The Mid-Pericarp Cell Layer in Soybean Pod Walls Is a Multicellular Compartment Enriched in Specific Lipoxygenase Isoforms[1]

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Specific lipoxygenase isoforms immunolocalize to the cytosol of a single cell layer in the soybean (*Glycine max* L.) pod wall. The cells of this layer, termed the mid-pericarp layer (MPL), are larger than adjacent cells and are highly branched. The entire MPL appears to form an elaborate interdigitated network within the pod wall. A particularly striking feature of the MPL is the presence of extensive regions of very thin, approximately 30 nm, cell wall, which connect the cells of the MPL. It was demonstrated that after mechanical wounding of the pod wall, 40-kD fluorescein-dextran was able to move throughout the MPL. In addition, when pod walls are cut, an exudate flows from the MPL that is highly enriched in lipoxygenase isoforms (approximately 40% of the total protein). The MPL of soybean pod walls may represent a novel multicellular compartment involved in defense of leguminous plants.

The soybean (*Glycine max* L.) pod is a simple, dry dehiscent fruit developed from a single pistil (Esau, 1977). The pericarp (pod wall) of soybean encloses and protects the developing seed, contains photosynthetic machinery to sustain itself and the seeds (Crookston et al., 1974), accumulates nutrients as a sink tissue that are later remobilized during seed development (Thorne, 1979; Staswick, 1989; Grimes et al., 1993; Dubbs and Grimes, 2000), and functions in seed dispersal via the process of pod shatter (Tiwari and Bhatia, 1995). Specific structures of the pod wall are related to these functions and can be studied by examining its anatomical, developmental, and biochemical properties.

The mid-pericarp layer (MPL) is marked by the presence of specific lipoxygenase (LOX) isoforms, VLX (vegetative LOX)A, VLXB, and VLXC in the cytosol of the MPL cells (Dubbs and Grimes, 2000). This layer lies in a plane parallel with the pod surface between the dorsal and ventral sutures and spans the length of the pod wall immediately outside the bundle sheath of minor veins. It does not fully circumnavigate the pod wall, but ceases at the lower cleft formed by the bundle cap of the dorsal and ventral sutures. The specific localization of VLXA, VLXB, and VLXC to the MPL and their apparent segregation from the major localization of the vegetative storage proteins (VSP), VSPα and VLXD, (Dubbs and Grimes, 2000) suggest a novel function(s) for this tissue not involving protein storage. In this report we further examine anatomical, developmental, and biochemical characteristics of the MPL.

RESULTS

Characterization of a Unique Cell Layer in Soybean Pod Walls

Immunolocalization using isoform-specific antibodies to VLXA, VLXB, and VLXC identifies a discrete single cell layer in the soybean pod wall. We term this layer, which runs the length of the pod wall within the mesocarp between the dorsal and ventral sutures, the MPL (Dubbs and Grimes, 2000). The MPL cells are typically more elongated and contain less than half the number of chloroplasts found in bordering mesocarp cells (Fig. 1A). Paradermal sections further reveal the MPL structure is distinct (Fig. 1B) in that the MPL cells are large and highly branched. Individual MPL cells appear to form tight fitting interconnections with other MPL cells using interdigitating arms.

A striking feature of the MPL cells is the variable thickness of their cell walls. Between the individual cells of the MPL are extensive regions where the cell wall is quite thin (approximately 30 nm; Fig. 2, A and B). These thin cell wall regions often appear extended, frequently bulging into the adjacent cell (Fig. 2C). Furthermore, these thin cell walls are occasionally found broken, allowing the cellular contents between adjacent MPL cells to mix (Fig. 2D).

The ontogeny of the MPL was examined using a developmental series, which included pre-anthesis and early postanthesis pods (Fig. 3, A–F). Staining with Stevenel's Blue/Safranin-O reveals a preferential staining of MPL vacuolar material (Fig. 3). This

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staining technique allows visualization of the cells destined to mature into MPL cells in pod walls as young as 2 d pre-anthesis (Fig. 3B). The ontological development of these MPL cells follows a distinct progression with the differential vacuole staining becoming most evident at 8 DPA (Fig. 3F). At this stage (8 DPA) pods are approximately 10% their final size and thin cell wall regions are evident (data not shown).

