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Leaf abscission in *Impatiens* (Balsaminaceae) is due to loss of highly de-esterified homogalacturonans in the middle lamellae[1]

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- *Premise of study:* Abscission zones (AZ) are sites where leaves and other organs are shed. Investigating the AZ by classical biochemical techniques is difficult due to its small size and because the surrounding tissue is not involved in abscission. The goals of this study were to determine whether AZ cell walls are chemically unique from the other cells of the petiole, perhaps making them more susceptible to enzymatic degradation during abscission and to identify which cell wall polysaccharides are degraded during abscission.
- *Methods:* A battery of antibodies that recognize a large number of cell wall polysaccharide and glycoprotein epitopes was used to probe sections of the *Impatiens* leaf AZ at several time points in the abscission process.
- *Key results:* Prior to abscission, the walls of the AZ cells were found to be similar in composition to the walls of the cells both proximal and distal to the AZ. Of all the epitopes monitored, only the highly de-esterified homogalacturonans (HG) of the middle lamellae were found to be reduced post-abscission and only at the plane of separation. More highly esterified homogalacturonans, as well as other pectin and xyloglucan epitopes were not affected. Furthermore, cellulose, as detected by an endoglucanase-gold probe and cellulose-binding module staining, was unaffected, even on the walls of the cells facing the separation site.
- *Conclusions:* In the leaf abscission zone of *Impatiens*, wall alterations during abscission are strictly limited to the plane of separation and involve only the loss of highly de-esterified pectins from the middle lamellae.

Key words: abscission zone; Balsaminaceae; cell wall; *Impatiens sultani*; homogalacturonan; immunocytochemistry; middle lamellae.

Abscission is the process by which plants shed organs such as leaves, flowers, and fruits (Abeles, 1968; Sexton and Roberts, 1982). Abscission occurs in a special layer of cells, known as the abscission zone (AZ), which develops at the base of the organ to be shed (Sexton and Roberts, 1982; Roberts and González-Carranza , 2007). The cells of the AZ are smaller than neighboring cells, especially in their axial dimension, with the result that they are physically less interdigitated with other neighboring AZ cells. This limited interdigitation reduces the amount of middle lamella/cell wall material that must be degraded before the organ can be shed, thus facilitating the abscission process. In many plant species, including *Impatiens*, the AZ forms before the actual process of abscission is initiated. Besides being physically shaped for abscission, the cells of the AZ appear to be primed to respond to plant hormones differently than neighboring non-AZ cells (Taylor and Whitelaw, 2001; Roberts et al., 2002).

One of the problems in the study of the AZ is its small size compared to the bulk of the tissue both proximal and distal to the AZ. Often there is no clear morphological marker to indicate the zone itself prior to the abscission event, or in many species, an AZ is not formed until the process of abscission has been induced. Thus, biochemical studies have been hampered in that it is difficult or impossible to ascertain whether a pure AZ fraction is obtained or whether the process of AZ formation or abscission itself is being investigated. Even if it were possible to extract clean AZs, the processes that occur in the AZ are often just between a very few cells and might go unnoticed compared to the bulk of other tissues that remains unaffected in the process.

Although *Arabidopsis* is often used as a model system for the investigation of many plant processes, *Arabidopsis* is not a useful system for the study of leaf abscission; its AZ is so small and difficult to discern that related *Brassica* species are now used instead (González-Carranza et al., 2002). One of the most well-studied systems is the abscission of leaves in *Impatiens*, investigated by Sexton and colleagues (Sexton, 1976, 1979; Sexton et al., 1977). With a simple manual de-blading of the leaf, a process of abscission commences, and by 24 h, the de-bladed leaf has abscised. Variability from sample to sample is very small, and the process is highly predictable, unlike natural abscission events. In *Impatiens* leaves, the AZ is formed prior to abscission, so that the de-blading simply induces the abscission event, not the induction of the AZ itself, which might confound the determination of which events

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are associated with the production of the AZ and the abscission itself. Sexton and his colleagues discovered several important aspects of abscission with this system. First, they found that, with the exception of cells in the vascular bundles, there was no breaking of intact cells, but rather the cells seem to be mostly separated at their middle lamellae. Second, even cells at the separation zone appeared to maintain their shape or were at best only slightly swollen. The fracture of cells in the xylem appeared to be roughly in the plane of the other cells in the AZ and may have been induced by the loss of cell–cell connections between the other cells in the AZ, so that the xylem elements fractured under increasing gravity-induced pressure. Also, tyloses form on the proximal side of these xylem elements to prevent water loss and seal off these elements, but not on the distal side.

These studies of Sexton and colleagues present a very concise and detailed description of the events of abscission in the *Impatiens* leaf petiole and were the state of the art at the time of their investigations. However, the studies did leave us with a number of unanswered questions as to the nature of the AZ. These questions include: (1) Are the cell walls in the AZ compositionally different than the surrounding petiolar cells such that this would facilitate abscission? (2) What compositional changes occur in the cell walls in the process of abscission that allow them to separate from each other? To answer these questions, we used a battery of antibodies and cytochemical probes to examine the composition of the AZ before, during, and after the abscission events using this very well-defined system. We were thus able to pinpoint the changes that occur in these AZ's very accurately. Surprisingly, our results indicate that the loss of highly de-esterified homogalacturonans from the middle lamellae on a single plane through the AZ is responsible for leaf abscission in *Impatiens*.

MATERIALS AND METHODS

Plant material and specimen preparation—*Impatiens* (*Impatiens sultani* cv. Shady Lady; Balsaminaceae) plants were purchased from a local supplier and grown in a sterile potting mixture. Plants were grown in a Conviron growth chamber under constant light (~750 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and temperature (22°C). Leaves were de-bladed with a razor blade or sharp scissors to induce the abscission process. The abscission zones were collected from the plant at 0, 8, 16, and 24 h after de-blading with a razor blade by cutting a shallow arc through the stem starting <1 mm above the leaf axil and ending <1 mm below the petiole. Samples were vacuum fixed in 3% (v/v) glutaraldehyde in 50 mmol/L PIPES buffer, pH 7.4 for 2 h. Following fixation, samples were washed three times with fresh 50 mmol/L PIPES and then dehydrated with ethanol at room temperature in 25%, 50%, 75% (2 h each), then overnight in absolute ethanol at −20°C in a freezer. Samples were then infiltrated with LR white resin (Polysciences, Warrington, Pennsylvania, USA) with increasing concentrations (25%, 50%, 75%, 100%, 24 h each step), also at −20°C. Specimens in 100% resin were removed from the freezer and placed onto a rocking platform for 24 h at room temperature. Specimens were placed into cylindrical polyethylene capsules (TAAB, Ted Pella, Redding, California, USA) and oriented so that the (truncated) petiole extended parallel to the bottom of the capsule (i.e., the AZ was perpendicular to the end of the capsule). A square of dental wax was floated on the resin (Bowling and Vaughn, 2008c), and the capsules were polymerized at 55°C until the wax melted, indicating that polymerization had taken place (1.5–2 h). Blocks were trimmed so that the sliver of stem was in the large edge of the pyramid, and the petiole was in the small edge. Sections (0.35 μm) were cut on a Reichert UltraCut E microtome and stained with 1% toluidine blue (in 1% sodium borate) and then mounted in Permount before being imaged on a Zeiss Axioskop with an Olympus Q-color 3 digital camera. Images were contrast-enhanced with the program ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and made into plates using the program GIMP (www.gimp.org; ver. 2.2).

