Structure and Histochemistry of the Micropylar and Chalazal Regions of the Perisperm–endosperm Envelope of Cucumber Seeds Associated with Solute Permeability and Germination

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ABSTRACT. The perisperm–endosperm (PE) envelope surrounding the embryo of cucumber (*Cucumis sativus*) acts as a barrier to apoplastic permeability and radicle emergence. The envelope consists of a single cell layer of endosperm whose outer surface is covered by noncellular lipid and callose-rich layers. We compared the structure and histochemistry of the radicle tip and chalazal regions of the envelope, because these regions differ in permeability. Seeds were treated with coumarin 151, a nonionic, fluorescent tracer with systemic activity. Treated seeds were imbibed and on seedcoat removal, the root tip area of the membrane-covered embryo accumulated the fluorescent tracer, but the tracer could not penetrate the envelope that bordered the cotyledons and chalazal region. The cone-shaped remnant of tissue opposite the micropylar region of the envelope was identified as nucellar tissue, the “nucellar beak.” The cuticular membrane and callose layer of the PE envelope were interrupted in the nucellar beak as well as in the chalazal region. Their role in permeability is apparently substituted by the presence of thick-walled suberized cells in the beak and chalaza. A canal was observed in the center of the nucellar beak that likely provided a conduit for the tracer to diffuse from the environment to the embryo. This canal was the remnant of pollen tube entry through the nucellus and was plugged with several cells, presumably residue of the suspensor. These cells degenerated just before cucumber seed germination. This remnant of the pollen tube canal presumably offers less mechanical resistance in the nucellar beak that might help facilitate radicle protrusion during germination. Cells of the outermost and basal regions of the nucellar beak as well as the walls of endosperm cells contained pectic material. Significant pectin methyltransferase activity was found in the lateral and cap regions of the PE envelope long before seed germination. Lack of callose in the envelope at the radicle tip suggests that callose does not act as a barrier to radicle emergence during cucumber seed germination.

Cucumber is a species whose seeds have a semipermeable barrier restricting transport of solutes. A thin membrane beneath the testa, the perisperm–endosperm (PE) envelope, acts as a barrier to apoplastic permeability of cucumber and other Cucurbitaceae seeds (Ramakrishna and Amritphale, 2005; Yim and Bradford, 1998). This envelope is reported to consist of a single cell layer of endosperm whose outer surface is covered by lipid and callose layers (Yim and Bradford, 1998). The PE envelope of seeds of the genus *Cucumis* limits transport of tetrazolium salts, abscisic acid (Ramakrishna and Amritphale, 2005), and prevents solute leakage from dead seeds (Welbaum and Bradford, 1990; Yim and Bradford, 1998). We have established that the cucumber seed envelope is impermeable to the nonionic, moderately lipophilic, fluorescent tracer, 7-amino-4-(trifluoromethyl) coumarin (coumarin 151) (Salanenka and Taylor, 2008), except in the region that covers the radicle tip area (Fig. 1A). Tao and Khan (1974) observed the permeation of chemicals into this radicle tip area in *Cucurbita maxima* seeds, although they did not study its causal factor. Tao and Khan (1974) suggested that the anatomical structure of the tip region of the seed might relate to enhanced permeability. Although the structure of the PE envelope was studied

(Ramakrishna and Amritphale, 2005; Welbaum and Bradford, 1990; Yim and Bradford, 1998), attention was not paid to particular regions of the PE envelope. This article focuses on the micropylar and chalazal regions of the PE envelope in cucumber seed. These two regions are positioned closely to one another in the fully anatropous ovule (Tillman, 1906), and these regions provide specialized functions in Cucurbitaceae seeds. The chalazal region of the PE envelope, located opposite the micropyle, is involved in transport of nutrients from maternal tissue into the developing embryo during seed development. The micropylar region acts as a barrier for radicle emergence during germination and is studied in many species such as tomato (*Solanum lycopersicum*) (Groot and Karssen, 1987; Nonogaki and Morohashi, 1996), lettuce (*Lactuca sativa*) (Ikuma and Thimann, 1963), pepper (*Capsicum annuum*) (Watkins et al., 1985), and tobacco (*Nicotiana tabacum*) (Leubner-Metzger et al., 1995, 1996). In seeds of Cucurbitaceae, mannan-rich endosperm and a callose layer of the PE envelope are considered to form the primary barrier to radicle emergence during germination. Weakening of the mannan-rich endosperm and callose in the micropylar region is necessary for radicle protrusion through the PE envelope (Ramakrishna and Amritphale, 2005; Welbaum et al., 1995, 1998).

