Plant Signaling & Behavior 6:11 1638-1643; November 2011; ©2011 Landes Bioscience

Cellulose synthesis in two secondary cell wall processes in a single cell type

Venugopal Mendu,[1] Jozsef Stork,[1] Darby Harris[1,2] and Seth DeBolt[1,2,*]

[1]Department of Horticulture; [2]Plant Physiology; Biochemistry and Molecular Biology Program; University of Kentucky; Lexington, KY USA

Key words: cellulose biosynthesis, mucilage, embryogenesis, seed development, seed coat, radial cell wall, secondary cell wall biosynthesis, cell shape

Abbreviations: CSC, cellulose synthase complex; CESA, cellulose synthase-A; WT, wild type; SEM, scanning electron microscopy; DPA, days post anthesis; CEF, cellulose elemental microfibril; TC, terminal complex

Plant cells have a rigid cell wall that constrains internal turgor pressure yet extends in a regulated and organized manner to allow the cell to acquire shape. The primary load-bearing macromolecule of a plant cell wall is cellulose, which forms crystalline microfibrils that are organized with respect to a cell's function and shape requirements. A primary cell wall is deposited during expansion whereas secondary cell wall is synthesized post expansion during differentiation. A complex form of asymmetrical cellular differentiation occurs in Arabidopsis seed coat epidermal cells, where we have recently shown that two secondary cell wall processes occur that utilize different cellulose synthase (CESA) proteins. One process is to produce pectinaceous mucilage that expands upon hydration and the other is a radial wall thickening that reinforced the epidermal cell structure. Our data illustrate polarized specialization of CESA5 in facilitating mucilage attachment to the parent seed and CESA2, CESA5 and CESA9 in radial cell wall thickening and formation of the columella. Herein, we present a model for the complexity of cellulose biosynthesis in this highly differentiated cell type with further evidence supporting each cellulosic secondary cell wall process.

Directional growth in plant cells is mediated, in part, by the constraint imposed on the cells internal turgor pressure by a rigid yet extensible primary cell wall. The primary structural elements of the cell wall are paracrystalline chains of β-1,4 linked glucose molecules that create a cellulose elemental microfibril (CEF).[1] Cellulose biosynthesis is essential for plant growth and development as illustrated genetically[2] and pharmacologically.[3] Several lines of evidence suggest that cellulose synthesizing organisms produce microfibrils from plasma membrane localized cellulose synthesizing complexes, namely terminal complexes (TC) due to the attachment between CEF and freeze fracture rosettes in the plasma membrane.[4] In the Arabidopsis genome, 10 cellulose synthase (*CESA*) genes have been identified based on sequence similarity originally to bacterial cellulose synthases[2,5] and subsequently to RSW1 (CESA1).[6] A combination of biochemical and genetic analyses have shown that at least three CESA subunits directly interact to form a CSC in primary[7,8] and secondary (xylem) cell wall formation,[9] but added complexity, such as ancillary associated proteins[10,11] is expected. Indeed, the diverse range of cell shapes and complexity of cell differentiation and functional requirement in certain cell types suggests added complexity in CESA functionality may exist (**Fig. 1**), but this is poorly understood at the genetic level. By theory, combinations of specific CESAs may be active in different cell types at different times and may be active in the same cell type at different stages, serving different functions.

Functional specialization of CESAs has been examined. Our current understanding suggests that CESA1 is a necessary component of primary wall synthesis in most tissues because *rsw1-1*,[2] a temperature sensitive allele caused disappearance of TCs from the plasma membrane at restrictive temperature. *CESA3* is co-expressed with *CESA1*, and homozygous *cesa3* alleles are also male gametophytic lethal.[7] CESA6 is required for elongation of hypocotyl cells in etiolated seedlings.[12,13] During xylem development, all three secondary wall CESAs (*CESA4*, *CESA7*, *CESA8*) are expressed in the same cells at the same time.[9] The secondary radial wall of seed coat epidermal cells requires at least *CESA2, CESA5* and *CESA9*.[14,15] Secondary cell wall mucilage biosynthesis in seed coat epidermal cells requires *CESA5* for attachment to the parent seed.[15,16] Currently, function of CESA10, which based on sequence homology is most similar to CESA1 [6,17] is not known.

