Occurrence of 9.5 Cellulase and Other Hydrolases in Flower Reproductive Organs Undergoing Major Cell Wall Disruption

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ABSTRACT

The occurrence of enzymes associated with bean leaf abscission was investigated in bean (Phaseolus vulgaris) flower reproductive organs in which catabolic cell wall events are essential during anther and pistil development. Cellulase activity was detected in high levels in both pistil and anthers of bean flowers before anthesis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with 9.5 cellulase antibody identified a protein in anthers and pistil with the same size (51 kilodaltons) and serologically closely related to the abscission cellulase. The accumulation of 9.5 cellulase protein in the anther is developmentally regulated and increases from undetectable levels at very young stages of anther development to high levels as the anther matures. In the pistil, the 9.5 cellulase was localized in the upper part of the pistil where the stigma and the staminal neck resided and was detected in the youngest developmental stage analyzed. Antibodies against basic chitinase, which accumulates to high levels in abscission zones after exposure to ethylene, identified a protein with the same size (33 kilodaltons) and serologically closely related, in both anthers and upper portion of the pistil. In contrast, a 45-kilodalton protein and the basic β-1,3-glucanase associated with abscission were undetected in bean reproductive organs. Interestingly, β-1,3-glucanase activity was detected in young bean anthers and decreased at anthesis, but the anther β-1,3-glucanase is serologically unrelated to the basic β-1,3-glucanase. Thus, it appears that the basic cellulase and chitinase occur in combination in many plant processes that require major cell wall disruption, whereas hemicellulases such as β-1,3-glucanase are specific to each process.

Plants modify the cell wall to a different extent in a variety of physiological events. During cell growth and expansion, only discrete modifications in the cell wall are sufficient to cause cell wall loosening. Instead, during abscission, major changes in the cell wall are necessary to cause cell separation. Major cell wall disruption also occurs in other plant processes, for example, at several steps in the development of the flower reproductive organs. Early during pollen differentiation, the callose wall that protects the meiotic cells is rapidly broken down and microspores are released into the anther locule (9). Later, the innermost layer of the anther wall, the tapetum, begins to break down and, as the walls are lysed, the cytoplasm is released into the locule (9). Finally, the anther lobes develop a fissure, the stamium, which allows the release of the mature pollen grains from the anthers (9). Similarly, during pollen-stigma interactions, cell wall loosening of the papillary cells at the surface of the stigma has been reported in Brassica (8).

The composition of the cell wall can differ markedly between different plants and among different organs within the same plant (2). However, the basic cell wall structure in all cases is similar and is composed of cellulose microfibrils embedded in a matrix of hemicelluloses, pectins, and proteins. Thus, cellulases and hemicellulases are bound to play an important role in the enzymatic modifications of all cell walls. Fry (10) has proposed that cell wall loosening during cell growth and expansion comes about by the action of an auxin-induced cellulase. Our work has demonstrated that cell wall disruption during ethylene-induced abscission involves the action of a cellulase, referred to as 9.5 cellulase (3, 7, 22, 24). In addition, we recently demonstrated that in bean abscission zones, along with cellulase, ethylene induced the accumulation of a 45-kD protein of unknown function and a group of PRPs2, including basic isoforms of β-1,3-glucanase and chitinase (4). We suggested that these PRP enzymes accumulated in the abscission zone in anticipation of a possible pathogen invasion through the disrupted cell wall of the abscission layer cells. We investigated whether the 9.5 cellulase and the PRP enzymes associated with abscission also occur during other plant processes in which major cell wall disruption takes place. In particular, we analyzed the occurrence and accumulation of 9.5 cellulase, basic β-1,3-glucanase, and basic chitinase in flower reproductive organs where cell wall dissolution is essential during development.

MATERIALS AND METHODS

Plant Material

Flower buds in progressive developmental stages were collected from the bean plants (Phaseolus vulgaris, cv Green-sleeves) grown in vermiculite for 1 month. With the aid of a dissecting microscope, flower buds were opened, sepals and petals were removed, and anthers, ovary, style, and stigma were collected and stored at −80°C. Bean abscission zone explants were prepared as described before (4).