Immunogold-silver light microscopy—Semithin sections (350 nm) were blocked with 1% (w/v) BSA in phosphate-buffered saline (PBS) for 30 min at room temperature. Primary antibody was applied to sections (at various dilutions from 100% to 1 : 8 in PBS-BSA) and incubated in a moist chamber for 3 h. Details of the antibodies, their specificities and sources are given in Table 1. Sections were rinsed and incubated with secondary antibody (15 nm gold, E-Y Laboratories, San Mateo, California, USA) diluted 1 : 20 in PBS-BSA for 1 h at room temperature. (The buffer for the 2F4 antibody was 20 mmol/L Tris [pH 8.2], 5 mmol/L $CaCl_2$, and 150 mmol/L NaCl throughout the blocking and incubation steps; this antibody does not recognize its epitope in the PBS-BSA buffer system.) After washing with double-distilled water, the sections were silver-enhanced (IntenSE, GE BioSciences, Piscataway, New Jersey, USA) for 20–30 min at room temperature. Sections were mounted in Permount, and images were collected as for light microscopic sections.

Some sections were treated with sodium carbonate, which chemically de-esterifies homogalacturonan, by placing a drop of 1 mol/L Na_2CO_3 for 1 h at room temperature on the slide. The slides were washed with double-distilled water, dried with compressed air, and immunolabeled as described already. After this treatment, the fracture-face wall again reacts with JIM5 because sodium hydroxide chemically converts esterified pectins into de-esterified (i.e., JIM5-reactive) forms, confirming the presence of esterified pectins in this wall following abscission. Some sections were enzymatically treated with a polygalacturonase as described by Marcus et al. (2008). This treatment allows for demasking of epitopes that would otherwise be hindered by the presence of homogalacturonans around other polysaccharides. Efficacy of the treatment was monitored by probing the treated sections with JIM5, as described.

Cellulose detection—Samples were probed with the 1→4-endoglucanase-gold probe, prepared as described by Sabba et al. (1999). Samples were obtained from the same block faces as used for immunocytochemical analysis and blocked for 30 min in PBS-BSA. After the blocking step, the sections were probed with the endoglucanase-gold probe for 4 h, washed with a spray of double-distilled water and then reacted with the IntenSe silver intensification kit. The sections were then washed with distilled water and dried with compressed air. The sections were mounted in Permount and observed by light microscopy as described for immunogold-silver. Preincubation of the probe in 1 mg/mL carboxymethylcellulose resulted in complete inhibition of binding to

TABLE 1. Antibodies employed in this study and references for their specificities.

Antibody	Reference
Homogalacturonans	
JIM5	Clausen et al., 2003
JIM7	Clausen et al., 2003
LM18	Verhertbruggen et al., 2009
LM19	Verhertbruggen et al., 2009
LM20	Verhertbruggen et al., 2009
2F4	Liners et al., 1989
CCRC-M38	W. Willats, personal communication
Rhamnogalacturonan I (and side chains)	
LM5	Jones et al., 1997
LM6	Willats et al., 1998
CCRC-M2	Freshour et al., 1996
CCRC-M7	Freshour et al., 1996
CCRC-M8	CCRC web site [a]
CCRC-M10	CCRC web site [a]
CCRC-M22	CCRC web site [a]
Xyloglucan	
LM15	Marcus et al., 2008
CCRC-M1	Puhlmann et al., 1994
Arabinogalactan protiens	
JIM13	Smallwood et al., 1994
LM2	Smallwood et al., 1996
Xylan	
LM10	McCartney et al., 2005
LM11	McCartney et al., 2005

[a] Complex Carbohydrate Research Center (CCRC), Monoclonal Antibodies for Plant Cell Walls, http://cell.ccrc.uga.edu/~mao/wallmab/Antibodies/antib.htm.

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the sample. Some samples were also examined by binding of a cellulose-binding module (Plant Probes, Leeds, UK) as described by Meloche et al. (2007). Images were captured using an Olympus Qcolor 3 camera as described.

Electron microscopic immunogold—Specimens from the same block face as used for the light microscopic analysis were sectioned with a diamond knife on a Reichert Ultracut E ultramicrotome at a section thickness of ~100 nm (pale gold-silver) and were mounted on uncoated 300-mesh gold grids. The grids were then floated on 5–10 μL drops of the following solutions 1% BSA in PBS, 30 min; primary antibody diluted as for the light microscopic sections or 100%, 3 h; PBS-BSA, 2.5 min × 4; 1:20 dilution of goat anti-rat IgG conjugated to 15 nm gold in PBS-BSA, 1 h. (The PBS-BSA solution used in these steps was filtered through a 0.20 μm filter prior to mixing any of these solutions). The grids were then washed with a stream of double-distilled water and dried on filter paper before being poststained with 2% uranyl acetate (2 min) and Reynold's lead citrate (30 s). Some sections were de-esterified by floating the grids on 100 μL drops of filter-sterilized 1 mol/L Na_2CO_3 for 1 h at room temperature before the incubation steps. The grids were washed with double-distilled water and then air dried on Whatman's #1 filter paper before processing through the described steps for immunolocalization. Specimens were observed with a Zeiss EM10CR electron microscope with an accelerating voltage of 60 kV. Specimens were observed in the orientation of gold particles closest to the beam. Photographs were taken on Kodak electron imaging film and then scanned for digital reproduction.

RESULTS

Immunocytochemical survey of pectins in the abscission zone of Impatiens prior to induction of abscission—The AZ of *Impatiens* is comprised of a layer of axially shortened cells that extend across the base of the leaf petiole and are easily distinguished morphologically from the more expanded distal and proximal cells of the petiole. Antibodies to various pectin (homogalacturonan [HG] and rhamnogalacturonan I [RGI]) epitopes were applied to sections of the AZ that had been collected from normal, healthy leaves that showed no signs of abscising. In all cases, these antipectin antibodies reacted evenly across the tissue section; i.e., cells in the AZ were not noticeably deficient in any of the polysaccharide epitopes recognized by these antibodies (Fig. 1; Table 1). Reactions of antibodies that recognized xyloglucans, AGPs, and extensin were much more variable on these sections: some of the antibodies reacted strongly, whereas others reacted weakly (e.g., CCRC-M1); however, in all cases, the reactions of cells in the AZ were the same as those of the surrounding petiolar cells. Thus, at least in terms of the epitopes recognized by the antibodies used in this study, the AZ is not compositionally unique.