Evidence for discreet microfibrils in a single cell type has recently been postulated[18] and recently shown in differentiating xylem cells of Populus[19] and in Arabidopsis shoot trichomes.[20] In the case of xylem cells, authors identified two independent CSCs, providing evidence that different CSCs could produce structurally discrete microfibrils, capable of partitioning during cell wall synthesis.[19] Functional specialization of CESAs was

*Correspondence to: Seth DeBolt; Email: sdebo2@email.uky.edu
Submitted: 07/28/11; Accepted: 08/11/11
DOI: 10.4161/psb.6.11.17709

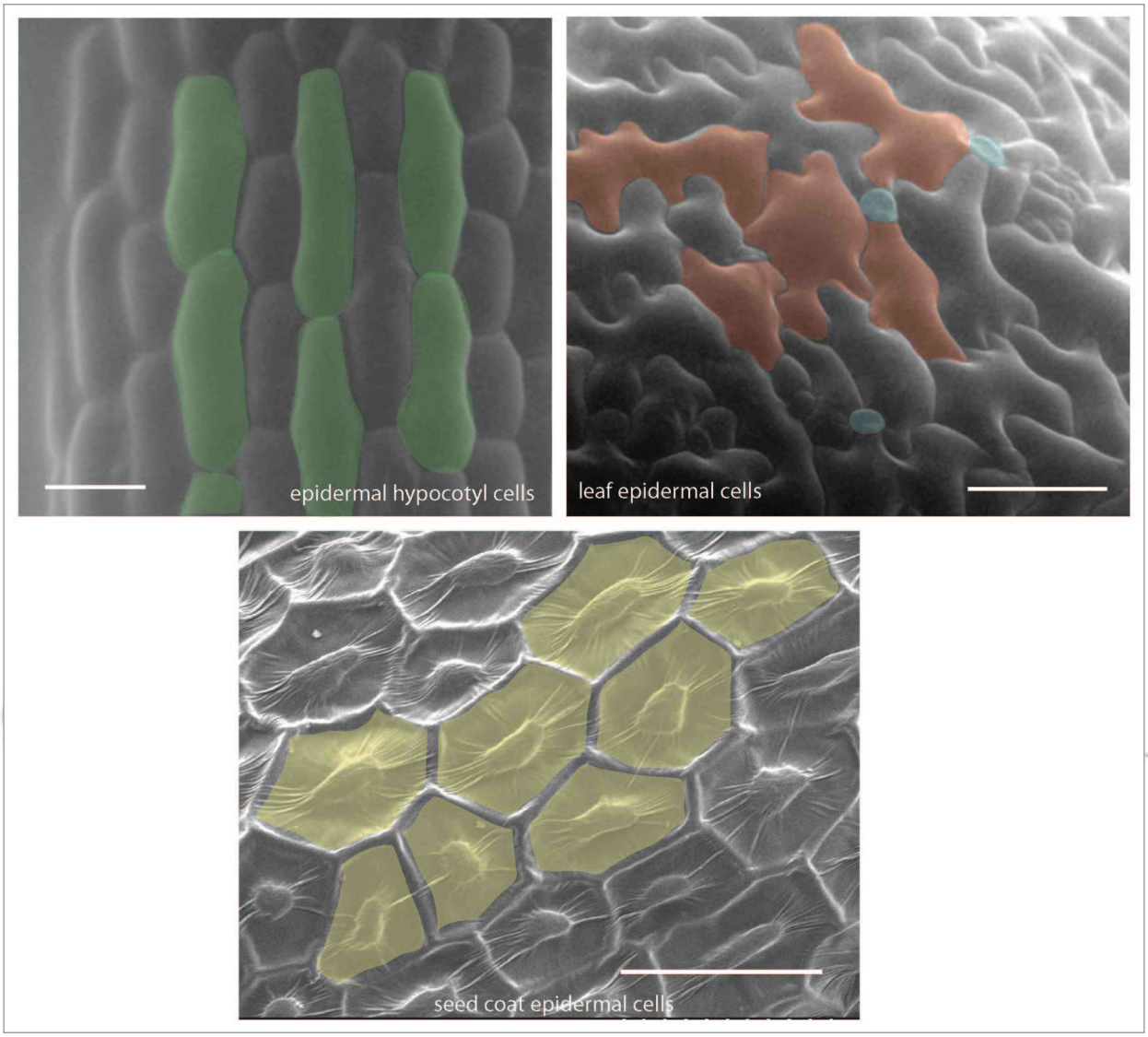

Figure 1. Scanning electron microscopy (SEM) micrographs showing variation in epidermal cell size and shape in different plant organs. Etiolated hypocotyls, leaves and seeds from Arabidopsis were sputter coated with gold-palladium using Hummer VI sputtering system (Anatech) and visualized using Hitachi-S-800 SEM. Scale bars = 30 μm.

recently explored using genetics in the epidermal seed coat,[14-16] which revealed that the distinct two secondary cell wall processes, mucilage synthesis and secondary cell wall reinforcement requiring specific CESAs spatially and temporally (7 vs. 9 d post-anthesis: DPA) (**Fig. 2**). Arabidopsis epidermal seed coat cells follow a complex developmental program.[21] After fertilization, the ovule outer integument cell layer differentiates into an asymmetrically structured cell type. Two of the main processes are thought to require cellulose biosynthesis.

