7.0, for 1.5 hr at room temperature, dehydrated in a graded ethanol series, and infiltrated in LR White acrylic resin (London Resin Co.) as described (Moore and Staehelin, 1988). The resin was cured in a vacuum oven at 50°C for 24 hr. For immunolabeling experiments, silver-gold thin sections were mounted on formvar- and carbon-coated nickel grids. The sections were blocked on drops of PBS-BSA-gelatin (20 mM sodium phosphate, 0.5 M sodium chloride, 1% (w/v) bovine serum albumin, 0.5% (w/v) gelatin [porcine skin, Bloom number 60, Sigma], pH 7.2) for 15 min. The sections were then incubated for 2 hr in primary antibody diluted in PBS-BSA-gelatin containing 0.2% (v/v) Tween 20, washed in PBST and incubated for 1 hr in protein A-gold diluted in PBST-BSA-gelatin. The grids were washed in a stream of PBST followed by water and then post-stained in 2% (w/v) aqueous uranyl acetate for 5 min and triple lead for 5 sec. Protein A-gold (12 nm and 17 nm) was prepared as described (Slot and Geuze, 1985). The thin sections were viewed in a Hitachi H600 or a Philips CM-10 electron microscope at 75 kV.

Isolation of Protoplasts and Vacuoles

Fully expanded primary leaves were deveined, immersed in 0.45 M mannitol, and sliced into strips of about 0.2 mm width. The strips were washed twice in 0.45 M mannitol and were then immersed in an enzyme solution (10 mL/g fresh weight) containing 1% (w/v) cellulase (Worthington Diagnostics), 1% (w/v) Rhozyme HP 150 (Genencor), 0.4% (w/v) macerase (Behring Diagnostics), in 0.45 M mannitol and 3 mM MES, pH 5.7. The preparation was vacuum infiltrated for 10 min and incubated at 30°C for 5 hr in a shaking water bath. The protoplasts were separated from undigested material and purified by flotation as described (Boller and Vögeli, 1984). They were counted in a hemacytometer. Protoplast yields based on chlorophyll determination (Arnon, 1949) were low, varying between 5% and 11%.

Vacuoles were released from protoplasts essentially as described (Matoh et al., 1987). All steps were carried out at room temperature. To lyse the protoplasts, 7 mL of a solution of 0.3 M mannitol, 20 mM HEPES (adjusted to pH 8 with 1 M Tris solution), 1 mM EGTA, and 0.5 mM CHAPS (Sigma) was added to 0.5 to 1.0 mL of protoplast suspension (2×10^6/ml). The progress of lysis was followed by light microscopy until about 90% of the protoplasts were lysed. The protoplasm aggregates formed during incubation were removed by gentle stirring with a wooden applicator stick. To purify the vacuoles, 4 mL of the crude vacuole preparation was gently mixed with 4 mL of 0.4 M mannitol containing 20% ficoll, 20 mM HEPES, pH 8.0, 1 mM EGTA, and 0.5 mM CHAPS. This suspension was overlayered with 3 mL of the same solution containing 4% ficoll and with 2 mL of solution without ficoll. The step gradient was centrifuged at 100*g* for 20 min. The vacuoles banding at the 0%/4% ficoll interface were collected, counted in a hemacytometer, and frozen in liquid nitrogen for later biochemical analysis.

Protein Determination and Enzyme Assays

Protein was determined by the Bradford assay (Bradford, 1976). Chitinase and β-1,3-glucanase activities were measured as described (Mauch et al., 1984).

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