

A new callose function

Involvement in differentiation and function of fern stomatal complexes

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Abbreviations: AF, actin filament; *A. nidus*, *Asplenium nidus*; 2-DDG, 2-deoxy-D glucose; ER, endoplasmic reticulum; GC, guard cell; MT, microtubule; TEM, transmission electron microscopy; VW, ventral wall

Callose in polypodiaceous ferns performs multiple roles during stomatal development and function. This highly dynamic (1→3)-β-D-glucan, in cooperation with the cytoskeleton, is involved in: (a) stomatal pore formation, (b) deposition of local GC wall thickenings and (c) the mechanism of stomatal pore opening and closure. This behavior of callose, among others, probably relies on the particular mechanical properties as well as on the ability to form and degrade rapidly, to create a scaffold or to serve as a matrix for deposition of other cell wall materials and to produce fibrillar deposits in the periclinal GC walls, radially arranged around the stomatal pore. The local callose deposition in closing stomata is an immediate response of the external periclinal GC walls experiencing strong mechanical forces induced by the neighboring cells. The radial callose fibrils transiently co-exist with radial cellulose microfibrils and, like the latter, seem to be oriented via cortical MTs.

Callose represents a hemicellulosic matrix cell wall component, usually of temporal appearance, which is synthesized by callose synthases, enzymes localized in the plasmalemma and degraded by (1→3)-β-glucanases.¹⁻⁴ It consists of triple helices of a linear homopolymer of (1→3)-β-glucose residues.⁵⁻⁷ The plant cell is able to form and degrade callose in a short time. On the surface of the plasmolyzed protoplast a thin callose surface film may arise within seconds.⁸ Callose is the only cell wall component that is implicated in a great variety of developmental plant processes, like cell plate formation,⁹⁻¹¹ microspore development,¹²⁻¹⁴ trafficking through plasmodesmata,^{15,16} formation and closure of sieve pores,¹⁶ response of the plant cells to multiple biotic and abiotic stresses,^{4,5} establishment of distinct “cell cortex domains”,¹⁷ etc.

Despite the widespread occurrence of callose, its general function(s) is (are) not well understood (reviewed in refs. 4 and 5). It may serve as: a matrix for deposition of other cell wall

materials, as in developing cell plates;⁹ a cell wall-strengthening material, as in cotton seed hairs and growing pollen tubes;¹⁸ a sealing or plugging material at the plasma membrane of pit fields, plasmodesmata and sieve plate pores;¹⁶ a mechanical obstruction to growth of fungal hyphae or a special permeability barrier, as in pollen mother cell walls and muskmelon endosperm envelopes.^{4,19,20} The degree of polymerization, age and thickness of callose deposits may cause variation in its physical properties.⁵

Evidence accumulated so far showed that a significant number of ferns belonging to Polypodiales and some other fern classes forms intense callose deposits in the developing GC wall thickenings.²¹⁻²⁸ This phenomenon has not been observed in angiosperm stomata, although callose is deposited along the whole surface of the young VW and in the VW ends of differentiating and mature stomata (our unpublished data; reviewed in refs 29 and 30).

Stomata are specialized epidermal bicellular structures (Fig. 1A) regulating gas exchange between the aerial plant organs and the external environment. Their appearance in the first land plants was crucial for their adaptation and survival in the terrestrial environment. The constituent GCs have the ability to undergo reversible changes in shape, leading to opening and closure of the stomatal pore (stomatal movement). The mechanism by which GCs change shape is based on: (a) the particular mechanical properties of GC walls owed to their particular shape, thickening, fine structure and chemical composition and (b) the reversible changes in vacuole volume, in response to environmental factors, through fairly complicated biochemical pathways.³⁰⁻³³

The present review is focused on the multiple-role of callose in differentiating and functioning fern stomata, as they are substantiated by the available information, including some unpublished data, and in particular in: stomatal pore formation, deposition of GC wall thickenings and opening and closure of the stomatal pore. The mode of deposition of fibrillar callose deposits in GC walls and the mechanism of their alignment are also considered.