Dye Uptake Experiments Indicate That the MPL Is a Multicellular Compartment

Individual MPL cells are separated by cell walls that are unusually thin (approximately 30 nm) in distinct regions (Fig. 2A). Electron microscopy suggests that these thin cell wall regions are fragile and that the cell contents of adjacent cells may be able to mix (Fig. 2D). Furthermore, when a pod wall is mechanically wounded by cutting across the pod, an exudate forms at the cut surface that appears to emanate from the MPL (data not shown). It seems likely that these regions of thin cell wall may rupture

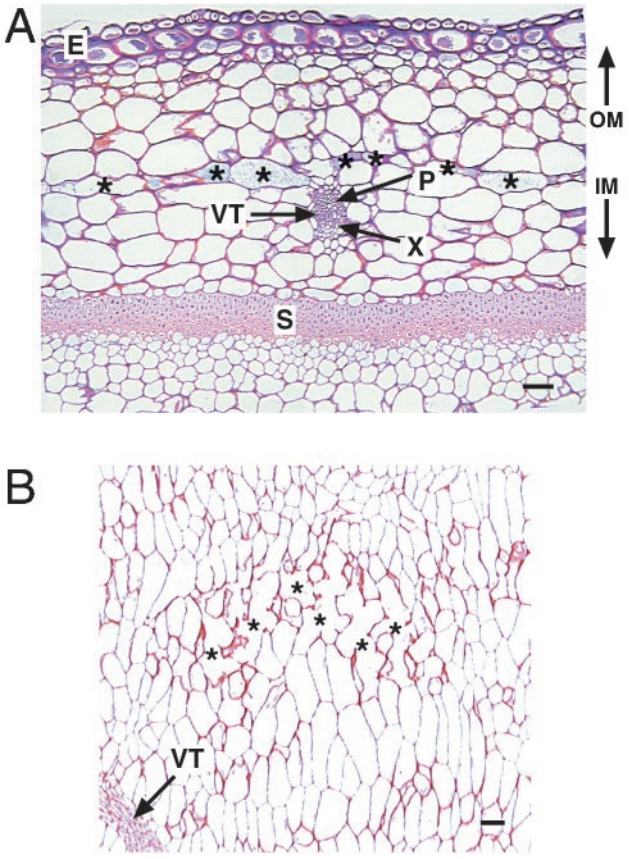

Figure 1. Anatomical structure of the MPL. A, Light micrograph of a cross-section through a pod wall 3 weeks after anthesis. B, Light micrograph of a paradermal section through a pod wall 3 weeks after anthesis. The asterisk marks cells in the MPL. OM, Outer mesocarp; IM, inner mesocarp; P, phloem; X, xylem; VT, vascular tissue. Bar = 250 μm.

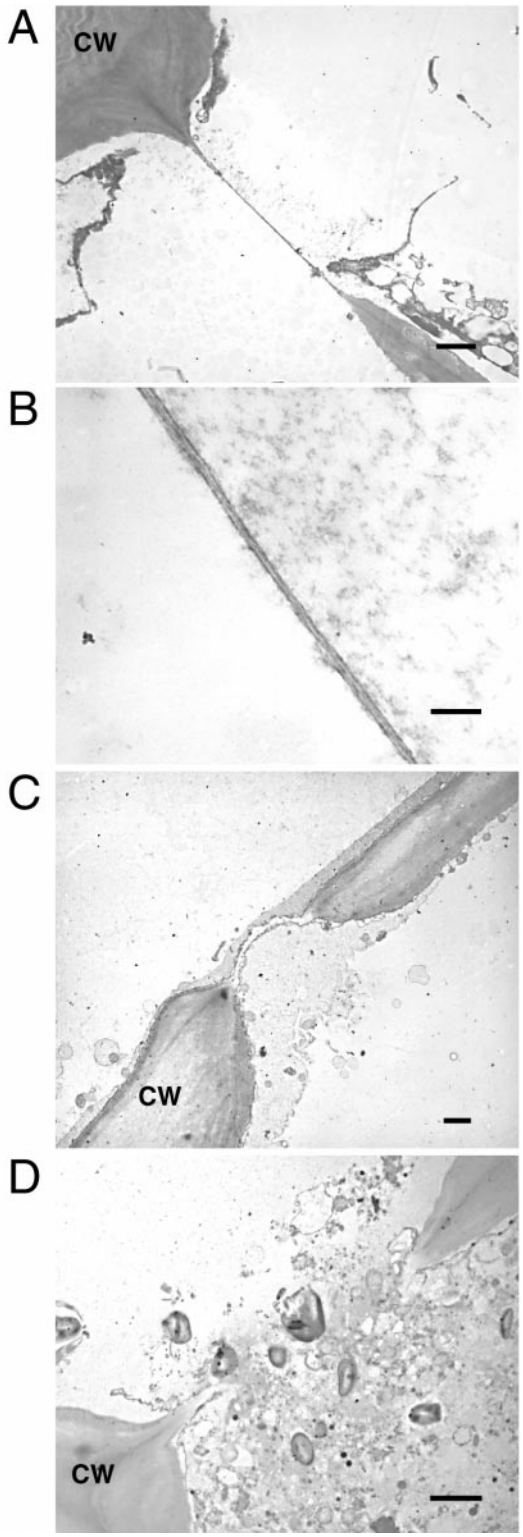

Figure 2. Cells of the MPL are connected by extensive lengths of thin (25–50 nm) cell wall. A, Transmission electron microscopy (TEM) showing a region of thin cell wall between two adjacent MPL cells. Bar = 2 μm. B, TEM showing a region of the thin wall in A at higher magnification. Bar = 200 nm. C, TEM of one of these thin cell wall regions bulging into an adjacent MPL cell. Bar = 2 μm. D, TEM showing a rupture of one of the thin cell wall regions and the mixing of cellular contents. Bar = 2 μm. CW, Cell wall.