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2 Abbreviations: PRP, pathogenesis-related protein; ry, youngest stage; in, intermediate stage; ba, before anthesis; of, flower opened.
Protein Extraction

Tissues were ground to a powder in liquid nitrogen with a small mortar and pestle, and proteins were extracted with a buffer (2.2 mL/g fresh weight) containing 20 mM Tris-HCl (pH 8.0), 3 mM EDTA, 0.5 mM NaCl. The mixture was homogenized at 0°C in a glass homogenizer and then centrifuged at 750g for 5 min. Proteins were precipitated by adding cold acetone to a final concentration of 80% (v/v). After incubation at −20°C for 16 h, the precipitate was collected by centrifugation at 23,000g for 15 min and resuspended in 0.8 mM MOPS, 0.5 mM KOH, 20% glycerol (pH 8.0). Protein content was measured in extracts according to the procedure described by Schaffner and Weissmann (20).

Antibody Production

Mouse polyclonal antibodies against chitinase, β-1,3-glucanase, and 45-kD protein were prepared as described before (4). Rabbit polyclonal antibodies against 9.5 cellulase were prepared as described by Durbin et al. (7).

Cellulase and β-1,3-Glucanase Activity Determinations

Activity of β-1,3-glucanase was determined by a modification of a colorimetric assay (5). The assay mixture contained 490 µL of 0.1% reduced laminarin in 10 mM potassium phosphate buffer (pH 5.25) and 10 µL of crude extract. After incubation at 37°C for 30 min, the amount of released reducing sugars was determined. Cellulase activity was determined on the crude extracts using a viscometric assay as described by Durbin and Lewis (6). The assay mixture contained 10 µL of crude extract, 90 µL of 20 mM Tris-HCl (pH 7.5), 0.5 mM NaCl buffer, and 20 µL of carboxymethyl-cellulose at pH 7.5. The mixture was incubated at room temperature.

SDS-PAGE and Immunoblotting Analysis

One-dimensional SDS-PAGE was performed according to the method of Laemmli et al. (15) using 12% (w/v) polyacrylamide gels. After electrophoresis, proteins were transferred electrophoretically to nitrocellulose sheets, blocked with 3% (w/v) gelatin, and incubated overnight at room temperature with the respective antibodies. Detection was carried out as described before (4).

Affinity Purification of β-1,3-Glucanase

Proteins with an affinity for β-1,3-glucan were selectively removed from bean abscission zone and bean anther extracts by addition of pachymann (18). The suspensions were incubated at 0°C for 1 h. After centrifugation and sequential washes with a high salt buffer at pH 8.0 and 5.0, respectively, the proteins bound to the insoluble substrates were released by boiling in SDS sample buffer consisting of 60 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol.

RESULTS

Analysis of Cellulase in Both Bean Explants Treated with Ethylene and Bean Flower Organs

A bean flower consists of a single pistil surrounded by 10 stamens (26). Figure 1A shows bean flower buds collected at various stages of development. In vy, the bud was 2 to 3 mm long, the style was linear and short, and the anthers were translucent. At (in), the bud was 4 to 6 mm long, the style was longer and curved in the apical portion, and the anthers were off-white. In the oldest stage of the bud, just (ba), the bud was 8 to 10 mm long, the style was long and coiled, the surface of the stigma revealed a sticky exudate (16), and the anthers contained many pollen grains and were ruptured or close to rupture. The developmental stages in and ba are comparable to those described by Webster et al. (26) in which in corresponded to green stage buds and ba corresponded to white stage buds. Between vy stage and anthesis, the pistil (Fig. 1B) and the surrounding filaments that support the anthers (Fig. 1C) extend greatly, forming a tightly coiled structure within the bud. When (of), the style length was about 17 mm. Pollination took place after anther dehiscence but before the flower was open (26).

To analyze cellulase activity, total proteins were isolated from the different flower organs collected at the ba stage. The pistil was cut into two parts indicated by arrows in Figure 1B. One cut was made just above the ovary and separated the ovary from the style. The second cut was made 3 to 4 mm below the stigma and separated the stigma and the distal portion of the style (stylar neck) from the rest of the style. The mean length of the style between the arrows was 10 to 12 mm. The anthers were separated from the supporting filaments at the position indicated by the arrow in Figure 1C. Activity was also determined in bean abscission zones, stem,
and petiole exposed to ethylene for 72 h. Results in Table I show that cellulase activity was highest in the abscission zone at the site where fracture occurs, although some activity was detected in the tissues adjacent to the abscission zone (3). High cellulase activity was also detected in both anthers and upper portion of the pistil (stigma + stylar neck) from bean flowers just before anthesis. Cellulase in the ovary and in the lower portion of the style was lower than in the stigma + stylar neck, suggesting that cellulase is localized in the stigma or in stylar neck or both.

