Occurrence and Localization of 9.5 Cellulase in Abscising and Nonabscising Tissues

Elena del Campillo, Philip D. Reid, Roy Sexton, and Lowell N. Lewis
Department of Plant Biology, University of California, Berkeley, California 94720

Nitrocellulose tissue prints immunoblotted with 9.5 cellulase antibody were used to demonstrate areas of cellulase localization within Phaseolus vulgaris explants on exposure to ethylene. The 9.5 cellulase was induced in the distal and proximal abscission zone and in the stem. In both abscission zones, the 9.5 cellulase was found in the cortical cells of the separation layer, which develops as a narrow band of cells at the place where fracture occurs. The enzyme was also found associated with the vascular traces of the tissues adjacent to the separation layer extending through the first few millimeters at each side of the separation layer. The two abscission zones differed in the way that cellulase distributed through the separation layer as abscission proceeded. In the distal zone, cellulase appeared first in the cells of the separation layer adjacent to vascular traces and extended toward the periphery. In the proximal zone, 9.5 cellulase accumulated first in the cortical cells that lie in the adaxial side and then extended to the abaxial side. In response to ethylene, 9.5 cellulase was also induced in the vascular traces of the stem and the pulvinus without developing a separation layer. The role of 9.5 cellulase in the vascular traces is unknown. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with 9.5 cellulase antibody identified the same 51-kilodalton protein in both abscising and nonabscising tissues. Therefore, the determinant characteristic of the abscission process is the induction of 9.5 cellulase by cortical cells in the separation layer, and this implies that these cells have a unique mechanism for initiating 9.5 cellulase synthesis.

INTRODUCTION

Abscission is a process that results in the shedding of a plant part. In the primary leaf of bean, the preferred model system to study abscission, there are two positions where shedding can occur: the distal abscission zone, at the base of the leaf blade in the pulvinus-petiole junction, and the proximal abscission zone, where the petiole joins the stem. Ultrastructural studies of abscission in Phaseolus have shown that tissue fracture takes place when both the middle lamella and the primary wall are disrupted (Webster, 1973; Sexton and Hall, 1974). This disruption occurs in a narrow layer of cells and reflects the appearance of cell-wall hydrolases. Lewis and Varner (1970) demonstrated a correlation between cellulase activity and breakstrength decline during the course of abscission. The increase in cellulase activity was due to the de novo synthesis of a cell-wall cellulase that is characterized by a basic isoelectric point of 9.5, an apparent molecular mass of 51 kD, and is referred to as 9.5 cellulase.

Previous studies to localize cellulase in bean (Sexton et al., 1980, 1981), focused on the proximal abscission zone and used 9.5 cellulase antibody to detect the enzyme in fixed tissue sections. By using a peroxidase-conjugated second antibody, this work showed a weak staining in the cortical cells lying at the junction of the stem and the petiole accompanied by a strong, nonspecific staining in the vascular tissue. In other experiments, the localization of 9.5 cellulase was deduced directly from the levels of cellulase activity measured in small fragments dissected from the abscission zone. The result of this study showed that activity was high in the cortical cells at the separation layer (Sexton et al., 1981), but the highest levels of activity were found in the stele of the abscission zone. These results indicated the presence of cellulase in the stele; however, the immunostaining technique failed to identify the activity as 9.5 cellulase because of the high degree of nonspecific staining.

Recently, it has been shown that tissue prints on nitrocellulose membranes reveal surprising amounts of anatomical detail (Cassab and Varner, 1987; Varner and Taylor, 1989). This technique can also be used to study the localization of proteins as long as they transfer to the nitrocellulose during the printing process. We have used this technique to describe the anatomical changes as abscission proceeds (Reid, del Campillo, and Lewis, 1990).
It was also shown that 9.5 cellulase, a salt-extractable enzyme, transferred to nitrocellulose during the printing process as revealed by an antibody immunostaining procedure. Here we use the tissue-print technique to examine and compare the cellular localization of 9.5 cellulase in both the distal and the proximal abscission zones of bean explants exposed to ethylene. Moreover, we have extended the studies to tissue segments that have cellulase but do not contain cells from the separation layer. The results show that 9.5 cellulase occurs in both abscising and nonabscising tissue.