In the present work, we focus on the structure and histochemistry of the micropylar and chalazal regions of the PE envelope of cucumber seed in relation to solute permeability and germination.

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Materials and Methods

Plant material

Mature seeds of 'Vlaspik' cucumber were obtained from Seminis, Inc. (Oxnard, CA) and were stored in plastic containers at 4 °C until needed. For embryological studies, seeds were collected from cucumber fruit at different stages of fruit development. Seeds were removed from fruit and washed vigorously in water to remove residual mucilaginous fruit tissue. Excess water was removed, and seeds were fixed for microscopy as described subsequently.

Permeability test and gross anatomical observations

Agarose was added to deionized water saturated with fluorescent tracer (coumarin 151) and microwaved. The solution (0.6% w/v) was poured into 9-cm petri dishes and cooled at room temperature. Seed coats were carefully removed, embryos immersed in the solidified agarose gel, and incubated at 25 °C for 12 h to study perfusion of tracer through the PE envelope. Seeds imbibed in the agarose gel were sectioned and examined under low magnification ($\times 3$ and $\times 10$) with a long-wavelength ultraviolet radiation source (365 nm). For gross seed anatomy, seeds were decoated and observed without further preparations with a stereomicroscope (SZX12; Olympus, Center Valley, PA) equipped with a SPOT Insight camera and software (Version 4.5; SPOT Imaging Solutions, Sterling Heights, MI).

Tissue preparation and fixation for microscopy

Mature dry seeds were fixed in a chromic acid–acetic acid–formalin mixture (CRAF III) and subsequently dehydrated and cleared in an ethanol–tertiary butanol (TBA) series as described by Ruzin (1999). Briefly, seeds selected for soundness and uniformity were fixed in CRAF III solution at room temperature for 12 h. The fixative was then rinsed in running water overnight. After dehydration in a graded ethanol–TBA series, followed by infiltration by absolute TBA, the specimens were embedded in a paraffin mixture (Tissue Prep; Fisher Scientific, Fair Lawn, NJ). Tissue sections (7 μ m) then were cut with a steel knife on a rotary microtome. Thereafter, tissue sections were processed for microscopic observations based on specific needs. Fresh seeds collected from developing cucumber fruit were fixed in a solution of 5% formaldehyde–10% glacial acetic acid–50% ethanol for 24 h and then embedded in paraffin as described previously.

Histochemical analysis

All specimens were examined using a microscope (BX60; Olympus) under brightfield or epifluorescence (exciter filter BP 360 to 370 nm, dichroic mirror 400 nm, barrier filter 420 nm for ultraviolet excitation; exciter filter BP 436 nm, dichroic mirror 460 nm, barrier filter 470 nm for blue–violet excitation) and images were recorded with an attached digital camera (Micro-publisher Camera and QCapture software; QImaging, Surrey, British Columbia, Canada). Fresh or fixed tissue was processed for a variety of histochemical tests. In most cases, sections were deparaffinized before stain application, except for toluidine blue O, for which paraffin-embedded tissue sections were first stained with dye before paraffin removal.

LIGNIN. A saturated phloroglucinol solution in 20% (v/v) HCl was first filtered to remove crystals and then applied to

sections. Sections in staining solution were mounted on glass slides, covered with coverslips, and then observed in visible radiation. Red–violet color indicated lignin (Jensen, 1962).

LIPIDS. Saturated solutions of either Sudan black B or a Sudan III–IV mixture in 70% (v/v) ethanol were applied to tissue sections and incubated up to 30 min. Cutin and suberin give a red–orange color with Sudan III–IV and a blue–black color with Sudan black B (Krishnamurthy, 1999). For fluorescent microscopy of lipids, neutral red (Lulai and Morgan, 1992), rhodamine B, and auramine O (Gahan, 1984) were applied to sections. Sections were stained with a 0.1% solution of neutral red in 0.1 M potassium phosphate, pH 6.5, for 1 min, rinsed briefly in buffer, and observed under blue–violet excitation. Other sections were covered with a 0.1% (w/v) rhodamine B aqueous solution for 30 min and then mounted with a coverslip using distilled water. Specimens were observed under ultraviolet excitation. Auramine O (0.01% solution) prepared in 0.05 M Tris–HCl buffer, pH 7.2, was applied to sections for 10 min, coverslipped, and viewed with blue–violet excitation.

TANNIN. A drop of saturated alcoholic vanillin was placed on fresh hand-cut sections, and several drops of concentrated HCl were added. Immediate development of a bright red color indicated the presence of tannins (Gardner, 1975).