Callose and Stomatal Pore Formation

The main tasks of GC differentiation are the formation of the stomatal pore and the development of the mechanism regulating its opening and closure.^{30,34} Stomatal pore is an intercellular space

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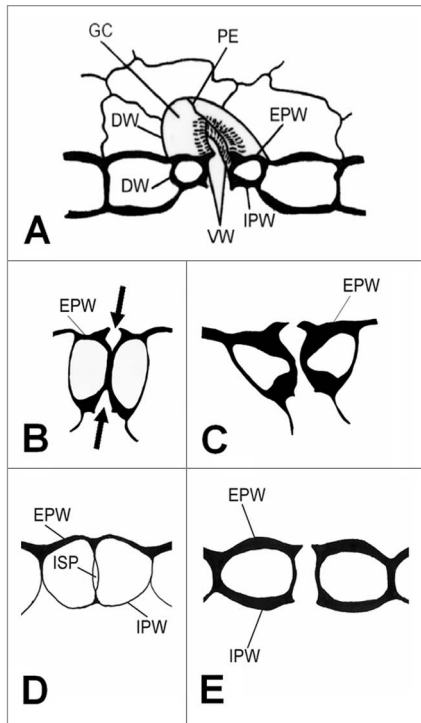


Figure 1. (A) Diagrammatic representation of an elliptical stoma. (B–E) Diagram to show the process of stomatal pore formation in angiosperms (B and C) and Polyopdiales ferns (D and E). The arrows in (B) indicate the forming stomatal pore. DW, dorsal wall; EPW, external periclinal wall; GC, guard cell; IPW, internal periclinal wall; ISP, internal stomatal pore; PE polar ventral wall end; VW, ventral wall.

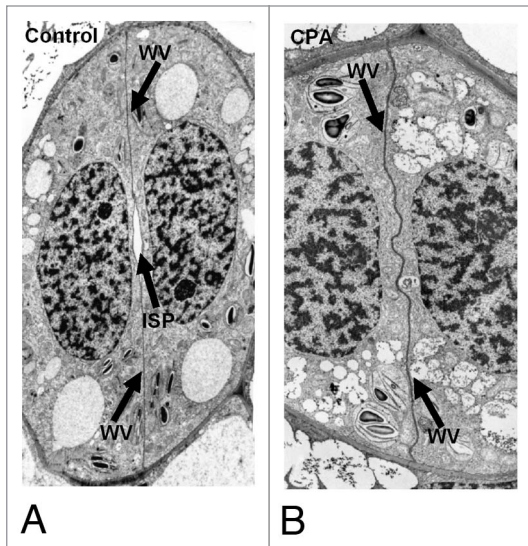


Figure 2. (A and B) TEM micrographs illustrating median paradermal views of post-cytokinetic *A. nidus* stomata. (A) Control stoma. (B) Stoma affected by 25 μ M CPA for 24 h, which lacks an “internal stomatal pore” (B; cf. A). ISP, internal stomatal pore; VW ventral wall. A: $\times 1,900$; B: $\times 2,300$.

forming schizogenously in the median region of the adjacent VWs separating the differentiating GCs, which brings into communication the labyrinth of the mesophyll intercellular spaces with the external environment (Fig. 1A). In most plants, this process begins from the external and internal periclinal GC walls and proceeds inwards (Fig. 1B and C; reviewed in ref. 30 and 31). In angiosperms, stomatal pore formation keeps pace with GC morphogenesis, a process largely controlled by the cortical MTs.^{35,36}

In ferns *Asplenium nidus*, *Adiantum capillus-veneris* and two *Anemia* species, an extraordinary mechanism of stomatal pore formation functions.^{24,25,37,38} Stomatal pore appears as an intercellular space at the centre of the adjacent post-cytokinetic VWs (“internal stomatal pore”; Figs. 1D and 2A), gradually broadening towards the external and internal periclinal GC walls. Finally, the periclinal walls over the “internal stomatal pore” are disrupted and the stomatal pore is completed (Fig. 1E). The “internal stomatal pore” formation starts before the deposition of any detectable cell wall material in the VW by the local apart movement of the adjacent plasmalemmata (Fig. 2A). MT and AF bundles, lining anticlinally the mid-region of the VW, seem to be implicated in this process.^{24,26,37}

In *A. nidus*, the “internal stomatal pore” formation is also related to the pattern of callose degradation in the nascent VW that accumulates considerable quantities of this glucan along its whole surface.²⁶ Callose degradation commences at the centre of the nascent VW and proceeds towards its periphery. This is coupled with the initiation of the “internal stomatal pore” at the mid-depth of the VW (Fig. 2A), which further broadens centrifugally towards the periclinal GC walls. Callose was also detected along the whole surface of the nascent daughter walls of the dividing ordinary protodermal cells, but its degradation is centripetal, i.e., it commences from the periphery and proceeds inwards. Increased ER quantities were found by TEM and immunolabeling of the HDEL proteins in the cytoplasm lining the median regions of the nascent VW of *A. nidus* and *Adiantum capillus-veneris* stomata (reviewed in ref. 26 and 37 and our unpublished data). Among others, they may be involved in synthesis of proteins implicated in the local callose formation and/or degradation and/or establishment of local Ca^{2+} gradients controlling the above processes.