First, the production of a donut-shaped apoplastic pocket filled with pectinaceous mucilage contains linear striations of cellulose.[22] Second, the formation of a thick secondary cell wall that covers the columella and extends to the radial boundary wall,[23] which is thought to assist in reinforcing the cell layer that must ultimately protect the embryo while withstanding the forces of dessication and hydration. Cellulose is thought to be a key component of both these secondary cell wall processes. CESA5 is essential for the pectinaceous mucilage halo to remain attached to the parent seed.[15,16] By laser scanning confocal microscopy of Calcofluor stained seeds from *cesa2-1, cesa5-1, cesa9-1, cesa2-1cesa9-1* and *cesa2-1cesa5-1cesa9-1* it is clearly visible that linear striations arising from the hydrated seed coat are largely intact, but the fluorescence arising from in between linear striations was greatly reduced in genetic background with loss of CESA5 (*cesa5-1* and *cesa2-1cesa5-1cesa9-1*) (**Fig. 3**). Three dimensional reconstructions of the triple mutant seed illustrate the lack of fluorescence between columella (**Fig. 3D** z-projection animation). By contrast, wild-type seed exposed to Calcofluor was so bright in three dimensional reconstructions (Data not shown) that it is most effectively illustrated from a cross sectional viewpoint (**Fig. 3**). Cross sections reveal disperse yet bright fluorescence arising between columella striations. We interpret this

as suggesting that cellulose is produced during mucilage production and integrates into the inner layer of mucilage,[15,16] where it mediates attachment. We propose that CESA5 is producing the fluorescence that is evident between columella in wild-type cross section based on Calcofluor fluorescence (**Figs. 2 and 3** for a schematic). If cellulose is integrating into the mucilage, it is plausible that we could detect variation in mucilage structure linked to cellulose using Fourier Transform Infrared spectroscopy (FTIR). To attempt test this hypothesis, generated spectra for wild-type and triple mutant mucilage and used this data to create a subtraction spectra documenting the differences between wild-type and triple mutant mucilage (**Fig. 4**). While qualitative in nature, FTIR spectroscopy has been used effectively to detect subtle changes in cell wall carbohydrate structures in mutants compared with wild type based on signature peaks.[24,25] Our observations identified several previously characterized peaks linked to crystalline cellulose as key differences between wild-type and triple mutant.[24,26] The peaks at wave numbers (WN) linked to cellulose that were different between wild type and triple mutant (via subtraction plot analysis) were 898, 930, 1,033, 1,058, 1,120 and 1,162 (cm^{-1}).[24] Within the FTIR subtraction plot, we could also identify different peaks between pectin (WN-989, 1,017, 1,122, 1,144 and 1,150) and xyloglucan (WN-1,078 and 945) [24] between the wild type and triple mutant spectra. These data, together with biochemical data[15] support histochemical analyses[27] and the mucilage adherence phenotype linked to CESA5 (**Fig. 4**).

Second, to explore the cellulose biosynthetic machinery in the secondary wall that reinforces the columella and radial wall, we examined T-DNA insertional alleles for individual, double and triple mutants for CESA genes.[15] While mutation in CESA6 (*procuste*) failed to cause aberration of cell shape or cause radial wall ridge reduction, CESA2, CESA5 and CESA9 cause suppression of radial wall integrity.[14,15] By examining and quantifying the radial wall height in sectioned seed coats of wild-type and mutant lines, we found an additive reduction in radial wall height thickening suggesting that at least CESA2, CESA5 and CESA9 are involved in secondary radial wall formation (**Fig. 2**).[15] The most severe allele among *cesa2-1, cesa5-1* and *cesa9-1* single mutants was *cesa9-1.* To examine this allele in greater detail, we excised whole seed from siliques at two developmental stages and imaged them via SEM (approximately 9–14 DPA and 18 DPA) (**Fig. 5**). Careful examination of micrographs from wild-type seeds excised approximately 9–14 DPA revealed that the secondary radial wall was forming as a circumference near the primary radial wall. Between neighboring cells, it was possible to distinguish a boundary ridge that remained partially fused to the thickening radial wall (**Fig. 5**). By 18 DPA, the radial wall appeared thicker and fused between neighboring cells and determinate ridges could not be distinguished. By contrast, the *cesa9-1* allele displayed a visually less pronounced radial wall (**Fig. 5**) at both 9–14 and 18 DPA. A notable observation was the visibility of the underlying amyloplasts in the *cesa9-1.* Consistent with past models[28-31] the secondary radial wall thickening encompasses the columella (**Fig. 2**) and based on this model, we predicted that the amyloplasts, which are typically present in the columella vicinity