RESULTS

Time Course of 9.5 Cellulase Production in the Distal and the Proximal Zones

Abscission of the distal and proximal zones of bean was induced by excision of the leaf blade and accelerated by exogenous ethylene. As abscission developed, there was an increase in cellulase activity in both the distal and the proximal abscission zones, as shown in Figure 1. Enzyme activity was determined at pH 7.5 because it had been shown (del Campillo, Durbin, and Lewis, 1988) that the acidic cellulase isoenzyme was not active at pH 7.5. The 9.5 cellulase accumulated in both abscission zones but was detected in the distal zone 12 hr to 15 hr earlier than in the proximal zone. Thus, when abscission was complete in the distal zone, the proximal zone was beginning to weaken but was still attached to the stem. Figure 2 shows the distribution of 9.5 cellulase activity within each anatomical part of the distal and the proximal abscission zones after 60 hr of ethylene. The highest level of 9.5 cellulase activity was found in the separation layer, but the enzyme was also detected on both sides of the distal and proximal separation layer. Protein extracts from the different anatomical parts of the distal and the proximal abscission zones were separated on SDS-PAGE and immunoblotted with 9.5 cellulase antibody. Figure 2 (inset) shows that the antibody identified the same 51-kD protein in the separation layer and in adjacent tissues of either abscission zone.

Similar results were obtained when bean plants of either Red Kidney or Greensleeves cultivars were utilized. Mauch and Staehelin (1989) reported that leaves from Greensleeves had a linear increase in chitinase and glucanase activity over the period of 3 days under 1 ppm ethylene without showing signs of senescence and abscission. In contrast to the response to ethylene in intact plants, the Greensleeves explants responded to ethylene. Both the distal and the proximal zones showed clear signs of weakening and a concomitant accumulation of 9.5 cellulase (data not shown). Greensleeves has the advantage that, during the events leading to abscission, the pulvinus did not change color from deep green to yellow and allowed us to study abscission in the absence of senescence. Therefore, Greensleeves explants were used for all succeeding experiments except as noted.

Localization of 9.5 Cellulase in the Distal Zone

Figure 3 shows tissue prints of longitudinal and serial cross-sections of the distal zone prepared from explants treated with ethylene for 24 hr. A diagram of the anatomy of the distal zone showing the site where cross-sections were made is also included in Figure 3. The longitudinal section showed strong staining predominantly in a narrow band of cells perpendicular to the longitudinal axis of the tissue, at the junction of the distal pulvinus and the petiole. The staining extended throughout the center of the pulvinus. The serial cross-sections of a similar explant showed that, in the pulvinus, 9.5 cellulase was exclusively localized in cells of the vascular system. There was no 9.5 cellulase

![Figure 1. Time Course of 9.5 Cellulase Activity in the Distal and the Proximal Abscission Zones of Bean Explants after Treatment with Ethylene.](image-url)
Figure 2. Distribution of 9.5 Cellulase Activity and Protein in the Distal and the Proximal Abscission Zones.

Ten pieces from the following anatomical regions were collected and extracted in a high salt buffer: distal pulvinus, DP; distal and proximal separation layer, SL; petiole, Pt; proximal pulvinus, PP; and stem, St.

(A) Immunoblot analysis of 9.5 cellulase. Proteins were extracted from each tissue, separated on 12% SDS-PAGE, electroblotted to nitrocellulose, and probed with 9.5 cellulase antibody.

(B) Levels of cellulase activity in the extracts per gram of fresh weight (GFW).

Figure 3. Localization of 9.5 Cellulase in the Distal Abscission Zone.

Bean explants were exposed to 25 ppm ethylene for 24 hr and then sectioned for tissue printing, followed by immunoblotting with 9.5 cellulase antibody.

(A) Tissue print of a longitudinal section of the distal abscission zone.

(B) Tissue prints made from cross-sections of a similar explant cut at 2-mm intervals along the axis of the explant.

(C) Diagram showing the anatomical features of the distal zone illustrating the sites where cross-sections were made. Bar = 400 μm.

in the cortical cells of the pulvinus. However, at the separation layer that developed at the junction of the pulvinus and petiole, cellulase appeared in the vascular cells as well as in the cortical cells adjacent to the vascular tissue. On the petiole side, cellulase was only found in the ring of vascular bundles and extended through the first few millimeters (2 mm to 3 mm) below the separation layer. Figure 4 shows a time-course analysis of the distribution of cellulase in the separation layer. The 9.5 cellulase began to accumulate at the center of the tissue on cells adjacent to the vascular tissue and spread to the periphery.