In *A. nidus* stomata treated with 2-deoxy-D-glucose (2-DDG) and tunicamycin, substances inhibiting callose synthesis,^{39,40} the newly formed VWs lacked callose as well as an “internal stomatal pore”.²⁶ Gradually, they become abnormally thickened, appeared electron-transparent and included membranous elements, probably because of the uncontrolled growth and the extensive out folding of the plasmalemma into the apoplast. Moreover, treatment with cyclopiazonic acid (CPA) that disturbs cytoplasmic Ca^{2+} homeostasis,⁴¹ inhibited both callose deposition and “internal stomatal pore” formation of *A. nidus* stomata (reviewed in ref. 26; also Fig. 2B; cf. 2A). The VW of the 2-DDG-, tunicamycin- and CPA-affected stomata displayed polysaccharides, other than callose, positive to PAS staining and fluorescing intensely after calcofluor staining. Inhibition of cellulose synthesis by coumarin and dichlobenil that promote callose synthesis^{42–44} also blocked

callose degradation in the nascent VWs of *A. nidus* stomata. The affected stomata retained for a relatively long time large callose quantities in the nascent VWs, a phenomenon accompanied by the absence of “internal stomatal pore”.²⁶ Therefore, both the absence and prolonged presence of callose in the nascent VW of the affected stomata inhibit “internal stomatal pore” formation.

The existence of considerable callose quantities in the cell plate and the early post-cytokinetic daughter walls probably offers mechanical support to the daughter plasmalemmata.^{9,11,45} In *A. nidus* stomata, the presence of callose in nascent VWs probably makes the adjacent plasmalemmata more rigid and difficult to be separated for the “internal stomatal pore” formation, while the possibility that callose, forming a gel “sticking” the partner VW plasmalemmata to one another cannot be excluded. This difficulty is overcome by the rapid local callose removal that seems to allow the anticlinal MT and/or AF bundles lining the adjacent plasmalemmata at the middle of the VW^{24,25} to mediate their movement apart from each other and thus to initiate the “internal stomatal pore”. The temporal and spatial coincidence between callose degradation and “internal stomatal pore” formation supports the above view.

In addition, the maintenance of large callose quantities in the aberrant VWs formed in the dichlobenil- and coumarin-affected stomata, which is possibly accompanied by elevated deposition of pectic materials in them,⁴³ probably keeps the plasmalemmata together preventing the “internal stomatal pore” formation. Moreover, the inhibition of callose synthesis possibly results in the earlier deposition of wall materials in the affected stomata,²⁶ allowing the development of connections between the partner VWs and the adjacent plasmalemma that makes the “internal stomatal pore” formation impossible.

Callose and Deposition of Local GC Wall Thickenings

The differentiating GCs of fern stomata,^{21,22,24,25,37,46} like all the kidney-like GCs,³⁰⁻³² form local wall thickenings at the sites of junction of the mid-region of the VW with the periclinal ones. Gradually, they expand covering the median region of the periclinal walls around the stomatal pore. In mature stomata, these thickenings disappear, mainly contributing to the asymmetrical expansion of the external and internal VW areas at the stomatal pore region. The cellulose microfibrils in the periclinal GC wall thickenings as well as in the rest of the periclinal walls are radially arranged around the stomatal pore, playing an essential role in GC morphogenesis-stomatal pore formation and especially in stomatal movement.³⁰

The GC wall thickenings in species of the fern genera *Asplenium*, *Angiopteris*, *Blechnum*, *Dryopteris*, *Marrattia*, *Nephrolepis*, *Ophioglossum*, *Polypodium*, *Pteris* and *Pteridium* accumulate large quantities of callose localized at the periphery and the interior of the rising wall thickenings (reviewed in ref. 21–23 and 25–27 and our unpublished data). At the margins of the developing GC wall thickenings of *A. nidus* large ER quantities are accumulated (Fig. 3C), which among others, may participate in the local callose deposition. Surprisingly, in the periclinal