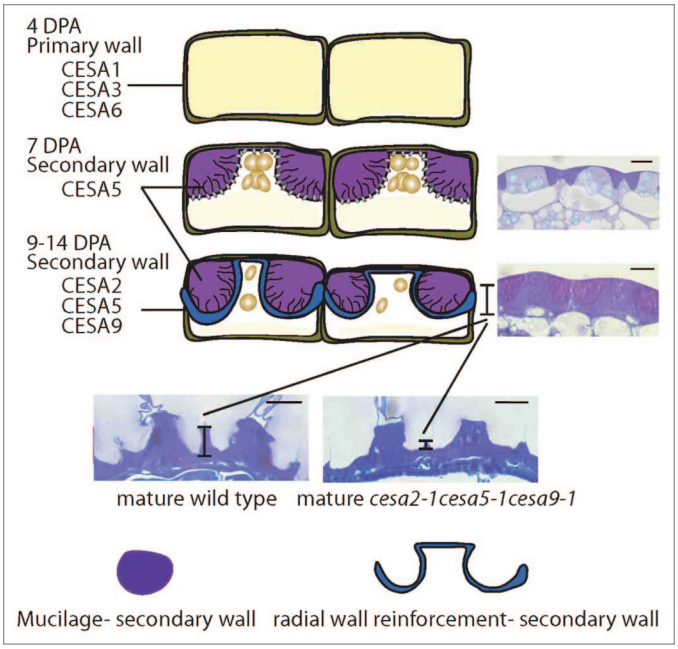

Figure 2. Schematic representation of secondary cell wall processes in the seed coat epidermal cells during seed development. During development seed coat epidermal cells undergo a complex developmental program formation leading to the formation of an asymmetrically differentiated seed coat cell. Two secondary cell wall processes occur. First, the mucilage biosynthesis and second, the formation of a thick secondary cell wall that covers the columella and extends to the primary radial wall. Our data[15] showed that at least three *CESA* genes (*CESA2, CESA5 and CESA9*) are necessary for the secondary cell wall processes. Triple mutant lacking these three *CESA*s showed a severe reduction or lack of radial wall formation and a defect in mucilage adherence to the parent seed.[15] Inset figures were obtained from original article Mendu et al. Scale bar = 10 μm.

during development, may be visible by SEM due to the thin outer tangential wall. Confirmation of this postulate further suggests that the cellulosic secondary cell wall encompasses the boundary radial wall and central columella (**Fig. 5**).[15,30] The reason this was not examined by section analysis[15] was that quantification of the radial wall was the most robust means to illustrate loss of secondary wall in mutant alleles, as columella naturally display shape variability.

Our data are consistent with the speculation that specific cell types may require functionally distinct cellulose deposition[18] and we question whether functional specification is a consequence of amino acid composition or transcriptional regulation. The developing seed coat represents an excellent system to dissect the composition of CSC in two separate secondary cell wall systems and an intriguing area for future exploration of mediating polarized deposition of cellulose. Moreover, while it has long been held that cellulose is the structural core of the plant extracellular matrix,[1,32] its association to adherence between the cell wall and parent cell has been complicated to examine. Our results illustrate that cellulose fulfills a matrix attachment role in cell wall biogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