NONESTERIFIED PECTIN. Ruthenium red, Alcian blue 8GX, and toluidine blue O were used to identify nonesterified pectin and other acidic polysaccharides. Sections were flooded with a 0.05% aqueous solution of ruthenium red for 20 min and observed for red color (Jensen, 1962). A 1% Alcian blue 8GX solution in 3% acetic acid (pH 2.5) was applied to tissue sections for 20 min, rinsed with water, and the sections were examined for blue color under visible radiation (Clark, 1981; Lev and Spicer, 1964). For metachromatic staining, a 0.05% toluidine blue O solution, prepared in citrate–phosphate buffer (pH 4.4), was applied to sections for 30 s before paraffin removal from sections (Sakai, 1973). Slides were rinsed in water for ≈ 2 min and air-dried. Paraffin was then removed with xylene and sections were examined for red–purple coloration, indicating acidic polysaccharides. Polyphenols were stained green–blue or dark blue.

CELLULOSE. Sections were stained with a 0.1% (w/v) aqueous solution of calcofluor white M2R for 1 min and observed under ultraviolet excitation for white–blue fluorescence (Hughes and McCully, 1975).

CALLOSE. Callose staining was performed as described by Currier and Strugger (1956). Sections were placed in 0.01% (w/v) aniline blue WS in 0.15 M potassium phosphate buffer, pH 8.2, for 30 min and examined under ultraviolet radiation. Aniline blue bound to callose has blue–white fluorescence.

SUBERIN AND PHENOLICS. A 0.1% (w/v) aqueous solution of berberin hydrochloride was applied to sections. After 20-min incubation, sections were mounted in a 0.5% (w/v) solution of crystal violet for 30 s and briefly rinsed. Sections were examined by epifluorescence microscopy with blue–violet excitation (Vaughn and Lulai, 1991).

DIFFERENTIATION OF CUTIN FROM SUBERIN. To determine whether cutin or suberin is included within the lateral region of the inner envelope of cucumber seeds, we used alkaline hydrolysis combined with chemical staining as described by Beresniewicz et al. (1995) with minor modifications. Isolated PE envelopes were boiled in 1 M NaOH for 30 min followed by an additional 10 min of boiling in chloroform–methanol (1:1 mixture). Samples were then embedded in paraffin and

sectioned as previously described. The sections were stained with phloroglucinol, berberine–crystal violet, and Sudan III–IV.

PECTIN METHYLESTERASE ACTIVITY ASSAY. To evaluate activity of pectin methylesterase (PME) in PE envelopes and radicle tips of cucumber seeds, PME was extracted with high-salt buffer (1 M NaCl), and a gel-diffusion assay for PME activity was done as described in Downie et al. (1998) with some modifications. A mixture containing 1% (w/v) of agarose in 0.1 M citrate/0.2 M dibasic phosphate buffer, pH 6.0, and 0.1% of pectin from citrus fruit (90% esterified; Sigma-Aldrich, St. Louis, MO) was boiled until agarose was completely dissolved. The solution was cooled to 50 °C. NaN₃ was added as an antimicrobial agent. The solution (8 mL) was poured into a petri dish (60 × 15 mm) and then solidified at room temperature. Wells, 2 mm in diameter, were made in the gel with segments of plastic pipette tips. PE envelopes, PE caps, or radicle tips (5 mm length) were isolated from cucumber seeds that had imbibed for 3 h or for 13 h on blotter paper moistened with deionized water. One hundred milligrams of each of the three isolated tissues were ground in liquid nitrogen in a mortar with a pestle, and then 2 mL of extraction buffer was added (1 M NaCl, 2.5 mM phenylmethylsulfonyl fluoride, 0.1 M citrate/0.2 M dibasic sodium phosphate, pH 6.0). The homogenate was centrifuged at 14000 g_n for 10 min at 4 °C and 5 µL of the collected supernatant was loaded into each well. The loaded dishes were covered with lids and incubated at 37 °C for 16 h. After incubation, the gel was stained with 0.05% (w/v) ruthenium red for 1 h and then rinsed in water. Diameters were measured of purple-stained zones radiating from each well that reflected the relative activity of the PME of each sample.

Results

Tracer diffusion through the perisperm–endosperm envelope

Coumarin 151 did not penetrate the region of the PE envelope that covered the cotyledons, including the chalazal region of cucumber seeds, after 12 h immersion in agarose gel containing the fluorescent dye. However, the fluorescent tracer did stain the embryo root tip, indicating that the tracer penetrated through the micropylar region of the PE envelope (Fig. 1A).