Localization of Cellulase in the Proximal Zone

In the proximal zone of bean explants, cellulase was detected 12 hr to 15 hr later than in the distal zone (Figure 1). Tissue prints of longitudinal and serial cross-sections, shown in Figure 5, were prepared from proximal zones of bean explants after 48 hr and 72 hr of ethylene treatment. A diagram of the anatomy of the two proximal abscission zones showing the sites where cross-sections were made is included in Figure 5. The longitudinal section showed that, after 48 hr in ethylene, cellulase was localized in cells that lie perpendicular to the axis of the petiole at the petiole-stem junction but only on the adaxial side. The 9.5
Figure 4. Distribution of 9.5 Cellulase in the Separation Layer of the Distal Zone as Abscission Proceeds.

Bean explants were exposed to 25 ppm ethylene for the indicated period of time and then sectioned for tissue printing, followed by immunoblotting with 9.5 cellulase antibody. For each time point, a distal zone, including the pulvinus and 2 mm to 3 mm of the petiole, was sectioned transversally at 2-mm intervals. The photographs show the cross-section nearest to the junction of the pulvinus and the petiole.

cellulase was also detected in the vascular system of the two proximal pulvini. The serial cross-sections prepared after 72 hr in ethylene showed that cellulase extended to the abaxial cortex, but remained more concentrated on the adaxial side. Cellulase also appeared in the stem side of the separation layer, in cells associated with the ring of vascular bundles through the first few millimeters below the separation layer. We could not determine whether cellulase accumulation started in the cortical cells adjacent to the vascular traces.

Induction of Cellulase in the Detached Pulvinus

To test whether 9.5 cellulase was synthesized by cells distant from the developing separation layer or simply diffused from cells in the separation layer to the vascular tissue, an experiment was performed in which the pulvinus was separated from the separation layer before the induction of 9.5 cellulase by exposure to ethylene. To ensure that abscission cells were excluded from the detached pulvinus segment, the cut was made 2 mm, 3 mm, and 4 mm away from the presumed separation layer. After the cut, the detached pulvinus was exposed to ethylene for 24 hr and analyzed by tissue printing. Figure 6A shows that cellulase was induced in the pulvinus cells independent of where the initial cut was made. Cellulase was located exclusively in the vascular tissue of the pulvinus. Figure 6B shows the distribution of cellulase in the segments that remained attached to the explant and included the remainder of the pulvinus, the separation layer, and the adjacent petiole. The 9.5 cellulase was found in the vascular tissue of the pulvinus and across the entire tissue section at the separation layer, as has already been shown for the intact distal zone.

Induction of Cellulase in the Detached Stem

When the bean stem was separated 5 mm below the proximal abscission zones and then treated with exogenous ethylene, a basic cellulase was induced (Linkins, Lewis, and Palmer, 1973). We wanted to determine whether this cellulase was the 9.5 cellulase or a related protein and to establish its tissue localization. Figure 7 shows the distribution of cellulase activity in the detached
Bean explants were exposed to 25 ppm ethylene for 48 hr and 72 hr and then sectioned for tissue printing, followed by immunoblotting with 9.5 cellulase antibody.

(A) Tissue print of a longitudinal section prepared from the proximal zone of a bean explant after 48 hr of ethylene treatment (cv Red Kidney).
(B) Series of tissue prints made from cross-sections after 72 hr of ethylene treatment.
(C) Diagram showing the anatomy of the two proximal abscission zones in bean explants, illustrating the sites where cross-sections were made.

Bar = 800 μm.

Figure 5. Localization of 9.5 Cellulase in the Proximal Zone.

stems after 2 hr in ethylene. Activity was higher at the top of the stem where the cut was made and decreased basipetally. SDS-PAGE, followed by immunoblotting with 9.5 cellulase antibody, showed that the stem extracts contained a 51-kD protein immunologically related to the 9.5 cellulase isolated from abscission zones as shown in Figure 7 (inset). A time-course analysis of the distribution of cellulase in the stem showed that dark-staining spots were detected in the vascular tissue of the stem after 24 hr in ethylene but only at the top of the stem, and, with longer exposure to ethylene, cellulase appeared farther down the stem, as shown in Figure 8. Figure 9 shows that cellulase was restricted to the vascular bundles of the stem and was not observed in either the pith or the cortex,
even after a long treatment with ethylene. The tissue prints also showed that the top of the stem lost tissue integrity after 96 hr in ethylene.