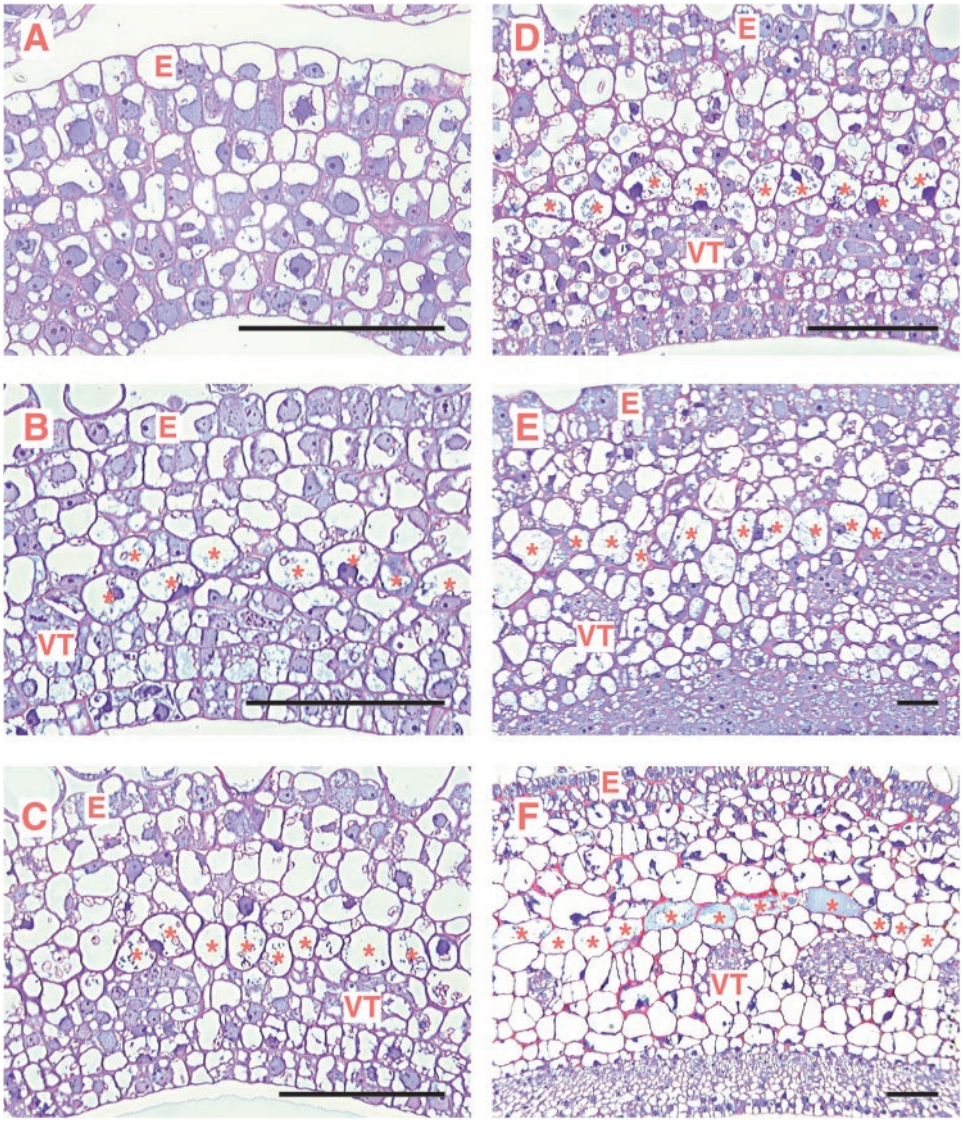

Figure 3. Ontogeny of the MPL. A developmental series of the soybean carpel/pericarp is shown with the sections stained by Stevenel's Blue/Safranin-O. This staining technique allows visualization of putative phenolic compounds that appear to accumulate in the MPL cells. A, Four days pre-anthesis. B, Two days pre-anthesis. C, Anthesis. D, Two DPA. E, Four DPA. F, Eight DPA. Note the preferential blue staining in the MPL cells. This staining pattern was followed in reverse chronological order to assist in the identification of cells in the process of differentiating into MPL cells. In all cases, the bar = 50 μm. E, Epidermis; VT, vascular tissue.

under specific conditions, resulting in the formation of a multicellular compartment.