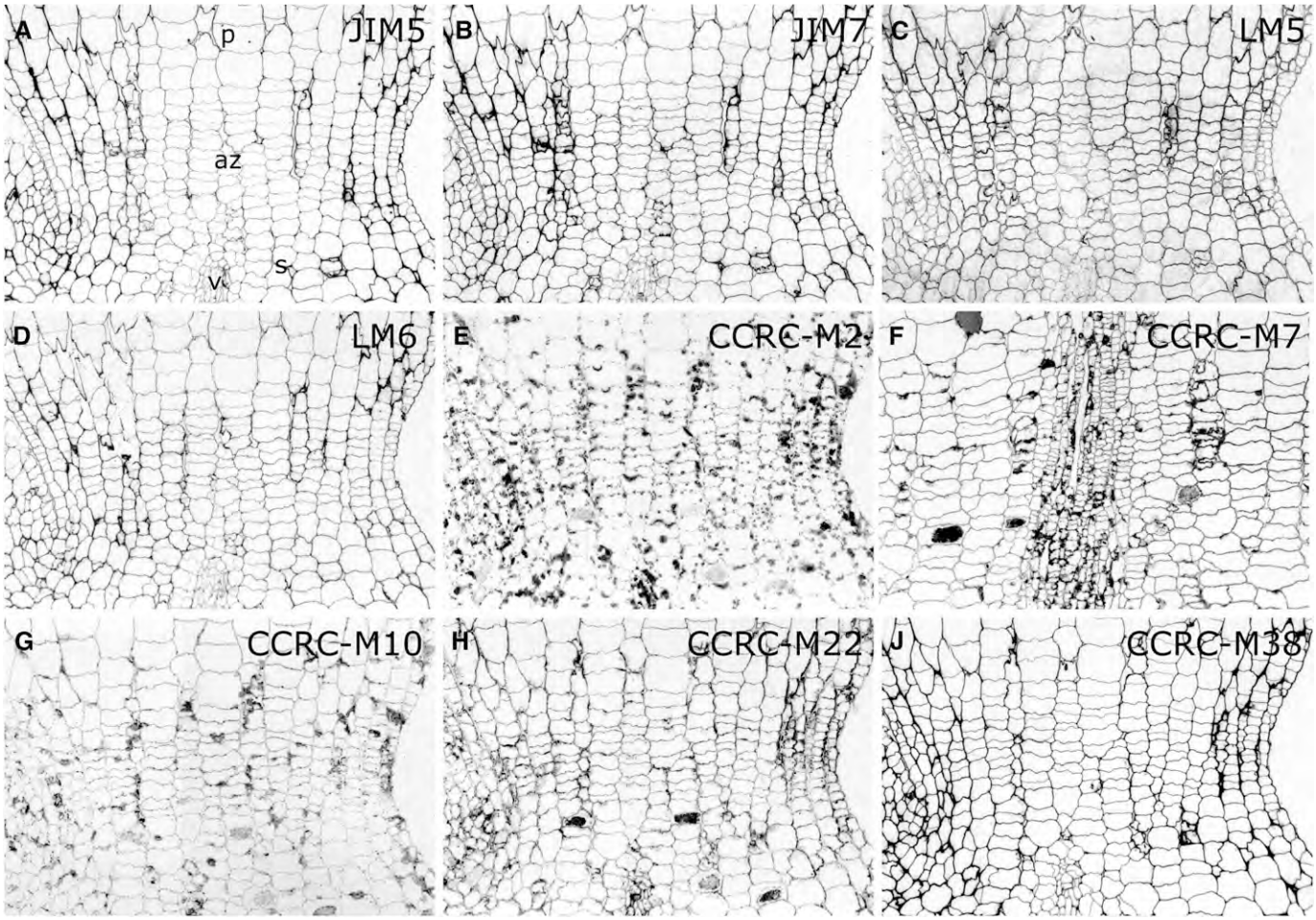


Fig. 1. The leaf abscission zone of *Impatiens* before the induction of abscission (time 0). Before induction of abscission, the abscission zone (az) of *Impatiens* is fully formed and can be identified by the smaller size (in both axial and transverse dimensions) of the cells of the abscission zone compared with other cells of the petiole (p) and stem (s). Sections of the noninduced abscission zone were labeled with a wide selection of antipolysaccharide antibodies, and none of them were found to be enriched in or excluded from the abscission zone in comparison to neighboring tissue. v = vascular bundle. Scale bar = 100 μm.