Gross morphology of the embryo and perisperm–endosperm envelope

The root tip of the embryo and the PE envelope covering the tip are subjacent to the micropyle of the developing ovule. This tip area of the PE envelope, hereafter referred to as the micropylar region, contained a conical tissue remnant (Fig. 1B) such as was observed in *Cucumis melo* seeds and described as unknown by Welbaum et al. (1995). In this study, the end of the ovule, seed, or PE envelope opposite from the root tip of the embryo was referred to as the chalaza region or chalaza zone (Fig. 1C).

The structure and histochemistry of the perisperm–endosperm micropylar region

The conical structure consisted of an outer zone of large thick-walled palisade-shaped cells, a cluster of small cells in the basal region that surrounded a squat central column of cells, and an intermediate filling tissue. The lateral region of this cone gradually diminishes in thickness to become the lateral PE envelope (Fig. 1D). Because of cone position and likely inclusion of nucellar tissue, we termed this cone the “nucellar beak.” A histochemical analysis of the various tissue regions

of the nucellar beak is presented in Table 1. The outer region is constructed of dead cells whose contents stained red with ruthenium red, purple with toluidine blue O, and blue with Alcian blue 8GX, all indicating presence of carboxylated polysaccharides (e.g., mucilage, pectin) (Fig. 1D). Filling tissue contains cells that are more rectangular and that lack mucilage. Small, polygonal-shaped cells of the basal region have walls that stain dark blue with toluidine blue O, pH 4.4, indicating polyphenols (Fig. 1D). These cells surround large squat cells, likely a suspensor remnant that plugs a canal in the center of the filling tissue of the nucellar beak (Fig. 1E). The cell walls of both filling tissue and the outer region had an affinity for lipid indicators (Sudan black B, Sudan III–IV, neutral red). These same walls also stained pink with phloroglucinol/HCl, fluoresced yellow after staining with berberine/crystal violet (not shown), and had strong autofluorescence (Fig. 1F), all suggesting presence of lignin and suberin.

The cells of the lateral region of the nucellar beak are more elongated and their outer tangential walls and the outermost portion of their radial walls contain lipids. These lipid-containing cells merge into those making up the lateral lipid membrane of the PE envelope and emit a yellow fluorescence with rhodamine B under ultraviolet excitation, whereas the interior, ligno-suberized cells of the nucellar beak had blue fluorescence (Fig. 1G). Therefore, rhodamine B allowed differentiation of cutin and suberin in ultraviolet radiation. The cutin layer in the lateral areas of the PE envelope ends in the basal zone of the nucellar beak (Fig. 1G). In the lateral part of the PE envelope, we observed white–blue fluorescence of aniline blue, indicating callose (Fig. 1H). Callose was not detected in the nucellar beak area and adjacent regions of the PE envelope. Instead, a faint brown coloration was observed in the nucellar beak and adjacent areas of the PE envelope (Fig. 1I–J).

Our embryological study established that the entire cone-shaped tissue remnant contains a beak-like part of the nucellus. Such a nucellar beak is found in developing seeds of many species of Cucurbitaceae (Tillman, 1906). We found that the cells of the nucellar beak were already ligno-suberized at the globular stage of embryo development (Fig. 1K–L). Based on staining with berberine/crystal violet, lignification of cell walls was stronger in the central part of the cap (Fig. 1L). As found in mature seeds, in developing seeds, the cuticle was visible in the lateral areas of the nucellus and ended in the basal zone of the nucellar beak (Fig. 1M).

The structure and histochemistry of the perisperm–endosperm chalazal region

Median longitudinal sections through the chalazal region of the PE envelope revealed the presence of large polygonal-shaped cells surmounted by a remnant of a vascular bundle. The cell walls of the chalaza stain a dark blue–black with Sudan black B (Fig. 2A) and pink–red with Sudan III–IV, all indicating presence of lipophilic material, presumably suberin. The walls of chalaza cells are autofluorescent under ultraviolet radiation and give a positive reaction with phloroglucinol, thus indicating lignin (Fig. 2B–C). Figures 2D through G show a series of longitudinal sections sequentially cut through the chalazal region. Off-center tangential sections cut through the basal part of the chalaza showed no, or only a few, chalaza cells in that region, whereas the callose layer was thick and covered with continuous cuticle located beneath the vascular bundle (Fig. 2D–E). Sections through the middle region of the chalazal

Fig. 1. (Continued)

Table 1. Histochemical reactions of outer, lateral, and basal tissue regions of the micropylar region of the perisperm–endosperm envelope in cucumber seeds.^z