To test this, dye uptake experiments were performed by administering F-dextran (40 kD) to freshly cut pod walls for 20 min. The large M_r of this F-dextran precludes the possibility of cell-to-cell movement via plasmodesmata. Figure 4A clearly shows that the majority of the 40-kD F-dextran is associated with the MPL and that the dye traverses several MPL cells. Confocal microscopy confirms that the F-dextran is interior to the MPL cells and is confined to the MPL layer (Fig. 4B). A small amount of the F-dextran non-specifically associates with the air spaces between the loose cells of the endocarp middle zone and tracheary elements. These experiments indicate that the MPL may function as a multicellular compartment. This multicellular compartment may exist in the absence of a wound, but clearly exists after this mechanical damage occurs.

To determine if any plasmodesmata connections exist between the MPL and surrounding cells, which would allow small molecules to move cell-to-cell, transverse uptake experiments were performed using PTS (8-hydroxy-1,3,6-pyrenetrisulfonate; 538 D). The PTS dye was found to distribute identically to the 40-kD F-dextran (data not shown). Even after 1 h, PTS did not move out of the MPL into adjacent mesocarp cells, establishing that either very few plas-

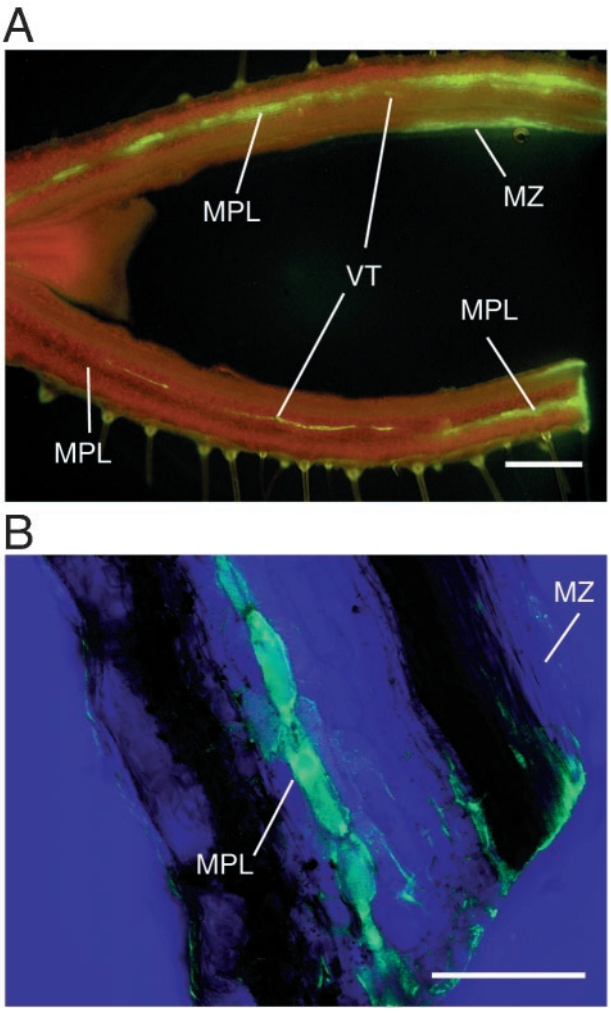

Figure 4. F-dextran (40 kD) moves throughout MPL cells after mechanical damage. A 3-week-old pod wall was cut at the middle locule and the cut end immersed into buffer containing 40-kD F-dextran and incubated for 20 min. A, Micrograph of the pod cross-section. Note the green fluorescence throughout the MPL region demonstrating that this 40-kD dye moved through numerous cells of the MPL. Bar = 500 μm. B, Confocal micrograph of individual optical sections totaling 2 μm of the pod wall. Note the strict localization of F-dextran to the interior of the MPL cells after the area where the cut was made. Bar = 200 μm. MPL, Mid-pericarp layer; MZ, endocarp middle zone; VT, vascular tissue.

modesmata are found in the MPL cells or that these plasmodesmata are blocked. Transmission electron microscopy revealed no obvious pit fields or plasmodesmata in the cells of the MPL or between the MPL cells and adjacent mesocarp cells. Additionally, callose staining of fresh, free-hand sections with aniline blue showed that only a few plasmodesmata were associated with the cells in the MPL region (data not shown).