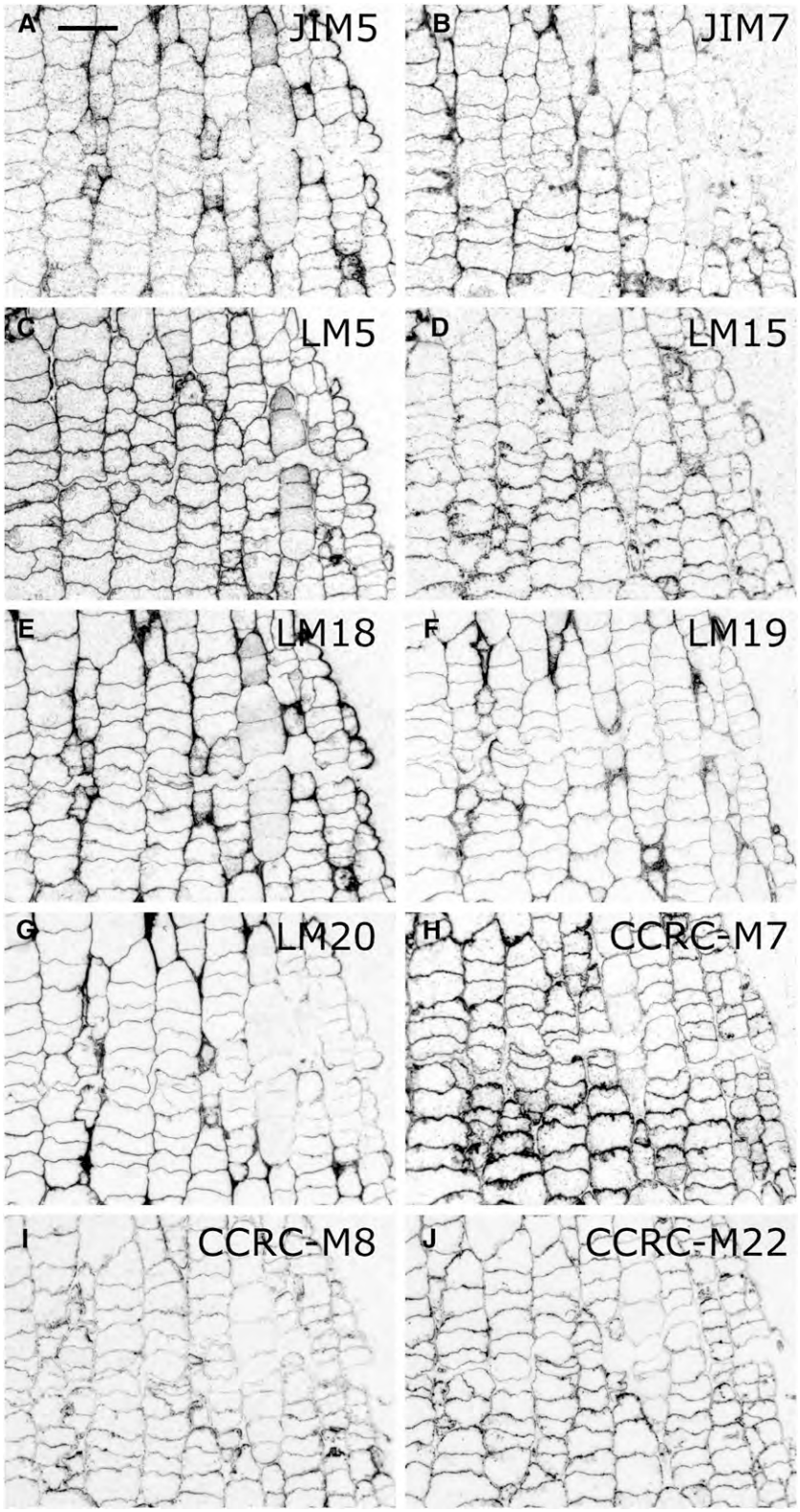


Fig. 2. The *Impatiens* leaf abscission zone (AZ) 16 h post-induction of abscission. At this point, cell separation has already begun (arrowheads). JIM5-reactive homogalacturonan (HG) appears to be removed from only the walls facing the separation site (a), while LM19 (f), LM20 (g), and JIM7 (b) appear to be lost from a larger area. In contrast, LM18 (e), which reacts with esterified HG, does not appear to be altered. Furthermore, the rhamnogalacturonan I (RGI)-reactive antibodies CCRC-M8 (i) and CCRC-M22 (j) appear to be slightly lighter in the walls of cells around the separation, while other anti-RGI antibodies (CCRC-M7; h) and anti-RGI-side chain antibodies (LM5; c) do not. The anti-xyloglucan antibody LM15 (d) shows no change in the cells of the AZ. Scale bar = 50 μm.

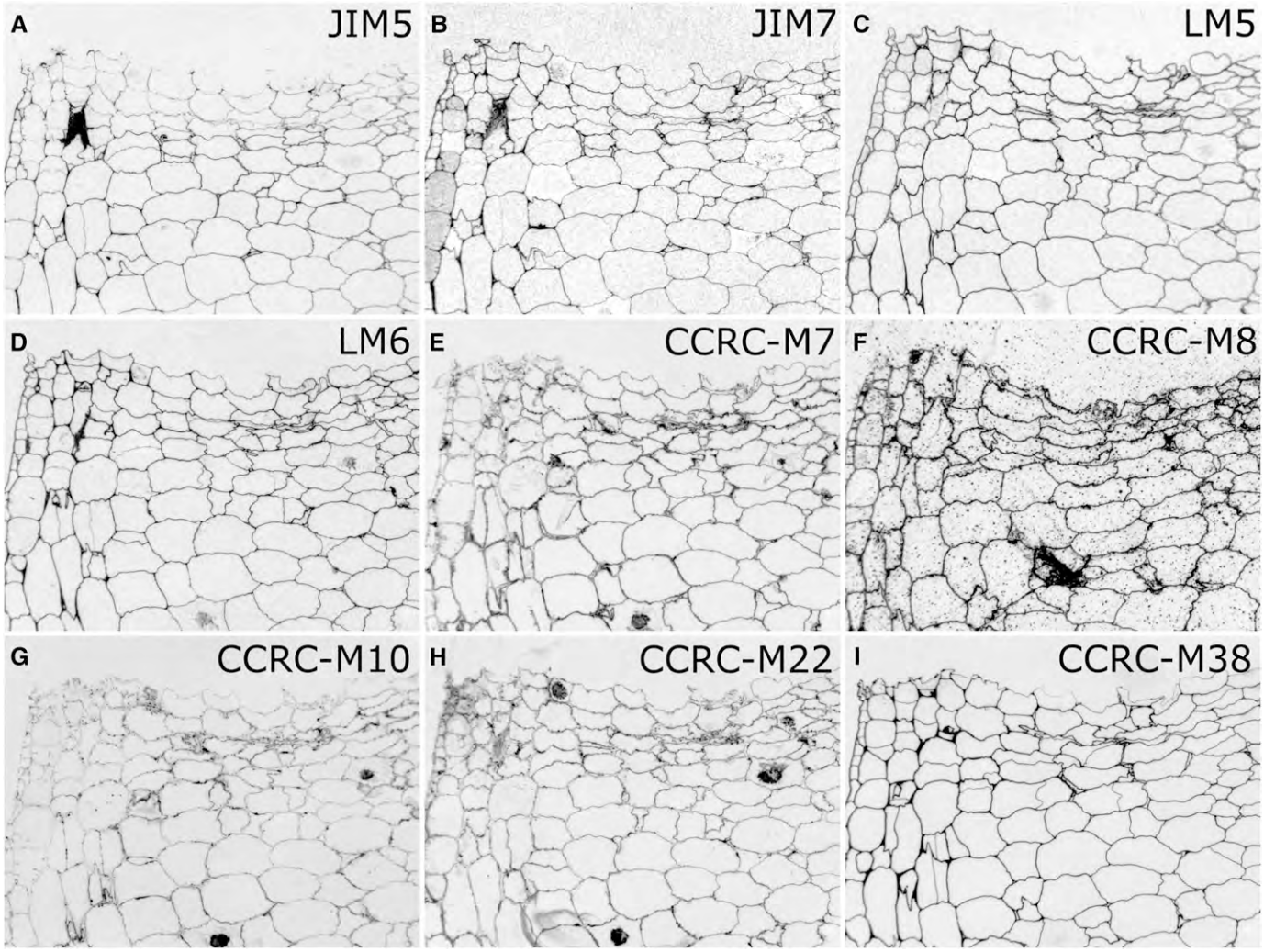


Fig. 3. The *Impatiens* leaf abscission zone (AZ) after abscission (24 h). Serial sections of the proximal surface of the leaf AZ were labeled with a selection of antibodies to homogalacturonan (HG) and rhamnogalacturonan I (RGI). JIM5-reactive HG appears to be the main polysaccharide degraded at the AZ during the process of abscission (a). All of the other antibodies tested labeled the walls of the cells facing the separation site (b–i). Scale bar = 100 μm.

Immunocytochemical survey of the abscission zone of Impatiens post-induction of abscission—As reported by Sexton and colleagues (Sexton, 1976, 1979; Sexton and Redshaw, 1981), removal of most of the leaf blade initiates the process of abscission in *Impatiens*. Abscission zones were collected and fixed at 8, 16, and 24 h after de-blading of leaves and were probed with a similar battery of antibodies as the time 0 specimens. At 8 h post-induction of abscission, no salient changes were evident in the walls of the cells of the AZ (data not shown). However, 16 h after the induction of abscission by de-blading, a noticeable fissure became apparent between the cells of the AZ extending from the adaxial edge of the petiole to the abaxial (Fig. 2). Highly de-esterified HG appeared to have been degraded from the walls of the cells at the edge of the fissure (JIM5, Fig. 2a; and LM19, Fig. 2f). Esterified forms of HG appeared to be lost from a larger region around the origin of the fissure (JIM7, Fig. 2b; LM20, Fig. 2g), but not as much from the cell walls of the fracture face. RGI epitopes appeared to be unaltered in the cell walls of the AZ, including the walls fronting the fissure (CCRC-M7, Fig. 2h; CCRC-M8, Fig. 2i; CCRC-M22, Fig. 2j). Similarly, antibodies to the galactan side chains of RGI showed that RGI is not being de-branched during abscission (LM5, Fig. 2c), which would be a sign of senescence or general wall softening (e.g., Bowling and Vaughn, 2008c; Pena and Carpita, 2004). Thus, among the pectin epitopes investigated, all of the changes involved changes in HGs not RGI.