To analyze the serological properties of the cellulase from flower organs, equal amounts of protein from organs of the bean flower (ba) were separated by SDS-PAGE and followed by immunoblotting with 9.5 cellulase antibody. As a positive control, proteins from the abscission zone exposed to ethylene were also loaded on an equal protein basis. Figure 2A shows that the antibody identified a protein of identical size (51 kD) in the abscission zone, the stigma + stylar neck, and the anthers. The small molecular mass fragments serologically related to 9.5 cellulase in both anther and stigma tissues are probably the result of protein degradation because they became more abundant after tissue or tissue extract storage. The faint upper band seen in both stigma and anther gels is an artifact caused by the presence in bean tissue extract of a very abundant protein of approximately 66 kD. No 9.5 cellulase-like protein was found in the ovary or the lower portion of the style.

Accumulation of 9.5 Cellulase in Anther and Stigma during Flower Development

To analyze the regulation of the cellulase protein in anthers and stigma during flower development, bean flowers were collected at the three previously described stages of bud development (Fig. 1A). Reproductive organs from open flowers were not collected as they were dried and contaminated with pollen. At the vy stage, both the anther and the stigma of a bean flower are fully differentiated but immature. Instead, at the ba stage, both reproductive organs have reached maturity. Anthers and stigma fractions were collected as previously described (Fig. 1, B and C). Proteins were extracted, resolved on SDS-PAGE, and immunoblotted with 9.5 cellulase antibody. Gels were loaded on an equal fresh weight basis. Figure 2B illustrates that 9.5 cellulase protein accumulated in the anther in a developmentally regulated manner, changing from very low levels at a very young stage to high levels at an intermediate and mature stage of anther development. Figure 2B also shows that, in the stigma fraction (which also included the stylar neck), cellulase protein was present at similar levels during all developmental stages tested.

Analysis of β-1,3-Glucanase and Chitinase in Bean Explants Treated with Ethylene and in Bean Flower Organs

In bean abscission zones, along with 9.5 cellulase, ethylene induces the accumulation of PRPs, including enzymes such as β-1,3-glucanase and chitinase (1, 3, 11). The ethylene-induced β-1,3-glucanase and chitinase are basic proteins with molecular masses of 36 and 33 kD, respectively, and both accumulate to high levels in bean abscission zones before tissue fracture (3, 11, 24). Figure 3 shows proteins from bean abscission zones exposed to ethylene and from organs of bean flowers (ba) subjected to SDS-PAGE followed by immunoblotting with antibodies raised against ethylene-induced β-1,3-glucanase (basic β-1,3-glucanase) and basic chitinase. Figure 3A illustrates that basic β-1,3-glucanase (36

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**Table 1. Cellulase Activity in Extracts of Bean Flower Organs Collected from Buds at the vy and ba Stages of Development and from Bean Abscission Zones and Stems after 72 h Exposure to Ethylene**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ethylene Exposure</th>
<th>Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscission zone</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Abscission zone</td>
<td>72</td>
<td>1616</td>
</tr>
<tr>
<td>Stem</td>
<td>72</td>
<td>59</td>
</tr>
<tr>
<td>Ovary</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Style (ba)</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Stigma + stylar neck (ba)</td>
<td>0</td>
<td>913</td>
</tr>
<tr>
<td>Anthers (vy)</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Anthers (ba)</td>
<td>0</td>
<td>350</td>
</tr>
</tbody>
</table>