Histochemical stain	Specification	Outer region ^y		Lateral region ^y		Basal region ^y	
		Cell wall	Cell contents	Cell wall	Cell contents	Cell wall	Cell contents
Visible radiation							
Toluidine blue O	Polycarboxylic acid: purple	–	–	–	–	–	+
	Polyphenols: green, blue–green	+	–	+	–	+	–
Ruthenium red	Polyanions (pectin): red	+	+	–	–	–	–
Alcian blue	Acidic mucopolysaccharides	+	+	–	–	+	–
Phloroglucinol-HCl	Lignin: red, pink	+	–	–	–	–	–
Alcoholic vanillin	Tannins: red	–	–	–	–	–	–
Sudan III–IV	Total lipids: red	+	–	+	–	–	–
Sudan black B	Suberin, cutin: blue–black	+	–	+	–	–	–
Aniline blue	Callose	–	–	–	–	–	–
Epifluorescence							
Autofluorescence		+	–	–	–	+	–
Calcofluor W2R	Cellulose	+/–	–	–	–	–	–
Neutral red	Lipids	+	–	+	–	–	–
Auramine O	Lipids	+	–	+	–	+	–
Berberin/crystal violet	Phenolics, suberin	+	–	+	–	+	–
Rhodamine B	Suberin: blue	+	–	–	–	–	–
	Cutin: yellow	–	–	+	–	–	–
	Other: red	–	–	–	–	+	–

^z+ = Positive staining; – = negative staining.

^yOuter region (OR), lateral region (LR), and basal region (BR) as shown in Figure 1D.

zone showed numerous layers of suberized chalaza cells, whereas cuticle and callose layers were interrupted in this region (Fig. 2F–G, arrowheads). Sections at the globular embryo stage of seed development did not reveal lignin or suberin in chalaza cell walls (not illustrated). However, cell walls in the chalazal zone gave a positive reaction with Sudan black B at the torpedo stage of embryo development, indicating suberin (Fig. 2H). Cell walls stained with Sudan black B also were examined by fluorescence microscopy that revealed significant lignin deposition at this stage (Fig. 2I). Sudan black B quenches autofluorescence of suberin.

Chemical nature of the lipid layer of the perisperm–endosperm envelope

According to the method described by Beresniewicz et al. (1995), cutin staining is lost after alkaline hydrolysis, but

suberin remains strongly stained with Sudan dyes. After alkaline hydrolysis, suberin exposes its lignin-like component and thus can be stained with phloroglucinol/HCl and berberine/crystal violet. After alkaline hydrolysis, we observed no positive staining of the lipid layer of the PE envelope on staining with Sudan dyes, phloroglucinol, or berberine/crystal violet (not illustrated).

Pectin in the perisperm–endosperm envelope and pectin methylesterase activity

Although this study mostly focused on distribution of callose and lipid layers in the PE envelope, we also examined additional histochemical features of the endospermic and nucellar tissues to further our understanding of cucumber seed germination. Cell walls of the endospermic tissue adjacent to the cotyledons and to the nucellar beak reacted positively with

Fig. 1. (A–C) Gross morphology of mature cucumber seeds. (A) Seed at the left was treated with the fluorescent tracer (coumarin 151), imbibed, and the perisperm–endosperm (PE) envelope removed to expose the surface of the embryo. The cotyledon tips (CT) lie opposite the chalaza zone of the seed; the radicle tip (RT) lies opposite the micropyle of the seed. Note fluorescent tracer penetrated the removed PE envelope only in the root tip area (arrowhead). Seed at the right was not treated with tracer. (B) Root tip area of the PE envelope in a decoated cucumber seed showing a tissue remnant located in the micropylar region (MR). (C) The chalazal zone (CZ) of the PE envelope at the end of the seed opposite from the micropyle. (D–J) Anatomy and histochemistry of tissues associated with the micropylar region of the PE envelope of dry mature seeds. (D) Longitudinal section through the tip of the envelope, stained with toluidine blue O, pH 4.4, viewed with visible radiation. The conical construction may be described as the nucellar beak (NB). Contents of the large palisade-shaped cells of the beak's outer region (OR) stained purple, indicating carboxylated polysaccharides. Filling tissue (FT) lacked carboxylated polysaccharides. Small polygonal-shaped cells of the basal region (BR) stained dark blue, indicating polyphenols, but the lateral region (LR) lacked stain (SR = suspensor remnant, EM = embryo). (E) Longitudinal section of the nucellar beak (NB) shows a remnant of the suspensor (SR) and pollen tube canal (C). Stained with auramine O and examined under ultraviolet excitation. (F) Cross-section of the nucellar beak viewed with ultraviolet excitation shows the remnant of the pollen tube canal (C) and autofluorescent suberized cell walls (SCW). (G) The cuticle (CU) of the PE envelope disappears (at arrowheads) in the nucellar beak (NB). Section stained with rhodamine B and examined with ultraviolet excitation. (H–J) Aniline blue fluorescence of the callose layer of the PE envelope observed with ultraviolet excitation. (H) White–blue fluorescence of callose (CAL) deposition in the lateral areas of the PE envelope. (I) The same specimen as in G but after aniline blue application. Note lack of fluorescence in the PE envelope adjacent to the micropylar region, although faint brown coloration (BC) is visible. (J) Similar lack of fluorescence in the micropylar region of the PE envelope but showing strong brown coloration (BC) at the base of the nucellar beak (NB). (K–M) Anatomy and histochemistry of the micropylar region at the globular stage of embryo development. (K) Suberized cell walls (SCW) of the nucellar beak (NB) stained blue–black with Sudan black B, seen in transmitted radiation. (L) The lignified cell walls (LCW) of the nucellar beak (NB) stained with berberin–crystal violet and observed by epifluorescence under blue–violet excitation. (M) The cuticle (CU) of the nucellus ends (arrowheads) at the base of the nucellar beak (NB). The cuticle, stained with rhodamine B, emits yellow fluorescence under ultraviolet radiation (GE = globular embryo).