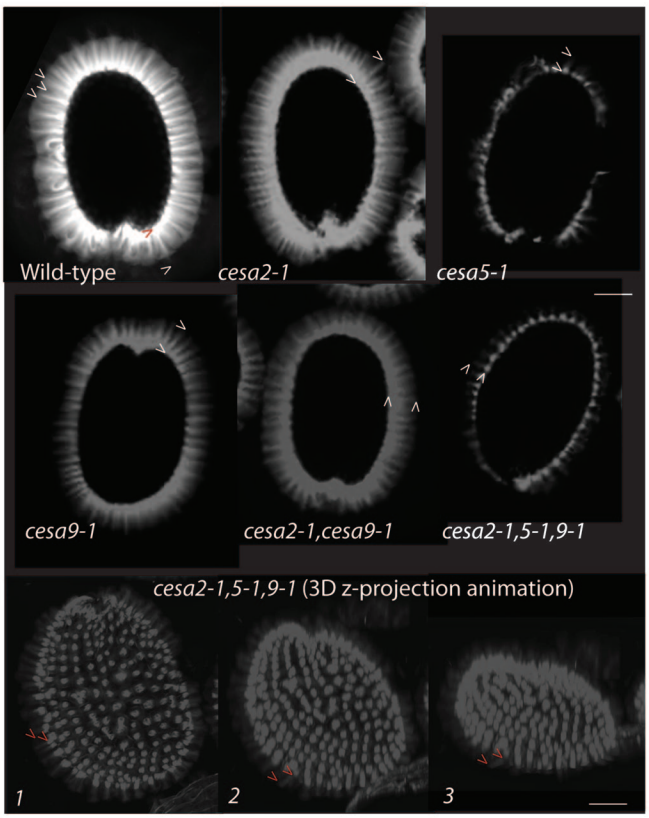

Figure 3. Absence of cellulose dependent florescence arising between columella striations in mutants involving *CESA5*. Analysis of Calcofluor white stained seeds showed lack of florescence between striations of fluorescence arising from regions coincident with columella in the mutant seeds involving CESA5 (*cesa5-1* and *cesa2-1cesa5-1cesa9-1*) compared with wild-type and other mutant seeds. The intensity and length of the florescence halo surrounding the seed was reduced in *cesa2-1, cesa9-1* and *cesa2-1cesa9-1* compared with wild-type. To visualize the calcofluor florescence, wild type and mutant seeds were stained with Calcofluor white for 5 min and observed under laser scanning confocal microscopy. Three dimensional reconstructions were made by taking images on a Z-series at 2 μm intervals and threading them into an animation. The images represent three different angles and illustrate the uniformity of the phenotype across a three dimensional plane. Scale bar = 250 μm.

Acknowledgments

This work was partially supported by the National Science Foundation grants EFRI-0937657 and NSF-IOS-0922947.