The data characterizing the MPL regions with thin cell walls (Fig. 2) and the F-dextran uptake (Fig. 4) were done with 3-week-old (+21 DPA) pod walls. Ontological characterization, however, suggests that the MPL layer is defined anatomically 6 to 8 DPA. To determine if this apparent anatomical maturity correlated with the ability of the MPL cell walls to rupture upon mechanical wounding, a series of 40-kD F-dextran uptake experiments were performed with pod walls of different ages. Pods were harvested at 6 and 7 DPA, cut, and incubated in 40-kD F-dextran to test whether the MPL in these pods were able to uptake the F-dextran. At 6 DPA, very little or no F-dextran uptake was observed in the MPL (Fig. 5A). Note here, however, that the MPL appears dark. This is due to the low amount of chlorophyll autofluorescence, which corresponds to the low number of chloroplasts in the MPL. At 7 DPA, however, the F-dextran was able to move throughout this cell layer (Fig. 5B, note the F-dextran staining in the MPL at the bottom of the cross-section, as well). The ability of F-dextran to move through the MPL wound-induced multicellular compartment remained consistent for several weeks (a representative experiment is shown in Fig. 5C). However, by 63 DPA (9 weeks), when the pods were beginning to senesce, only a small amount of F-dextran was observed in the MPL (Fig. 5D).

Lipoxygenases Are Enriched in the MPL Exudate

When fresh pods are cut, an exudate forms that appears to emanate from the MPL. Since immunolocalization indicates that VLXA, VLXB, and VLXC are preferentially associated with the cytosol of the MPL cells (Dubbs and Grimes, 2000), it was determined if mechanical wounding of pod walls would release significant quantities of LOX in the MPL exudate. Exudate was collected from cut pods and subjected to biochemical analysis via SDS-PAGE and LOX assays.

Coomassie Brilliant Blue staining of total soluble proteins from pod wall exudate, total pod walls, and a leaf (for comparison) indicate that mechanically wounded pod walls exude a significant amount of LOX (Fig. 6). Immunoblotting further shows that this exudate is enriched in VLXA, VLXB, and VLXC (Fig. 6), although other proteins are present in this pod wall exudate. The other proteins, such as VSPα, found in this exudate are probably either present in the MPL in lower quantities or they originate from other cells immediately adjacent to the wound site.

The data suggest that mechanical wounding of soybean pod walls results in the exudation of MPL contents, including lipoxygenases, from this multicellular compartment. To verify that the wound exudate contains active lipoxygenases, LOX activity was assayed in this exudate and compared to the LOX activity in extracts of whole pod walls. The specific activity of LOX in whole pod walls, using linolenic acid as a substrate, peaks at approximately 175 nkat/mg protein at approximately pH 6 (Fig. 7A). In comparison, however, LOX-specific activity from the wound exudate peaks at approximately 700 nkat/mg