After 24 h, the abscission process was complete (or nearly so) in all of the samples collected. In most cases, the distal portion of the leaf petiole separated from the proximal portion of the petiole either before or during the collection and fixation process. Both the proximal and the distal tissues were immunolabeled with the same battery of antipolysaccharide antibodies as were used on the specimens from the other times. Non- or low-esterified HGs (recognized by JIM5 and 2F4), which are normally a large component of the middle lamella, appeared to be lost predominantly from the wall facing the separation site (i.e., the fracture face wall), while the level of these HGs appeared nearly normal on the other sides of the cell (JIM5, Figs. 3a, 4a). A similar pattern of reaction was noted with the antibodies 2F4 (which recognizes highly de-esterified HGs that are

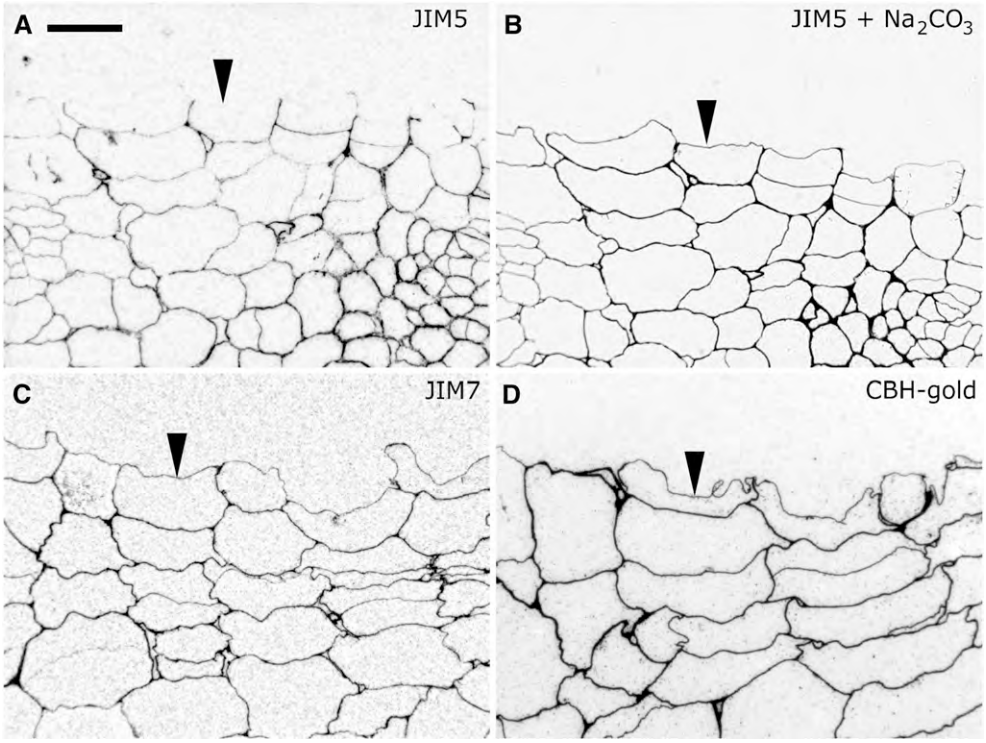
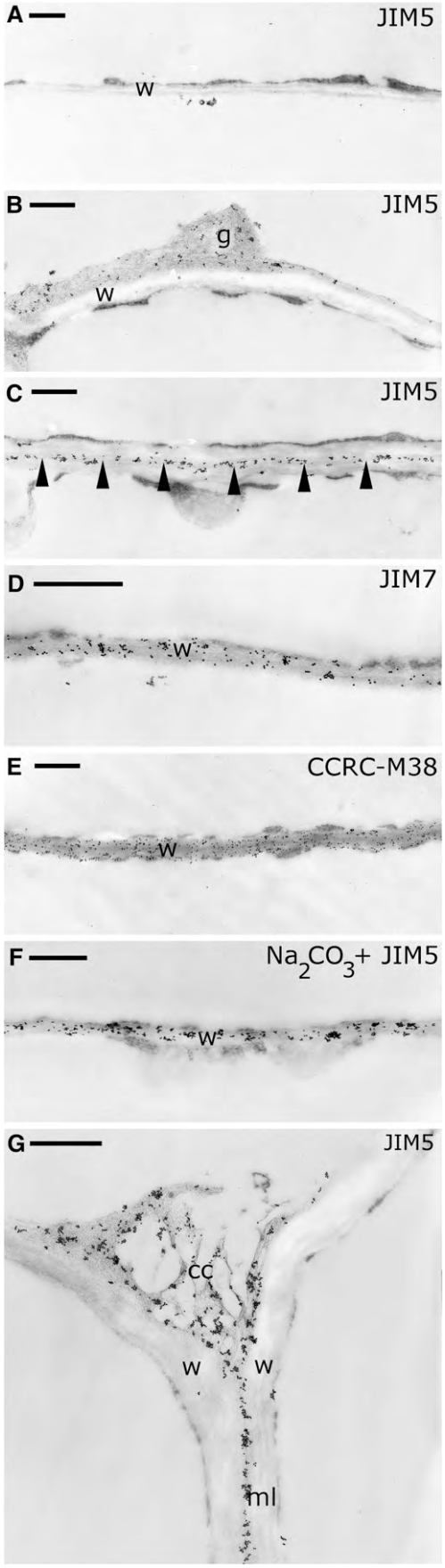


Fig. 4. Sections of the proximal abscission zone (AZ) were treated with sodium carbonate to chemically de-esterify the homogalacturonan (HG) component of the walls and compared with untreated sections. (a) Before treatment with sodium carbonate, the anti-de-esterified HG antibody JIM5 bound very strongly to the walls/middle lamellae of the cells of the petiole base, but missing entirely from the wall/middle lamellaeat the face of the separation surface. (b) After treatment with sodium carbonate, all of the partially esterified HG was converted to de-esterified HG, and the JIM5 antibody labeled all of the cell walls very completely and strongly. Arrowheads on (a) and (b) show analogous areas from serial sections to facilitate comparison. The reaction of the sections with (c) JIM7 and cellobiohydrolase-gold (to detect cellulose; d) are shown for comparison. Scale bar = 50 μm.

calcium-bridged; Liners et al., 1989) and LM19 (which reacts similarly to JIM5; Verhertbruggen et al., 2009). However, antibodies to other polysaccharides showed no change in reactivity compared to the time 0 AZ (Fig. 1) described already. In particular, the fracture face wall reacted with JIM7 (Figs. 3b, 4c) and LM18 (reacts similarly to JIM7; not shown), meaning that more highly esterified HGs remain in these walls. Indeed, upon treatment of the sections with sodium carbonate, which chemically de-esterifies HG, the outermost wall facing the separation face again had reacted with JIM5 (Fig. 4b). The antibody CCRC-M38 recognizes HG with a range of esterification states (W. Willats, University of Copenhagen, Denmark, personal communication), and it, too, was unaffected. Sections probed with endoglucanase-gold showed that cellulose is still present in the walls of the AZ cells, including the fracture face wall (Fig. 4d). Similar results were obtained using the cellulose-binding module CBM3a (not shown).