References

1. Brown RM Jr. Cellulose microfibril assembly and orientation: Recent developments. J Cell Sci Suppl 1985; 2:13-32; PMID:3867669.
2. Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, et al. Molecular analysis of cellulose biosynthesis in Arabidopsis. Science 1998; 279:717-20; PMID:9445479; DOI:10.1126/science.279.5351.717.
3. Heim DR, Skomp JR, Tschabold EE, Larrinua IM. Isoxaben inhibits the synthesis of acid insoluble cell wall materials in *Arabidopsis thaliana*. Plant Physiol 1990; 93:695-700; PMID:16667525; DOI:10.1104/pp.93.2.695.
4. Giddings TH, Brower DL, Staehelin LA. Visualization of particle complexes in the plasma membrane of *Micrasterias denticulata* associated with the formation of cellulose fibrils in primary and secondary cell walls. J Cell Biol 1980; 84:327-39; PMID:7189756; DOI:10.1083/jcb.84.2.327.
5. Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM. Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proc Natl Acad Sci USA 1996; 93:12637-42; PMID:8901635; DOI:10.1073/pnas.93.22.12637.
6. Richmond TA, Somerville CR. The cellulose synthase superfamily. Plant Physiol 2000; 124:495-8; PMID:11027699; DOI:10.1104/pp.124.2.495.
7. Persson S, Paredez A, Carroll A, Palsdottir H, Doblin M, Poindexter P, et al. Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in Arabidopsis. Proc Natl Acad Sci USA 2007; 104:15566-71; PMID:17878302; DOI:10.1073/pnas.0706592104.
8. Desprez T, Juraniec M, Crowell EF, Jouy H, Pochylova Z, Parcy F, et al. Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 2007; 104:15572-7; PMID:17878303; DOI:10.1073/pnas.0706569104.
9. Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR. Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc Natl Acad Sci USA 2003; 100:1450-5; PMID:12538856; DOI:10.1073/pnas.0337628100.
10. Gu Y, Kaplinsky N, Bringmann M, Cobb A, Carroll A, Sampathkumar A, et al. Identification of a cellulose synthase-associated protein required for cellulose biosynthesis. Proc Natl Acad Sci USA 2010; 107:12866-71; PMID:20616083; DOI:10.1073/pnas.1007092107.
11. Gu Y, Somerville C. Cellulose synthase interacting protein a new factor in cellulose synthesis. Plant Signal Behav 2010; 5:1571-4; PMID:21150290; DOI:10.4161/psb.5.12.13621.
12. Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, et al. Procuste1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. Plant Cell 2000; 12:2409-24; PMID:11148287.
13. Desprez T, Vernhettes S, Fagard M, Refrégier G, Desnos T, Aletti E, et al. Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform cesa6. Plant Physiol 2002; 128:482-90; PMID:11842152; DOI:10.1104/pp.010822.
14. Stork J, Harris D, Griffiths J, Williams B, Beisson F, Li-Beisson Y, et al. Cellulose synthase9 serves a nonredundant role in secondary cell wall synthesis in Arabidopsis epidermal testa cells. Plant Physiol 2010; 153:580-9; PMID:20335403; DOI:10.1104/pp.110.154062.
15. Mendu V, Griffiths J, Persson S, Stork J, Downie B, Voiniciuc C, et al. Subfunctionalization of cellulose synthases in seed coat epidermal cells mediate secondary radial wall synthesis and mucilage attachment. Plant Physiol 2011; 157:441-53; PMID:21750228; DOI:10.1104/pp.111.179069.
16. Sullivan S, Ralet MC, Berger A, Diatloff E, Bischoff V, Gonneau M, et al. Cesa5 is required for the synthesis of cellulose with a role in structuring the adherent mucilage of Arabidopsis seeds. Plant Physiol 2011; 156:1725-39; PMID:21705653; DOI:10.1104/pp.111.179077.
17. Carroll A, Specht CD. Understanding plant cellulose synthases through a comprehensive investigation of the cellulose synthase family sequences. Front Plant Sci 2011; 2:5; DOI:10.3389/fpls.2011.00005.
18. Carpita N, Vergara C. A recipe for cellulose. Science 1998; 279:672-3; PMID:9471727; DOI:10.1126/science.279.5351.672.
19. Song D, Shen J, Li L. Characterization of cellulose synthase complexes in populus xylem differentiation. New Phytol 2010; 187:777-90; PMID:20546138; DOI:10.1111/j.1469-8137.2010.03315.x.
20. Betancur L, Singh B, Rapp RA, Wendel JF, Marks MD, Roberts AW, et al. Phylogenetically distinct cellulose synthase genes support secondary wall thickening in Arabidopsis shoot trichomes and cotton fiber. J Integr Plant Biol 2010; 52:205-20; PMID:20377682; DOI:10.1111/j.1744-7909.2010.00934.x.
21. Western TL, Skinner DJ, Haughn GW. Differentiation of mucilage secretory cells of the Arabidopsis seed coat. Plant Physiol 2000; 122:345-56; PMID:10677428; DOI:10.1104/pp.122.2.345.
22. Macquet A, Ralet MC, Kronenberger J, Marion-Poll A, North HM. In situ, chemical and macromolecular study of the composition of *Arabidopsis thaliana* seed coat mucilage. Plant Cell Physiol 2007; 48:984-99; PMID:17540691; DOI:10.1093/pcp/pcm068.
23. Arsovski AA, Haughn GW, Western TL. Seed coat mucilage cells of *Arabidopsis thaliana* as a model for plant cell wall research. Plant Signal Behav 2010; 5:796-801; PMID:20505351; DOI:10.4161/psb.5.7.11773.
24. Kacuráková M, Capek P, Sasinková V, Wellner A, Ebringerová A. Ft-ir study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses. Carbohydr Polym 2000; 43:195-203; DOI:10.1016/S0144-8617(00)00151-X.