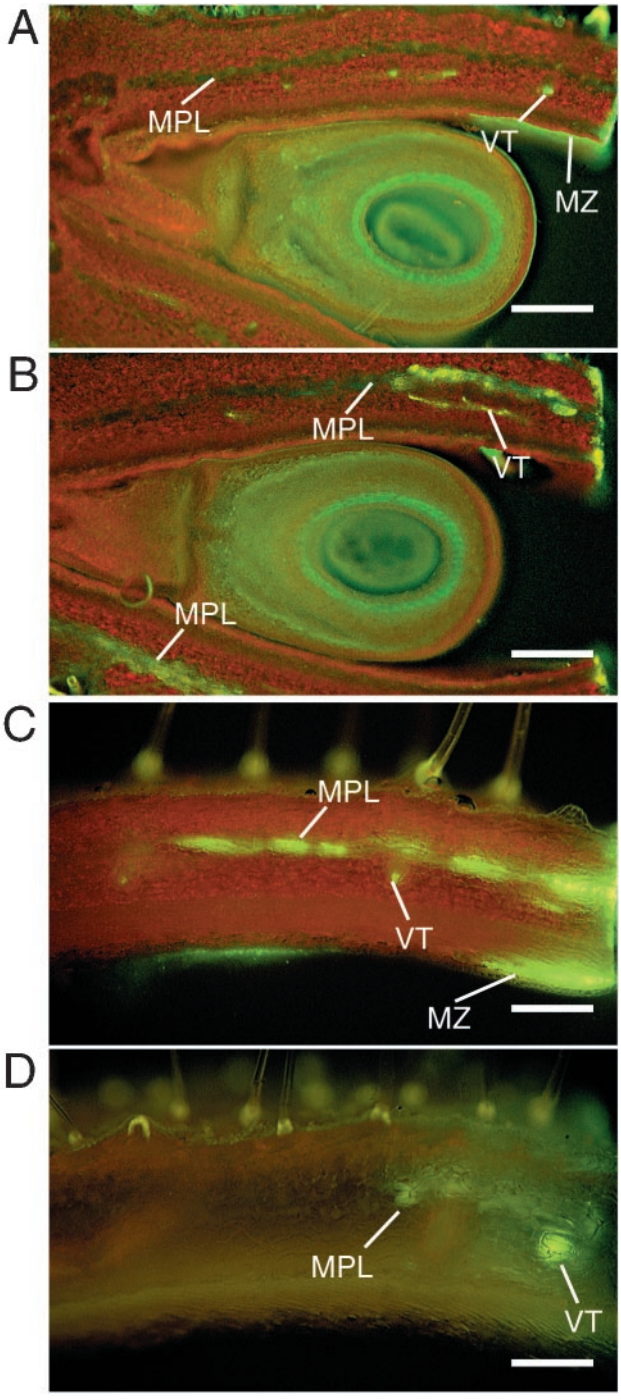

Figure 5. Assessment of the developmental stage in which the MPL cells become competent to move 40-kD F-dextran after mechanical wounding. Pods were harvested at 6, 8, 21, and 63 DPA, cut, incubated in 40-kD F-dextran for 20 min, and then analyzed using fluorescent microscopy. A, Six DPA. Note absence of green F-dextran staining in the MPL region. Only slight fluorescence is evident in vascular tissues. B, Eight DPA. Some fluorescence is evident in a few MPL cells immediately adjacent to the wound site, but dye movement is restricted after approximately 500 μm. C, Twenty-one DPA. F-dextran is competent to move throughout the MPL after mechanical wounding. D, Sixty-three DPA. F-dextran movement is severely restricted. At this stage, pods are senescing. Bar = 250 μm. MPL, Mid-pericarp layer; MZ, endocarp middle zone; VT, vascular tissue.

protein (Fig. 7B). LOX activity is thus approximately 4-fold higher in the wound exudate.

DISCUSSION

In soybean pod walls, specific LOX isoforms (VLXA, VLXB, and VLXC) preferentially localize to the cytosol of a single cell layer that we term the MPL (Dubbs and Grimes, 2000). Eiben and Slusarenko (1994), using an antiserum that probably detects multiple LOX isoforms, showed a preferential association of LOX with a cell layer located in the middle of the

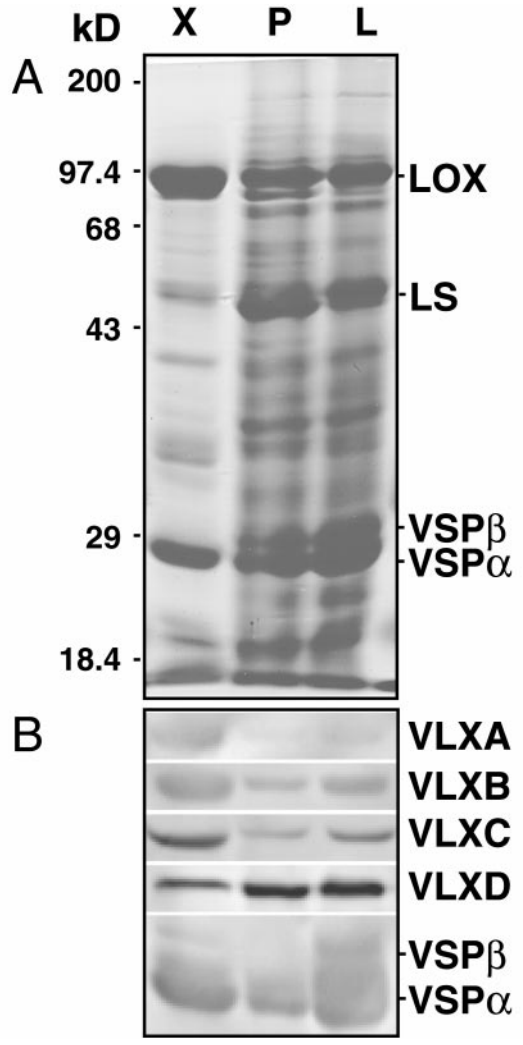

Figure 6. Characterization of protein and VLX composition in MPL exudate versus pod walls and leaves. Exudate was collected from cut pod walls (X) and compared to proteins extracted from whole pod walls (P) and a leaf from a plant subjected to daily pod removal for 5 weeks (L). A, Proteins from these three sources after SDS-PAGE separation and Coomassie Brilliant Blue staining. Note the high amount of LOX present in this exudate. B, Proteins from these same sources after SDS-PAGE and immunoblotted with antisera specific for the indicated VLX isoforms and VSPα. Note the enrichment of VLXA, VLXB, and VLXC in the exudate, whereas VLXD is enriched in whole pods.