Sections of these same block faces were probed with these antibodies and examined with the electron microscope (Fig. 5). Again, JIM5- and 2F4-reactive material was absent from the separation face, but other faces of these cells had middle lamellae that were strongly reactive. The only exceptions to the complete loss of HGs along this surface appeared on the distal side of the AZ, where small clumps of de-esterified pectins appeared as globules reactive with JIM5, CCRCM-38 and 2F4 along the distal surface, although they were not found along the proximal surface of the AZ. Thus, the loss of de-esterified HGs appeared to occur only on a single cell surface and not along all edges of these cells. The absence of globules on the proximal face of the abscission site might indicate that the enzymatic degradation of the middle lamellae is initiated at the proximal side, not the distal.

The presence of xyloglucan in the AZ was similarly assessed by probing sections with the antibodies LM15 (recognizes non-fucosylated xyloglucan; Marcus et al., 2008) and CCRC-M1 (recognizes fucosylated xyloglucan; Freshour et al., 1996). As with the pectin epitopes described above, we found that xyloglucan was also present in equal amounts in both AZ and non-AZ tissues (Fig. 6A, LM15; CCRC-M1, data not shown). At 16 h, when an obvious separation had begun to form at the AZ, no obvious loss of xyloglucan was evident (LM15, Fig. 2j). At 24 h, when abscission was complete, the cell wall facing the separation zone displayed a relatively unaltered amount of LM15-reactive xyloglucan (Fig. 6b). Overall, both anti-xyloglucan antibodies displayed relatively little affinity for the sections of *Impatiens* compared to other tissues we have prepared in a similar manner. One possible explanation for this low level of xyloglucan labeling is that pectins may be masking the epitopes recognized by these anti-xyloglucan antibodies; so the sections were treated with polygalacturonase to "unmask" these epitopes (Marcus et al., 2008). However, even after this "unmasking", CCRC-M1 displayed little affinity for the *Impatiens* sections, indicating that levels of fucosylated xyloglucan were relatively low in these petiolar cells. (Efficacy of the polygalacturonase treatment was confirmed by the lack of recognition by JIM5 and JIM7 after the treatment.) The LM15 antibody, however,



reacted noticeably more with the tissue after treatment of the sections with polygalacturonase to remove masking pectin (Fig. 6d), but the overall pattern of labeling remained the same: the wall facing the separation zone contained xyloglucan, and the cells outside of the AZ were labeled in a similar manner to those in the AZ itself.

Xylem breakage—Following abscission, sections of the petiole to either side of the AZ (i.e., both the proximal and distal regions) were probed with the antixylan antibodies LM10 (Fig. 7a, 7c) and LM11 (not shown). These sections were lightly stained with toluidine blue so that other cellular structures would be obvious because only the xylem cells are labeled with these antibodies. The cells of the vascular bundle in general, and xylem cells in particular, appeared to be disrupted or torn at the plane of separation, rather than cleanly separated like the neighboring parenchyma cells (Fig. 7a, 7b). Furthermore, unlike other workers (Lee et al., 2008), we did not observe any increased label of xylan or xyloglucan in the AZ. All antixylan labeling was associated with the xylem elements, just as in other tissues of *Impatiens* (Vaughn, 2006). Sexton (1979) reported that tyloses formed in the proximal side of the AZ, but not the distal zone on tissue explants. We have confirmed that finding on freshly harvested tissue (Fig. 7d). These proximal tyloses are strongly labeled with the HG antibody CCRC-M38, although this same antibody does not label the helical thickenings of the xylem cells themselves (Fig. 7d). Previously, this antibody was also found to label tyloses in trees (hackberry and sweetgum; Bowling and Vaughn, 2008b). Because HGs are a prominent component of mucilages, it is likely that the presence of HGs indicates some sort of hydration of these tyloses. Oddly, none of the other HG antibodies reacted with the tyloses, further indicating that CCRC-M38 has a different specificity to HG than the other anti-HG antibodies.

DISCUSSION

The nature of the walls of AZ cells prior to abscission—The cells of the *Impatiens* AZ are morphologically distinct from the petiolar cells on either side of the AZ. The AZ cells are shorter in their axial dimension and are arranged in tight layers with little interdigitation between the layers of cells. There has been speculation that the walls of AZ cells must be chemically distinct from the walls of other cells because these are the only walls that are degraded during

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Fig. 5. High-magnification (TEM) analysis of the abscission zone (AZ) of *Impatiens* post-abscission. The de-esterified homogalacturonan (HG) component of the middle lamellae is totally lost from the exterior face of the AZ (a) except for occasional remnants (b). In contrast, walls that are even one cell removed from the plane of separation show a normal level of JIM5-reactive de-esterified HG in the middle lamella (arrowheads) (c). More highly esterified (JIM7-reactive) HG is not lost from the outer wall of the plane of separation (d), nor is the CCRC-M38-reactive polysaccharide component (e). After treatment of section with sodium carbonate, which chemically de-esterifies HG, the JIM5 antibody now labels the wall very strongly (f), demonstrating the high specificity for de-esterified HG displayed by the abscission-induced polygalacturonase. At cell corners, fibrils of JIM5-reactive HG can still be seen, although the cell corner appears partially degraded (g). w = cell wall, g = middle lamella remnant, cc = cell corner, ml = middle lamella. Scale bars: a–c, and e = 0.5 μm; d, f, and g = 0.25 μm.