Fig. 2. (Continued)

Alcian blue, ruthenium red, and toluidine blue O. This suggests the presence of acidic polysaccharides (pectins) that are likely accumulated in the middle lamella (Fig. 2J). Besides endosperm, positive staining for acidic polysaccharides with Alcian blue was observed in the cell walls of the basal region of the nucellar beak (Fig. 2K). In the dry mature seed before germination, the dead cells of this region were tightly compressed. After 13 h of seed imbibition, but before visible germination, the cells of this basal region appeared decompressed and expanded (Fig. 2L). Because pectin was present in the region of the PE envelope, and because pectin degradation may be critical in seed germination, this tissue was tested for activity of pectin-degrading enzymes. This test assessed PME activity in the radicle tip and PE envelope. Our gel diffusion assay showed that PME activity was present in the PE envelope but not in the radicle tips either after 3 or 13 h of cucumber seed imbibition. No substantial increase in PME activity in the PE envelopes or PE caps was detected after 13 h of seed imbibition.

Discussion

Seeds of cucumber and other species within the Cucurbitaceae have a semipermeable envelope surrounding the embryo. This study focused on the anatomy of the micropylar and chalazal regions of the envelope of cucumber seeds as a result of their differential permeability to a nonionic, systemic compound. Histochemical analysis suggests that the lipid layer of the cucumber PE envelope contains cutin rather than suberin. The lipid layer stained with auramine O was localized in the outer periclinal cell wall and in the outer anticlinal cell wall (as "pegs") of adjacent epidermal cells, a typical construction of plant cuticles. Considering that the lipid layer of the PE envelope is derived from the epidermis of the nucellus (Ramakrishna and Amritphale, 2005), the lipid layer of the envelope can be referred to as a nucellar cuticle. The cuticle extends throughout the length of the longitudinal section of the cucumber seed envelope, but it ends in the chalazal and micropylar regions. The disappearance of cuticle in the chalazal region was observed in seeds of *Malus domestica* (Forino et al., 2000), *Citrus paradisi* (Espelie et al., 1980), and *Hordeum vulgare* (Collins, 1918). Similar to the situation in *C. paradisi*, cells of the chalazal region of the cucumber PE envelope are suberized. Suberization of the chalazal region in *C. paradisi* apparently is required to isolate the embryo from the importation of nutrients through the vascular traces during the late stage of seed development (Espelie et al., 1980). The lack of suberization in the chalazal region during the globular

stage of cucumber seed development, when the embryo still needs a source of nutrition, conforms to this explanation.

Ligno-suberization occurs in the walls of cells within the tissue remnant in the micropylar region of the cucumber PE envelope. Such a tissue remnant was observed in melon (*C. melo*) seeds, but its origin was not determined (Welbaum et al., 1995). According to our embryological study and that of Singh (1955) for *C. melo*, this remnant is the long nucellar beak formed after development of the megaspore within the ovule. This beak extends outward toward the micropyle. Our results show that, unlike the case of the chalazal region, suberization of the micropylar end of the PE envelope had already occurred by the globular stage of embryo development. Ligno-suberization of the micropylar region apparently starts shortly after fertilization of the egg within the ovule, quite possibly triggered by cell degradation during pollen tube growth. The nucellar beak is not covered by a cuticle in the micropylar end of the envelope. Such discontinuity of cuticle at the micropylar end was reported for the ovules of corn (*Zea mays*) (Heslop-Harrison et al., 1999), barley (*H. vulgare*) (Collins, 1918), sugar beet (*Beta vulgaris*) (Bruun and Olesen, 1989), and peach (*Prunus persica*) (Arbeloa and Herrero, 1991). We also did not detect a callose layer in the micropylar region, and the callose layer in the chalazal region was interrupted. Therefore, cell wall suberization, and not callose or cuticle deposition, is responsible for the permeability characteristics of the PE envelope in the chalazal and micropylar regions.