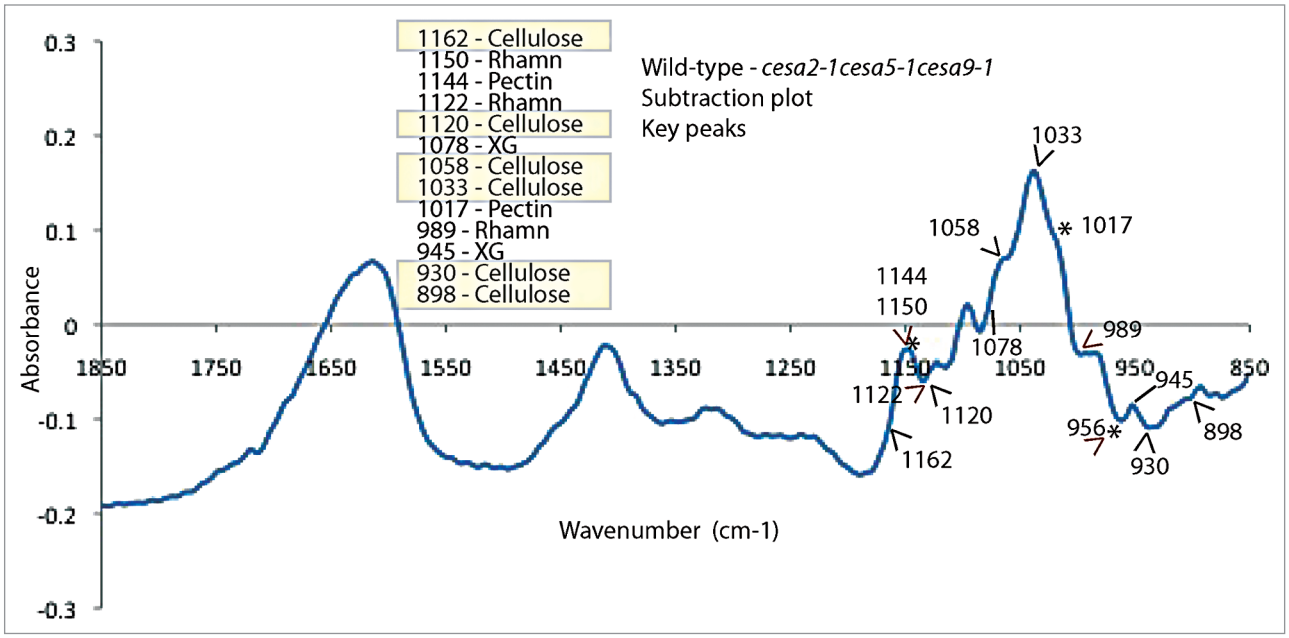

Figure 4. Analysis of mucilage composition using Fourier Transform Infrared analysis (FTIR) showed differences in cellulose, hemicelluloses and pectin peaks. FTIR was performed on the ammonium oxalate extracted mucilage for structural changes. Analysis of subtraction plot between wild type and *cesa2-1cesa5-1cesa9-1* triple mutant showed differences in peaks related to cellulose (898, 930, 1,033, 1,058, 1,120 and 1,162, hemicellulose (WN-1078 and 945) and pectin WN-989, 1,017, 1,122, 1,144 and 1,150). Samples were prepared by dissolving exactly 2 mg mucilage pellet in 50 μL water with 20 mg of KBr, the overnight dried (50°) mixture was stored in a desiccator. Pellets for FT-IR were made by using 2 mg mucilage-KBr mixture with 23 mg of fresh KBr, pressed into 7-mm pellets and collected spectra for wild type and mutants using a Thermo Nicolet Nexus 470 spectrometer (ThermoElectric Corporation) over the range 4,000–800 cm^{-1}. For each spectrum, 200 scans were performed at a resolution of 8 cm^{-1}. Subtraction plot between wild type and triple mutant was made using OMNIC software (Thermo Nicolet) and spectral differences were cross referenced with Kacuráková et al. to identify peaks linked to cellulose (indicated by black caret), xyloglucan (black line) and pectin (asterisk and red caret).

25. Mouille G, Robin S, Lecomte M, Pagant S, Höfte H. Classification and identification of Arabidopsis cell wall mutants using fourier-transform infrared (ft-ir) microspectroscopy. Plant J 2003; 35:393-404; PMID:12887590; DOI:10.1046/j.1365-313X.2003.01807.x.
26. Abidi N, Hequet E, Cabrales L, Gannaway J, Wilkins T, Wells LW. Evaluating cell wall structure and composition of developing cotton fibers using fourier transform infrared spectroscopy and thermogravimetric analysis. J Appl Polym Sci 2008; 107:476-86; DOI:10.1002/app.27100.
27. Blake AW, McCartney L, Flint JE, Bolam DN, Boraston AB, Gilbert HJ, et al. Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding modules in prokaryotic enzymes. J Biol Chem 2006; 281:29321-9; PMID:16844685; DOI:10.1074/jbc.M605903200.
28. North H, Baud S, Debeaujon I, Dubos C, Dubreucq B, Grappin P, et al. Arabidopsis seed secrets unravelled after a decade of genetic and omics-driven research. Plant J 2010; 61:971-81; PMID:20409271; DOI:10.1111/j.1365-313X.2009.04095.x.
29. Nowack MK, Ungru A, Bjerkan KN, Grini PE, Schnittger A. Reproductive cross-talk: Seed development in flowering plants. Biochem Soc Trans 2010; 38:604-12; PMID:20298229; DOI:10.1042/BST0380604.
30. Haughn G, Chaudhury A. Genetic analysis of seed coat development in Arabidopsis. Trends Plant Sci 2005; 10:472-7; PMID:16153880; DOI:10.1016/j.tplants.2005.08.005.
31. Windsor JB, Symonds VV, Mendenhall J, Lloyd AM. Arabidopsis seed coat development: Morphological differentiation of the outer integument. Plant J 2000; 22:483-93; PMID:10886768; DOI:10.1046/j.1365-313x.2000.00756.x.
32. Green PB. Mechanism for plant cellular morphogenesis. Science 1962; 138:1404-5; PMID:17753861; DOI:10.1126/science.138.3548.1404.