**DISCUSSION**

In this report, we have studied the occurrence and distribution of 9.5 cellulase in the two abscission zones of the primary leaves of bean by using antibodies specific to 9.5 cellulase to detect the enzyme on nitrocellulose tissue prints. In the distal abscission zone, 9.5 cellulase was localized in a discrete band of cells at the pulvinus-petiole junction, denoted as the separation layer, as well as in the stele of the pulvinus and the petiole adjacent to the separation layer. Within the stele of the pulvinus, cellulase was located exclusively in the central vascular tissue and extended as far as the divergence of the vascular traces in the base of the leaf blade where the lamina was cut. Within the stele on the petiole side, cellulase was located exclusively in the ring of vascular bundles on the periphery of the petiole and extended through the first few millimeters below the separation layer. Thus, cellulase accumulated in cortical cells only in the separation layer where fracture developed and appeared first in those cells adjacent to the vascular tissue and then extended toward the periphery as abscission proceeded. Early work (Brown and Addicott, 1950) noted that, in the abscission zone of bean trifoliate leaves, a centrally located lacuna with intact peripheral tissue developed after 24 hr of treatment with ethylene. Other work (Webster, 1973) observed that cell separation began first in those cells adjacent to the vascular tissue and pith. Thus, it appears that, in the distal zone, as abscission develops, cellulase synthesis and accumulation proceed through the cortex from the center to the periphery.

Our results from the distribution of cellulase in the proximal zone showed that cellulase accumulated in the separation layer but also extended to the vascular tissue at both sides of the separation layer. We also showed that cellulase accumulated first in the cortical cells of the adaxial side of the petiole. These results are consistent with the results of Sexton et al. (1981), who found that cellulase activity increased first in the adaxial cortex and spread 8 hr later to the abaxial cortex. These authors also reported that there were high levels of cellulase in the stele. The results obtained with tissue prints have confirmed those initial observations and allowed further resolution of the localization of 9.5 cellulase within the stele because we have shown that 9.5 cellulase accumulated in the vascular bundles but not in the pith parenchyma associated with the vascular bundles. These results are also consistent with those reported by Tucker et al. (1988).
Figure 7. Distribution of 9.5 Cellulase Activity and Protein through Bean Stem Segments Exposed to Ethylene.

(A) A bean stem was cut 5 mm below the node and exposed to ethylene for 120 hr. The stem was dissected into pieces (7 mm each) from the top through the first 3 cm. Each piece of the stem was extracted and analyzed by SDS-PAGE, followed by immunoblotting with 9.5 cellulase antibody.

(B) Cellulase activity levels through the first 3 cm of the stem after 120 hr of treatment with ethylene.

cellulase cDNA clone, they demonstrated that 9.5 cellulase mRNA was expressed most abundantly in the abscission zone but was also found in the stem and the petiole tissue adjacent to the separation layer.

The cellulase found predominantly in the cortical cells of the separation layer and the cellulase found in the vascular traces of the adjacent tissues of both the distal and proximal zone had identical molecular mass and were both recognized by the 9.5 cellulase antibody raised against the cellulase purified from the proximal zone. The main difference found between the distal zone and the proximal zone was the temporal appearance of cellulase, which preceded abscission, and the way cellulase spread through the cortex in the separation layers. In the distal zone, cellulase began to accumulate in the center of the tissue and spread from the vascular traces toward the periphery. In the proximal zone, cellulase began to accumulate in the adaxial cortex and spread to the abaxial cortex. Although we cannot explain why these differences occur, it is important to remember that the proximal zone is anatomically more complex than the distal zone. The occurrence of axillary buds near the proximal zone and the presence of the stipule at the base of the node could perhaps influence the spreading of the enzyme and contribute to the difference observed in the development of the separation layer between the two zones. Understanding how this difference occurs will help to identify factors that regulate 9.5 cellulase expression.