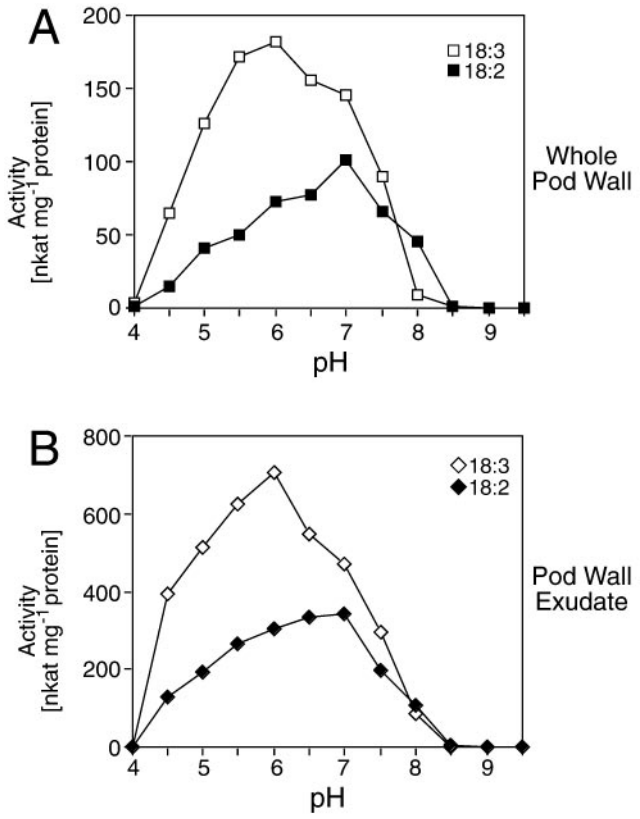

Figure 7. Biochemical characterization of lipoxygenases present in pod wall extracts and MPL exudates. A, pH profile of LOX activity extracted from whole pod walls using both 18:3 (□) and 18:2 (■) as substrates. B, pH profile of LOX activity from MPL exudate.

Phaseolus vulgaris pericarp. Thus, the MPL may be associated with various leguminous plants and, since specific LOX isoforms are associated with this cell layer, its function may be related to the biochemical activity of these LOXs.

In addition to the fact that specific LOX isoforms are present in the cytosol of MPL cells, three other features distinguish this cell layer. First, in paradermal sections the cells of the MPL of soybean can be seen as being larger and more branched than cells in the surrounding mesocarp. The MPL cells thus form an elaborate network of cells with tight fitting interconnections. Second, the cell walls between MPL cells have extensive regions where the cell wall is only approximately 30 nm thick. Electron microscopic examination of these regions of thin cell wall indicated that they can be compromised or ruptured, allowing intermixing of the cellular contents. Third, there are few, if any, plasmodesmata connecting the cells of the MPL. The MPL thus serves as a symplastic barrier between regions of the pod wall. These apparently unique features of the MPL imply a novel function for the MPL separate from that of other mesocarp tissues.

Several possible functions might be postulated for this cell layer. The MPL is positionally reminiscent of the leaf paraveinal mesophyll cell layer and shows similar immunolocalization of VLXA, VLXB, and VLXC in the cytosol (Stephenson et al., 1998; Fischer et al., 1999; Dubbs and Grimes, 2000). Because of its apparent similarity to the paraveinal mesophyll cell layer in soybean leaves, this cell layer might serve a storage function. However, VLXD, the major soybean LOX isoform involved in storage, is not associated with the MPL and most of the VSPα is associated with the exocarp. Hence, a storage role for the MPL seems unlikely. The regions of thin cell wall between MPL cells could represent points of physical weakness that might aid during pod shattering. The oppositely elongated sclerified cells in the endocarp and the hypodermis are believed to result in unequal shrinkage during pod wall senescence, causing pod walls to shatter along the two sclerified bundle caps (Fahn and Zohary, 1955; Tiwari and Bhatia, 1995). However, these sclerified tissues develop after pod elongation (Esau, 1977), whereas the MPL differentiates very early in pod development and prior to these sclerified tissues. Thus, the MPL does not appear to play a singular or major role in pod shatter.

The MPL does enclose the developing seeds and may be involved with their protection during development. Release of MPL cellular contents, including high levels of LOX, at the point of wounding may both flush and seal the wound site similar to resin release (Steele et al., 1998), which would prevent the intrusion of surface microbes. LOXs and their products have been implicated in wound response and in response to pest attack in a number of different species (Creelman et al., 1992; Grimes et al., 1992; Croft et al., 1993; Fenton et al., 1994; Bunker et al., 1995; Saravitz and Siedow, 1995, 1996). Hatanaka et al. (1992) suggested that LOXs in tomato fruit exist in a latent form, separate from their substrate, and are only activated upon injury when they acquire access to their substrates. The early development of the MPL, its persistent susceptibility to mechanical damage, the presence of active LOXs within MPL cells, and its existence as a multicellular compartment after wounding support a role for the MPL in plant defense.

MATERIALS AND METHODS

Plant Material

Soybean plants (*Glycine max* L. Merrill cv Wye) were grown as outlined in Dubbs and Grimes (2000). Early developmental stages of pods were determined using the protocol of Peterson et al. (1992).