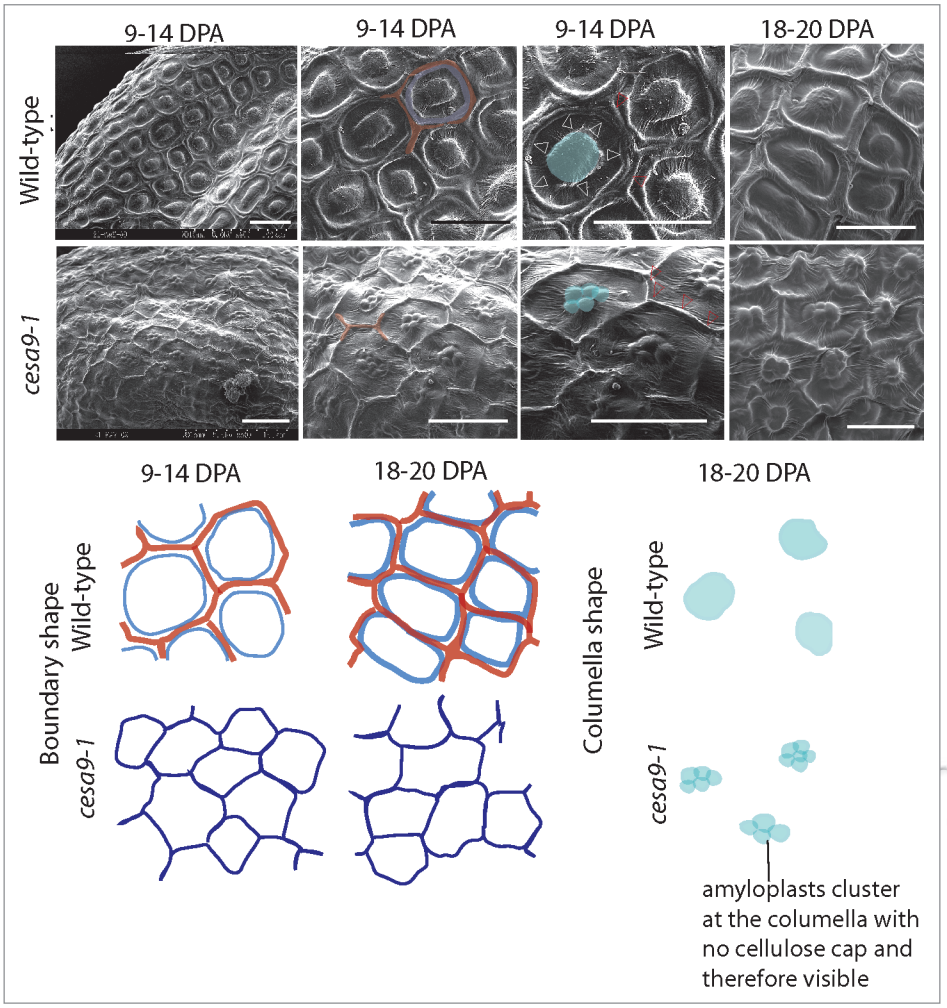

Figure 5. Radial wall ridge development in wild type and *cesa9-1* seed coat cells. Seeds were excised from siliques at approximately 9–14 and 18–20 DPA, sputter coated and visualized under SEM (Hitachi-S-800). Analysis of radial wall development using SEM at 9–14 DPA showed formation of secondary radial wall next to the primary cell wall and a thicker fused wall at 18 DPA in wild type while *cesa9-1* showed less pronounced secondary radial wall at 9–14 DPA and thinner wall at 18 DPA. Scale bar = 30 μm.