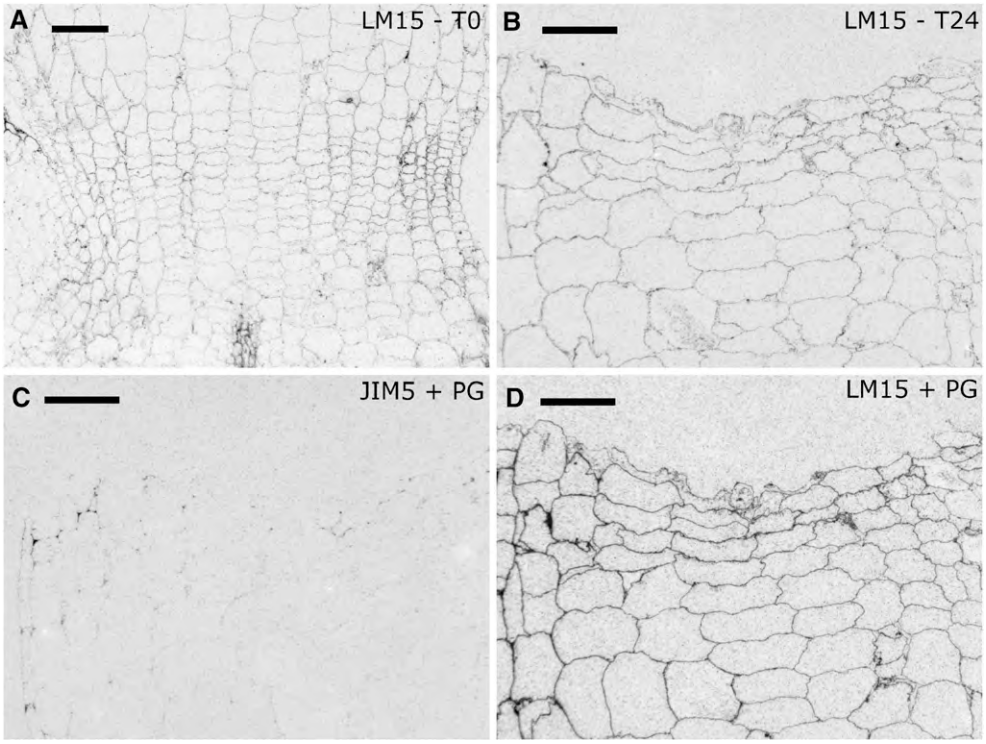

Fig. 6. Investigation of xyloglucan (XG) at the abscission zone (AZ). Before the induction of abscission, the anti-XG antibody LM15 reacts with all of the walls near the AZ (a). After abscission, there appears to be no reduction in the amount of XG on the cells facing the separation site (b). Due to concerns that masking pectins may be confounding the XG-antibody reactions, sections were treated with polygalacturonase (PGase) to remove pectin and probed again with LM15. Sections treated with PGase were labeled with JIM5 to confirm the removal of pectin (c). In PGase-treated sections, LM15 labeling is higher, but does not appear to be removed to any great extent from the cells facing the separation site (d). Scale bars = 100 μm.

abscission (Henderson et al., 2001; Rose et al., 2003). Surprisingly, we found no gross differences in the chemical composition of the walls of any of the cells in the petiole or in the AZ, despite probing sections with nearly 30 different antibodies. Thus, it appears that, before abscission starts, only the morphology of these cells (and most likely their ability to respond to exogenous hormonal signals) distinguishes them from cells outside the AZ.

Post-abscission changes in the walls of the cells of the AZ— As early as 1871, researchers suspected that dissolution of the middle lamella was responsible for abscission (Wiesner, 1871 in Facey, 1950). Facey (1950) used cytochemical staining techniques to determine that pectin was being lost from the leaf abscission zone. Since that time, many others have reported that it appears that dissolution of the middle lamella plays a major role in the abscission process (Sexton et al., 1977; Rose et al., 2003), so that along the abscission surface, the actual abscission site is between cells, not through them. The only exception to this appears to be the xylem elements, which appear to fracture roughly on the plane created by the separation of the cells in the AZ. Furthermore, there has been speculation that the morphologically distinct cells of the AZ might in fact have a cell wall and/or middle lamella (ML) composition that differs from other plant cells and that these walls could be more readily degraded, thus facilitating abscission (Henderson et al., 2001; Rose et al., 2003). This latter speculation appears not to be true, however. The ML of the cells in the AZ before induction of abscission was compositionally identical to cells outside the AZ.

In this study, we confirmed earlier observations on the primary role of the ML in the abscission process, but with the monoclonal anti-polysaccharide antibodies now available, we were also able to pinpoint the specific polysaccharide changes responsible for tissue separation. Following abscission, the only changes in the composition of the walls of AZ cells were actually in the middle lamellae, not the wall proper, between the cells at the plane of separation. Middle lamellae from the other surfaces of these same cells were unaffected. Furthermore, the only pectin molecules that were affected were the highly de-esterified HGs (i.e., those recognized by JIM5, LM19, or 2F4). More highly esterified HGs (JIM7- and LM18-reactive) and RGI (e.g., polysaccharides bearing epitopes recognized by CCRC-M2, CCRC-M7, CCRC-M22, and RGI side-chain polysaccharides, recognized by LM5 and LM6) were unaffected, although these other pectin epitopes were predominantly found within the wall, not in the middle lamella. Additionally, despite reports of cellulase activity in leaf abscission zones (Sexton et al., 1981; González-Carranza et al., 2002; Mishra et al., 2008), we observed no obvious changes in cellulose labeling using endoglucanase-gold cytochemistry (Fig. 4d). It is possible that the well-documented presence of expansin and endoglucanase activity reported in abscission zones is responsible for the wall loosening and breakage of the xylem elements only rather than the more mild changes that occur in the other AZ cells. Thus, the one change in the AZ cell wall composition appears to occur just in the middle lamellae and even there only to the cells at the separation surface itself. Moreover, we have now examined dehiscence zones from seed capsules of nine species, including another species of *Impatiens*, and all of them involve