A canal located along the longitudinal axis of the suberized nucellar beak may play a significant role in the permeability of the micropylar region of the PE envelope. The canal was identified as the pollen tube entry canal, formed when the pollen tube enters the micropyle and crushes the cells in its path before fertilization (Singh, 1955). We observed a short, truncated suspensor at the globular stage of embryo development. The group of cells seen in the basal part of the micropylar region of mature seeds likely is the remnant of this suspensor. A suspensor remnant in the micropylar region was described by Singh (1955) for mature melon seeds, Collins (1918) for barley grain, and Serrato-Valenti et al. (2000) for *Phacelia tanacetifolia*. The persistence of the suspensor in mature seeds is rare among numerous other species and its presence or function is not well understood. The suspensor usually degenerates during the later period of embryo development in angiosperms (Yeung and Meinke, 1993). Any metabolic activity of suspensor remnants in mature seeds remains to be identified. Interestingly, we noted that the suspensor degrades before germination of cucumber seeds. We did not investigate whether the degradation of the suspensor is caused by mechanical obliteration

Fig. 2. (A–G) Anatomy and histochemistry of the tissues associated with the chalazal region of the perisperm–endosperm (PE) envelope of dry, mature cucumber seeds. (A) Median section through the cotyledons and thick chalazal zone (CZ) of the envelope, stained with Sudan black B, indicating presence of suberin (COT = cotyledon, VB = vascular bundle). (B) Median section of the chalazal zone (CZ) stained with rhodamine B shows bright blue fluorescence of ligno-suberized walls of interior cells. The cuticle (CU) and callose (CAL) layers in the lateral PE envelope end in the suberized chalazal zone (VB = vascular bundle). (C) Lignified cell walls (LCW) of the chalazal zone stained red with acidified phloroglucinol (CAL = unstained callose layer, VB = vascular bundle). (D–G) Series of sections through the chalazal region (CZ) of the PE envelope shows gradual cuticle (CU) disappearance, from off-center sections in D to sections of the thickened CZ in G. Arrowheads show cuticle and callose (CAL) interruption (COT = cotyledons, EN = endosperm, VB = vascular bundle). (H–I) Chalazal region at the torpedo stage of embryo development. (H) Section stained with Sudan black B indicating suberized cell walls (SCW). Observed under visible radiation. (I) Similar to H, but fluorescent under ultraviolet excitation, indicating lignified cell walls (LCW) (VB = vascular bundle). (J–K) Longitudinal sections through the nucellar beak (NB) region in the root tip area of decoated, dry mature seeds (J–K) and seeds imbibed for 13 h on moistened blotter paper before germination (L). (J) Section stained with ruthenium red. Red color of cell walls of endosperm (EN) indicates carboxylated polysaccharides. (K) Section stained with Alcian blue 8GX. The basal region (BR) of the nucellar beak (NB) is stained blue, indicating pectins (EN = endosperm layer). (L) Changes in the basal region (BR) of the nucellar beak (NB) of imbibed seeds, just before germination, included cell decompression and cell expansion (SR = degraded suspensor remnant). Section stained with the metachromatic dye toluidine blue O (EN = endosperm layer).

during radicle protrusion or by programmed cell death. Serrato-Valenti et al. (2000) hypothesized that the presence of the suspensor in mature seeds might create structural conditions that facilitate radicle protrusion through the embryo seed coverings during seed germination. Indeed, in cucumber seeds, both the remnant of the suspensor and the pollen tube canal in the nucellar beak could offer less mechanical resistance to radicle emergence through the micropylar region of the PE envelope during germination.

Our results illustrating permeability of the PE envelope in the radicle region to coumarin 151 appears as an anomaly, because no leakage occurs from nonviable seeds during imbibition. Imbibing nonviable mature muskmelon seeds resulted in extensive swelling that was attributed to the accumulation of osmotically active compounds from the dead embryo that was trapped by the PE envelope (Welbaum and Bradford, 1990). Thus, the PE envelope acts as a sac with complete integrity that is impermeable to solute diffusion. The same phenomenon was observed in lettuce: nonviable seeds imbibed 23% more water than did viable seeds with a concomitant increase in volume (Hill and Taylor, 1989). In the current study of cucumber, coumarin 151 perfused into the radicle region during imbibition. We examined other fluorescent tracers that were charged molecules or more hydrophilic in nature, and none of these compounds moved into the embryo (Salanenka and Taylor, unpublished data). Therefore, the osmotically active, water-soluble compounds would not leak from nonviable seeds, even in the radicle region, thus still maintaining integrity of the PE envelope. The selective permeability of the distal region of the canal may be the result of pectins, ligno-suberins, or the material causing the faint brown coloration that was not identified by our histochemical stains.