Light and Transmission Electron Microscopy

Approximately 1-mm-thick sections were made of the pod wall approximately 3 mm down from and including the dorsal suture. Tissue was fixed for 2 d on a rotator in 1.25% (v/v) glutaraldehyde plus 2% (v/v) paraformalde-

hyde in 50 mM PIPES (1,4-piperazinediethanesulfonic acid) buffer (pH 7.2). Tissue was washed three times over 1 h in buffer alone, then dehydrated and infiltrated with L.R. White resin in a Pelco 3450 Lab Microwave Processor (Ted Pella, Redding, CA), following the procedure of Giberson and Demaree (1995) and Giberson et al. (1997). Alternatively, tissue was post-fixed in 2% OsO_4 overnight, and then processed as above into Spurrs resin.

Sectioning was done using a microtome (Ultracut-R, Reichert, Cambridge Instruments, Nussloch bei Heidelber, Germany). Thick sections (600-nm) were allowed to dry on uncoated microscope slides then stained with 0.5% (w/v) Safranin-O for 1 min or stained with undiluted Stevenel's Blue for 1 min at 60°C, then rinsed and counter-stained with 0.5% Safranin-O for 1 min. Images were obtained using an Ortholux microscope (Leitz, Midland, Ontario) with a digital camera (DXC-5000, Sony, Tokyo). Thin (100-nm) sections were made and placed on nickel grids, then stained for 3 min in 2% (w/v) uranyl acetate:1% (w/v) potassium permanganate (2:1), rinsed, and examined in an electron microscope (JEM-1200ex, JOEL, Tokyo). Paradermal sections were made of the pod wall in the plane of the MPL. Confirmation that the paradermal sections contained the MPL was made by VLXA, VLXB, and VLXC antibody localization as described previously (Dubbs and Grimes, 2000).

Protein Procedures

Pod wall exudate from 3- to 5-week-old pods was collected by making a single cross-cut through the compressed area distal from the middle seed. The cut surface was quickly blotted on filter paper to reduce contamination from cells at the point of cutting. Exudate was collected with a capillary tube as it formed on the cut surface and was transferred into a microcentrifuge tube containing 25 mM Tricine (*N*-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl] Gly; pH 7.5), 1% (w/v) insoluble polyvinylpolypyrrolidone, 1 mM EDTA, 10 mM β-mercaptoethanol, 10 μM leupeptin, 1 μM pepstatin, and 0.57 mM phenylmethylsulfonyl fluoride. Samples were centrifuged for 10 min at 15,000*g* in a 4°C microcentrifuge. Protein concentration of the supernatant was assayed with protein assay reagent (Bio-Rad, Hercules, CA; standardized using bovine serum albumin). Aliquots of these extracts were stored at −80°C until analysis of LOX activities. For SDS-PAGE, extracts were mixed (1:1, v/v) with 2× Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min. Total soluble protein extract from lyophilized whole pod walls was performed using the method outlined in Dubbs and Grimes (2000). SDS-PAGE, electroblotting, immunoblotting, protein quantification, and LOX assays were performed as previously described (Fischer et al., 1999; Dubbs and Grimes, 2000).

Fluorescent Dye Uptake

Fluorescein-dextran (40 kD; Molecular Probes, Eugene, OR) and PTS (538 D; Sigma, St. Louis) were used at 1 mg/mL perfusion media (14 mM MES [2-(*N*-morpholino)-ethanesulfonic acid]-KOH, 2 mM K phosphate [pH 6.8], and 1.3 mM $CaCl_2$, adjusted to 300 mOsm with polyethylene glycol according to Wang and Fisher [1994]). Fresh weights of whole pods were determined for each sampling period. The center locule was isolated by making cross-cuts through the two adjacent compressed regions. The ventral one-half of the locule was separated from the dorsal one-half by a lateral cut. The cut surfaces of the ventral side were blotted onto filter paper and a small amount of fluorescent dye was pipetted onto the longitudinal cut. The tissue was then placed, longitudinal side down, inside a covered Petri plate at 100% relative humidity. After 20 min the piece of tissue was rinsed in running tap water for 1 min, then free-hand cross-sections were made and mounted on a microscope slide in perfusion media (300 mOsm). Early postanthesis pods were collected daily and tested to ascertain the earliest age of competency for dye uptake. Following this determination, pods of progressing developmental stages were sampled at weekly intervals to determine the age at which dye uptake diminished. Fluorescent dye uptake was monitored using a microscope (Ortholux II, Leitz) with epi-illumination and imaged using a digital camera (DXC-5000, Sony). Tissue specificity and subcellular localization of the fluorescent dye was determined using an inverted microscope (Eclipse TE300, Nikon, Tokyo) as part of a MRC1024 Confocal Scanning Microscope system (Bio-Rad). Detection of dye was done using a single fluorochrome method and the image analysis software (Laser Sharp 3.2, Bio-Rad).

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