This report identifies and localizes the occurrence of 9.5 cellulase in cells that are not involved in abscission. In response to ethylene, 9.5 cellulase was expressed exclusively in the vascular traces of the stem after it was separated from the node. Similarly, 9.5 cellulase was expressed in the vascular tissue of the pulvinus that was separated from the cells that constitute the separation layer. The function of 9.5 cellulase in cells of the vascular tissue away from the separation layer is unknown but appears to be independent of the abscission process. Therefore, the determinant characteristic of the abscission process is the induction of 9.5 cellulase by cortical cells in the separation layer and implies that those cells have a unique mechanism for initiating 9.5 cellulase synthesis. It could be argued that cellulase participates in the break-
down of the vascular tissue during abscission. Morre (1968) has noted that the breakstrength at the petiole-pulvinus junction reaches a minimum 24 hr before natural separation occurs and suggested that fracture occurs when the thick cell walls of the vascular traces are severed. However, our results with tissue prints indicate that cellulase could be detected in the vascular traces of the pulvinus or the stem long before any apparent loss of structural rigidity occurred. In addition, Horton and Osborne (1967)

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Our results do not allow us to distinguish whether 9.5 cellulase is localized in either the xylem or the phloem or both. If cellulase were associated with the xylem vessels, it could be involved in the development of tyloses. The xylem vessels of the abscission zone of bean become occluded with tyloses as abscission proceeds (Scott et al., 1967; Poovaiah, 1974). However, tyloses were found in

Figure 9. Higher Magnification of Tissue Prints Immunolabeled with 9.5 Cellulase Antibody of the Top of the Stem.
(A) After 0 hr in ethylene.
(B) After 24 hr in ethylene.
(C) After 48 hr in ethylene.
(D) After 96 hr in ethylene.
Bar = 500 μm.
the petiole side, presumably to interfere with water loss; yet we found that cellulase was clearly present all across the pulvinar stele. We suggest that cellulase is produced by the phloem and participates in the thinning of the phloem sieve tubes to facilitate mobilization of plant reserves toward a lower part of the plant. Our future work will seek more information on this important point.

METHODS

Plant Material

Seeds of Phaseolus vulgaris, cvs Red Kidney and Greensleeves, were germinated in moist vermiculite in the greenhouse. Plants were grown for 10 days to 12 days until the first trifoliate was just emerging. Explants were prepared as described in Reid et al. (1974), placed in a 24.4-L plexiglass chamber, and exposed to 1 L/min ethylene in air (5 mL/L).

Tissue Prints

Nitrocellulose tissue prints were made with sections cut by hand. Cross-sectioning was always carried out basipetally through the explant, and sections were arranged in succession over the nitrocellulose by means of a fine tweezer. Immunoblotting and photography were performed as described previously (Reid et al., 1990).

Protein Extraction and Cellulase Enzyme Assays

Extracts from each anatomical region were prepared by collecting 10 segments, 2 mm to 5 mm thick, of the appropriate region from each of five equally treated plants. The sections were ground to a powder in liquid nitrogen with a small mortar and pestle and extracted with 200 μL of a solution containing 20 mM Tris, 3 mM EDTA, and 0.5 M NaCl. The mixture was incubated for 30 min at 0°C and then centrifuged in a microcentrifuge for 10 min. Cellulase activity was determined on the supernatant by using a viscometric assay as described previously (Durbin and Lewis, 1988). Assays were performed at pH 7.5 to eliminate activity of the acidic 4.5 cellulase (del Campillo et al., 1988).

Protein Gel Electrophoresis and Protein Blotting

Tissue extracts were prepared as described above, and protein was precipitated by adding pre-cooled acetone to a final 80% concentration. After incubation at −20°C for 1 hr, the precipitate was collected by centrifugation, resuspended in Laemmli sample buffer (Laemmli, 1970), and fractionated by SDS-PAGE (12%). When electrophoresis was complete, the proteins were transferred electrophoretically to nitrocellulose sheets and incubated with 9.5 cellulase antibody. We used goat anti-rabbit IgG (Fc) alkaline phosphatase conjugate as second antibody (Promega Biotec, Madison, WI) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride as color development reagents (Bio-Rad Laboratories, Richmond, CA).

Detached Pulvinus and Stem Experiments

The distal pulvinus was cut at 2 mm, 3 mm, and 4 mm away from the separation layer, whereas the rest of the pulvinus and the adjacent petiole remained attached to the explant. The detached segment of the pulvinus was laid on top of 1% agar containing 100 μg/mL ampicillin, and the rest of the explant was set in a styrofoam cup containing 25 mL of water. Both parts were exposed to 25 ppm ethylene for 48 hr.

The stem was separated from the nodal pulvinus as previously described and exposed to 25 ppm ethylene (Linkins et al., 1973). No lanolin paste was applied to the cut surface.

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