Enzymatic weakening of the envelope tissue in the micropylar region was discussed as a key factor required for seed germination of Cucurbitaceae species (Ramakrishna and Amritphale, 2005; Welbaum et al., 1998). For example, endo- β -mannanase was reported to be putatively involved in PE envelope weakening in seeds of Cucurbitaceae, particularly in the endosperm component (Ramakrishna and Amritphale, 2005; Welbaum et al., 1998). Endo- β -mannanase was hypothesized to break down mannans of walls of endosperm cells of cucumber and muskmelon seeds, thus facilitating radicle protrusion. Endo- β -(1,3)(1,4)-glucanase (Welbaum et al., 1998) and β -glucanase (Ramakrishna and Amritphale, 2005) activities were detected in muskmelon or in cucumber seed tissues. These enzymes were hypothesized to be involved in degradation of the callose layer and possibly in the weakening of the PE envelope. In contrast, we found no callose deposition in the micropylar region or in the adjacent area of the cucumber PE envelope. This indicates that callose is unlikely to serve as a barrier to radicle emergence through the cucumber seed envelope.

The walls of endosperm cells and cells of the basal region of the nucellar beak reacted positively with Alcian blue, ruthenium red, and toluidine blue O that suggests the presence of acidic polysaccharides, possibly pectins. Pectins are structural components of the primary cell wall and middle lamella in plants. It was reported that activity of pectolytic enzymes such as polygalacturonase and pectin methylesterase was coincident with germination of tomato and yellow cedar (*Chamaecyparis nootkatensis*) seeds (Downie et al., 1998; Ren and Kermode, 2000; Sitrit et al., 1999). We found pectin methylesterase activity in the PE envelopes of both dry and imbibed cucumber

seeds, but not in the radicle tissue. These results are similar to those reported by Ramakrishna and Amritphale (2005) on β -mannanase activity found within the PE envelopes of cucumber and melon seeds. Similar to β -mannanase, substantial PME activity was present exclusively in the PE envelopes long before radicle emergence; thus, it was not coincident with seed germination. Because PME activity was found in plant cells undergoing programmed cell death (Arunika et al., 2007), it is possible that PME activity in the PE envelope of cucumber seeds accompanies cell wall degradation during endosperm senescence. De-esterification of pectin by PME promotes the activities of other pectin degrading enzymes such as polygalacturonase and pectin lyase (Wakabayashi et al., 2000). Future work should focus on pectolytic activity of these enzymes to conclude whether pectin degradation is involved in the mechanism of PE envelope weakening. The enrichment of endospermic cell walls with pectin is noteworthy, because it may serve as a specific adaptation of the cucumber seed endosperm that provides protection and osmotic isolation of the embryo from the surrounding environment. Such pectin-rich walls are not unique to cucumber seeds. Pectin-rich walls were found in the semipermeable envelope of orchid seeds (*Sorbalia dichotoma*) (Prutsch et al., 2000). They suggested that pectin material with high matrix potential serves as a water sink, protecting the seed embryo against both osmotic stress during hydration and rapid desiccation.

In summary, we propose that ligno-suberization of cells, rather than callose or cuticle deposition, is responsible for permeability in the micropylar and chalazal regions of the PE envelope of cucumber seeds. Differences in permeability of these regions are determined by the presence of a canal or central channel in the micropylar region of the PE envelope that facilitates solute passage. The canal apparently provides a conduit for the fluorescent tracer to diffuse from the imbibing medium to the embryo. Pectin may be of sufficient amounts in the cell walls of the endosperm to provide additional osmotic isolation of the embryo or to serve as a moisture source that protects the embryo from desiccation. Pectin degradation may facilitate cucumber seed germination, because we found pronounced pectin methylesterase activity in cucumber PE envelopes. Further evaluation of other pectin-degrading enzymes is necessary before making a conclusion in regard to the involvement of pectin degradation in the putative weakening of the PE envelope of Cucurbitaceae seeds. We propose that the inherent structural organization of the micropylar region of the PE envelope contributes, at least partially, to the mechanism of cucumber seed germination.

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