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Figure 2. A, Immunoblot analysis with 9.5 cellulase antibody of proteins from abscission zones, lane 1, and proteins from organs of bean flowers collected just before flower opening (ba) and dissected as follows: ovary, lane 2; style, lane 3; stigma containing the stylar neck, lane 4; anthers, lane 5. Proteins were subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane. The molecular masses of marker proteins are indicated on the left in kilodaltons. Lanes were loaded on an equal protein basis. B, SDS-PAGE followed by immunoblotting with 9.5 cellulase antibody of proteins from anthers and stigma of bean flowers collected at the vy, in, and ba stages of development. Lanes were loaded on an equal fresh weight basis. The black points mark the position of the 9.5 cellulase (51 kD).
The β-1,3-glucanase has long been recognized to play an important role during microsporogenesis (23). We further examined the occurrence of β-1,3-glucanase in bean anthers by measuring activity in extracts of anthers at the vy and ba stages of flower development. The activity in the abscission zones, petiole, and stem of bean explants exposed to ethylene for 72 h provided a positive control. Table II shows that the levels of β-1,3-glucanase activity in bean abscission zones, stem, and petiole exposed to ethylene were very high after ethylene treatment and very low or undetectable before ethylene treatment. Treatment with ethylene induced β-1,3-glucanase activity 10-fold in all vegetative tissues tested. β-1,3-glucanase activity was also detected in anthers at both developmental stages, but the levels appeared to be higher in very young anthers. β-1,3-glucanase activity was also detected in stigma + stylar neck but was undetectable in the ovary and the style. Overall, the levels of β-1,3-glucanase activity in the anther were much lower than the levels found in the abscission zone after exposure to ethylene. The differences in protein content between anthers (17 mg/g fresh weight) and abscission zone (2 mg/g fresh weight) made the differences in β-1,3-glucanase specific activity more pronounced.

To analyze the immunological characteristics of the β-1,3-glucanase in the anther extracts, the immunoblot analysis with basic β-1,3-glucanase antibody was repeated after another extracts were enriched in β-1,3-glucanase content by addition of pachymann, a water-insoluble β-1,3-glucan. The proteins retained in the matrix were then removed with SDS sample buffer. As a control, the same procedure was applied to extracts from bean abscission zones that had been exposed to ethylene for 0, 24, 48, and 72 h.

Figure 4A illustrates proteins from crude extracts of anthers and abscission zones before addition of pachymann and proteins that were retained by the pachymann, subjected to SDS-PAGE, and stained with Coomassie. Results indicated that, in anthers and abscission zones before ethylene treatment, two proteins with affinity for β-1,3-glucans were removed by adsorption to pachymann. Similarly, in the bean abscission zone before ethylene treatment, at least three to four proteins were retained by pachymann. In contrast, in extracts of bean abscission zones treated with ethylene for 24 h, only one protein with a molecular mass of 36 kD was retained. Figure 4B shows proteins retained by the pachymann subjected to SDS-PAGE followed by immunoblotting with basic β-1,3-glucanase antibody. The antibody reacted strongly with the 36-kD protein of the abscission zone. Furthermore, the intensity of the immunolabeled band was proportional to the amount of β-1,3-glucanase loaded initially to the pachymann column. It should be noted that 5 units of β-1,3-glucanase from anthers at the vy or ba stages of development gave no signal, whereas 5 units of β-1,3-glucanase from the abscission zone gave an easily detected signal.

**DISCUSSION**

It has been previously demonstrated that a cellulase referred to as 9.5 cellulase participates in cell wall dissolution during bean leaf abscission (3, 7, 22, 24). Along with cellulase,
bean abscission zones accumulate high levels of PRPs, including β-1,3-glucanase and chitinase (1, 3, 11). These PRP enzymes are believed to protect plants from pathogens. We recently demonstrated that PRPs accumulated in the abscission zone before 9.5 cellulose accumulation and suggested that they accumulate in anticipation to a possible pathogen invasion through the disrupted cell wall of the abscission layer cells (4).

Here, we have investigated whether the 9.5 cellulase and the PRP enzymes associated with abscission also occur during other catabolic cell wall processes in which major cell wall disruption takes place. In particular, we investigated whether these enzymes occur in the anthers during the process of pollen formation and in the pistil during the development of the tissues that provide for pollen reception and penetration (9). We showed by activity determinations and by immunoblotting analysis with 9.5 cellulase antibody that a cellulase immunologically related to the abscission cellulase and with identical size (51 kD) occurs in the anthers and pistil of bean flowers. Within the pistil, the enzyme is found in the upper portion of the style, which includes the stigma and the stylar neck. The level of 9.5 cellulase in the anthers is developmentally regulated and reaches a maximum between anther maturity and anther dehiscence. These results are in agreement with our previous studies with sweet pea anthers that showed increasing levels of cellulase activity as the anthers approached flower opening (21). In the upper portion of the pistil, which included the stigma and the stylar neck, the 9.5 cellulase occurs in the youngest stage, when the style is short and straight, and the activity remained unchanged during further development. A similar distribution of cellulase activity was obtained by Konar and Stanley (14) using the gynoeccium of Hemerocallis fulva (day lily).