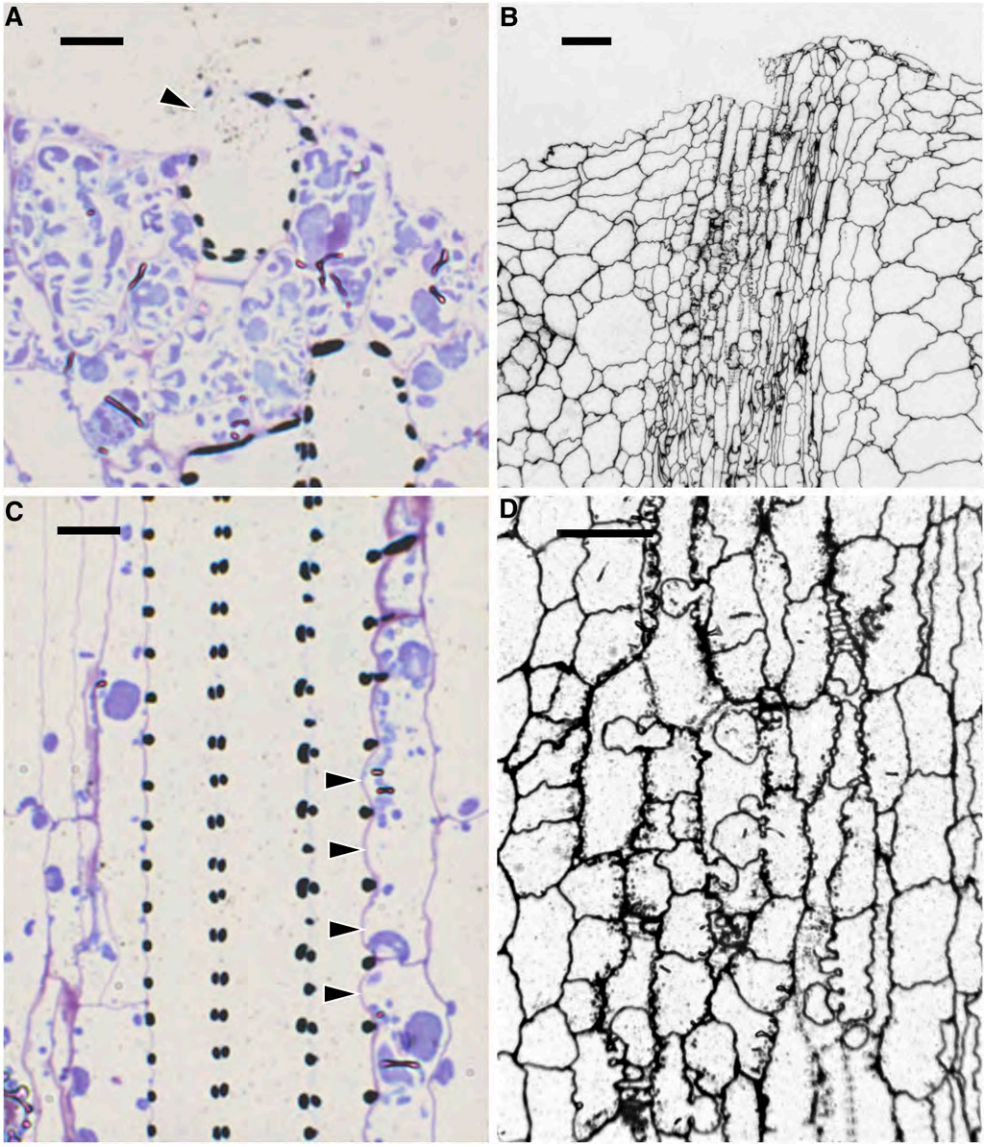

Fig. 7. The vascular bundle at the site of abscission in *Impatiens*. Sections of the abscission zone (AZ) were probed with either the anti-xylan antibody LM10 (a and c) or the anti-*Arabidopsis* seed mucilage antibody CCRC-M38 (b and d). At the separation surface, the vascular bundle can be seen to separate in a more irregular line than other cells of the petiole (b). At higher magnification, individual cells of the xylem appear to be torn (arrowhead) rather than separated cleanly at the ML (a). A light staining of immunolabeled sections with toluidine blue puts the heavily labeled (black) helical thickenings of the xylem in context with surrounding cells. In the distal xylem, slight invaginations into the xylem elements can be detected (c, arrowheads), while in the proximal xylem a highly developed system of tyloses is formed (d). Scale bars: a and c = 10 μm, b = 50 μm, and d = 25 μm.

the specific loss of highly de-esterified HGs from the middle lamellae (K. Vaughn and A. Bowling, unpublished manuscript), indicating this is a general mechanism for not just abscission but many if not all cell–cell separation processes in plants.

Comparison with the results of Sexton on Impatiens and with other AZ systems—Sexton and colleagues (Sexton, 1976, 1979; Sexton et al., 1977) described in very clear terms the series of events that occurred during the induction of abscission in *Impatiens* leaves. From their experiments, they concluded that abscission was confined to a change in just the middle lamellae of the cells of the AZ. In that region, the cells remained intact, but the middle lamellae appeared to be lost across the entire AZ surface on both distal and proximal surfaces. The only exception to this rule was the xylem elements, which appeared to be fractured roughly along the plane at which the cells of the AZ had separated. Xylem elements on the proximal side of the AZ developed tyloses that would aid in slowing the passage of water to the soon-to-be detached organ. Although the antibodies to cell wall components were not available at the time of the earlier Sexton studies, Sexton et al. (1984) later used the periodic acid-thiocarbohydrazide-silver proteinate (PATAg) technique to selectively stain the vicinyl OH groups of polysaccharides such as cellulose and pectin. These workers observed a loss of all of the middle lamella-reactive polysaccharides with relatively no alteration in the wall itself. What we have observed here with the same system not only confirms the results of Sexton and colleagues, but also identifies that the abscission event

appears to be solely the result of the loss of highly de-esterified HGs that occur predominantly in the middle lamellae. No other polysaccharides, or at least ones to which we have probed (Table 1), are changed as a consequence of abscission.

Our results also correlate well with studies done in several different plant species, using a wide variety of techniques. Using an immunocytochemical technique similar to ours, only the JIM5-reactive polysaccharides present in the middle lamellae were specifically degraded during abscission in the very small AZ of *Azolla* (Uheda and Nakamura, 2000). Although these workers did not use any other antibodies (except JIM7) to determine that this was the exclusive change in these cell types, their result with JIM5 appears to be the same as for the leaf AZ of *Impatiens*. Henderson et al. (2001) reported a higher concentration of HGs and an inducible polygalacturonase (PGase) in the abscission zone of oil palm. Their conclusion was that the oil palm AZ is enriched in HG to facilitate its degradation by PGase upon induction of abscission. Recent work on the abscission of leaves in tomato has shown that polygalacturonases play a significant role in abscission in this plant as well, because knocking out PGase activity caused a delay in abscission and an increase in the break strength of the AZ (Jiang et al., 2008). Conversely, overexpression of polygalacturonase in apple trees caused a reduced break-strength and premature abscission of leaves (Atkinson et al., 2002). Furthermore, a significant increase in the amount of PGase mRNA was seen after 24 h in the leaf AZ of oilseed rape (González-Carranza et al., 2002). There is also evidence to suggest a role for expansins in abscission (Cho and Cosgrove, 2000; Belfield et al., 2005). We have observed expansin in xylem elements of *Impatiens* and other systems (K. Vaughn, unpublished data), which may indicate a role in the wall loosening of vascular tissue during the abscission process.

When the floral AZ of poinsettia was monitored with anti-polysaccharide antibodies, much as we described here, a totally different pattern of changes was observed (Lee et al., 2008). There was an increase in xylans and xyloglucans (perhaps in cells also enriched in lignin) and alterations to pectins in cells both proximal and distal to the abscission zone. In particular, arabinan (LM6) and galactan (LM5) side chains of RGI were lost during abscission. The loss of these two RGI side-chains has previously been reported in fruit softening (Pena and Carpita, 2004) and in the softening of cells in Virginia creeper adhesive discs (Bowling and Vaughn, 2008a). Surprisingly, we found no change in any RGI-related epitopes in the AZ of *Impatiens*. Furthermore, we did not detect any change in the level of xylan or xyloglucans, nor did the cells away from the AZ appear to change in any way. Thus, it would appear that the abscission of leaves in *Impatiens* (herein) and *Azolla* (Uheda and Nakamura 2000) is a distinctly different event than the floral abscission in poinsettia.

The floral AZ of poinsettia arises de novo just prior to abscission, and the whole process of AZ formation and abscission takes 5–8 d (Lee et al., 2008). The difference in abscission events seen in poinsettia might also be due to the presence of much woodier tissues in a plant that persists for more than one season as opposed to the fleshy, seasonal tissue of *Impatiens*. It appears that *Azolla* and *Impatiens* may represent a simple type of AZ, where a predeveloped AZ exists and breaks upon a single cell surface with only the highly de-esterified pectins of the middle lamellae being lost during the process. Alternatively, it may be that the events observed by Lee et al. (2008) were due to the fact that they analyzed the floral AZ, while the *Azolla* and *Impatiens* studies were conducted on the leaf AZ. There have been reports that abscission in leaves, flowers, and fruits may proceed by different pathways. In the peach tree, for example, cellulase was detected strongly in the leaf abscission zone, while PGase was the predominant enzyme secreted into the fruit AZ (Bonghi et al., 1992). Future work will be required to determine whether the mechanism by which flowers, and fruit abscise differs from that in leaves.

In conclusion, we have found that the process of leaf abscission in *Impatiens* depends almost solely upon the breakdown (loss) of the highly de-esterified HGs in the middle lamellae from cells along the single surface of the separation layer. No other polysaccharides (of the ones we have monitored) appear to be markedly affected in either the cells of the abscission zone or in cells proximal and distal to the AZ. The only exception to this process are xylem cells, which appeared to be sheared-off at a plane near to that of the separation layer, and there is correspondingly less precision in the separation of this layer than in the other AZ cells (Andre et al., 1999), even though there does appear to be a layer of axially shortened xylem/phloem elements at the AZ plane.

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