Another glucanase, present in the abscission zones as abscission progresses, is the basic form of β-1,3-glucanase (1, 4). Abeles and Forrence (1) found that β-1,3-glucanase production correlated with the dissolution of callose plugs in the sieve elements of the phloem. Callose deposits are found in inactive sieve elements and they also develop during pollen development as a protective wall to the meiotic cells. This callose wall is later rapidly broken down, allowing the release of the microspheres into the anther locule (9). Stieglitz and Stern (23) showed that the rapid dissolution of the callose wall of the microspheres correlated with a sharp peak in activity of β-1,3-glucanase. This activity was localized in the somatic region of the anthers and not in the meiotic cells.

Consistent with previous results, we show here that β-1,3-glucanase activity is present in bean anthers at the vy stage of development and then declines as anthers approach dehiscence. The level of β-1,3-glucanase activity measured in the anther at the vy stage was low, suggesting that microsporogenesis was almost finished at this bud stage. Extracts of bean anthers were enriched in β-1,3-glucanase using a pachyman affinity column and then analyzed and compared with the basic β-1,3-glucanase induced by ethylene in bean abscission zones. Results clearly demonstrated that these enzymes are serologically unrelated. Our results suggest that the basic β-1,3-glucanase induced by ethylene in bean vegetative tissue and the β-1,3-glucanase from bean anthers correspond to different genes.

Along with β-1,3-glucanase, chitinase is induced in coordination in bean explants induced to abscise with ethylene (4, 25). In addition, another protein of 45 kD and unknown function accumulates in the abscission zone. This protein is serologically related to the chitinases because it shares partial sequence homology in the N-terminal region (4). In this work, we found that, in bean anthers and the upper portion of the pistil containing the stigma + stylar neck, a 33-kD protein serologically related to the basic chitinase also occurred. These results agree with those published by Neale et al. (19), showing that basic forms of chitinase are expressed in tobacco flowers organs at various stages during their development, and with results published by Lotan et al. (17), showing the expression of chitinases PR-P,Q in anthers and ovaries of tobacco flowers. The 45-kD protein was absent in all flower reproductive organs.

Overall, we have identified other tissues of the bean plant in which 9.5 cellulase occurs. We previously demonstrated that 9.5 cellulase occurs in the cells of the separation layer of bean abscission zones as well as in the vascular traces of bean explants outside the separation layer (3). Here, we show that this cellulase also occurs during anther development and occurs in the stigma and the stylar neck of bean flowers. The role of cellulase in flower reproductive organs is unknown, but its pattern of accumulation during anther development...
suggests that it could be involved in either the dissolution of the tapetum or the formation of the stromium. The role of cellulase in the upper portion of the pistil is also unknown. The high level of cellulase from v to b stage indicates that cellulase participates in a process occurring continuously throughout pistil maturation. The stigma of Phaseolus vulgaris produces a stigmatic exudate containing lipids, amino acids, phenols, sugars, and proteins (16). In fact, the amount of protein present in this exudate is very high, 6 to 12 mg/mL (16). In Trifolium pratense L, this exudate is produced in the stigma head when the overall length of the pistil is <0.6 mm (13). Later, this exudate is secreted into the intercellular spaces to create a secretory path for the pollen tubes (12), and the cells of the stigma surface layer undergo disruption (12). Thus, it is possible that 9.5 cellulase occurs in the exudate and participates in the dissolution of the cell wall of the stigma surface cells to facilitate pollen tube penetration during fertilization.

Along with 9.5 cellulase, we have identified the occurrence of a basic chitinase in the anthers and the upper portion of the pistil containing the stigma and the styril neck. Our results suggest that 9.5 cellulase and basic chitinase occur in combination in plant processes involving a major disruption in the cell wall, whereas hemicellulases such as β-1,3-glucanase appear to be more specific for each individual process.

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LITERATURE CITED