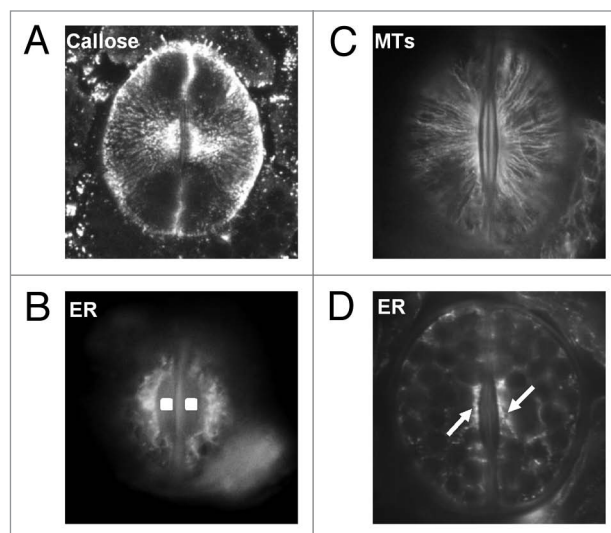


Figure 3. (A) Callose immunolabeling in a differentiating stoma of *A. nidus*. Confocal-laser-scanning-microscope image constructed from seven consecutive optical paradermal sections. The callose fibrils are radially arranged around the stomatal pore. x850 (B) MT immunolabeling in a closed *A. nidus* stoma. The MTs underneath the periclinal walls are radially arranged around the stomatal pore (cf. A). x1,000. (C and D) Immunolabeling of HDEL ER proteins in a differentiating (C) and a mature closed stoma (D) of *A. nidus*. In the stoma shown in (C) distinct ER aggregations are localized at the margins of the wall thickenings (squares in C) deposited at the stomatal pore region. The arrows in (D) point to local ER aggregations in the cytoplasm adjacent to the walls at the stomatal pore region. C: x1,000; D: x750.

GC walls of *A. nidus* stomata callose is deposited in the form of fibrils radially arranged around the stomatal pore (reviewed in ref. 27; see also Fig. 3A). Examination of the figures included in the articles of Peterson et al.²¹ and Waterkeyn and Bienfait²³ revealed the existence of radial callose fibrillar arrays in the external periclinal GC walls of the ferns *Ophioglossum lusitanicum*, *Ophioglossum crotalophoroides*, *Angiopteris teysmanniana* and *Marattia fraxinea*. The differentiating stomata of the ferns *Nephrolepis exaltata* and *Blechnum* sp. also display radial callose arrays (our unpublished data).

Therefore, in the periclinal GC walls of above ferns radial fibrillar arrays of two glucans (cellulose and callose) are organized. The radial callose fibrils probably reinforce the radial cellulose microfibrils to force differentiating GCs to assume a kidney-like form. The deposition of shapeless callose deposits in periclinal GC wall thickenings might interfere with the tangential periclinal GC wall expansion, thus worsening GC morphogenesis. Fibrillar callose has been also found in differentiating cotton fibers⁴⁷ and in protoplasts regenerating cell wall.⁴⁸⁻⁵⁰ Micelle-like and fibrillar callose structures are also produced during in vitro callose synthesis.⁵¹⁻⁵³

The radial pattern of fibrillar callose arrays in the periclinal walls of *A. nidus* GCs copies that of the underlying cortical MTs (reviewed in reference 27, also Fig. 3A; cf. 3B). This mutual alignment closely resembles that between cellulose microfibrils and cortical MTs in the kidney-shaped GCs.³⁰ The oryzalin induced MT disintegration in *A. nidus* stomata disturbs the pattern of

callose deposition, which becomes shapeless.²⁷ Therefore, similarly to cellulose microfibrils, callose fibrils are probably aligned via cortical MTs. Alternatively, the radial pattern of callose fibril deposition could be controlled by the preexisting radial cellulose microfibrils in the periclinal GC walls. However, the findings that: (a) in dichlobenil- or coumarin-affected *A. nidus* stomata lacking radial cellulose microfibrils, radial callose arrays were deposited and (b) in some oryzalin-affected stomata the deposition of callose was disturbed, despite the presence of intact radial cellulose microfibril systems,²⁷ show clearly that the latter are not implicated in callose fibril orientation. The cortical AFs underlying the periclinal GC walls²⁵ do not participate in this process. Experimental AF disintegration in *A. nidus* stomata did not affect the pattern of radial callose fibrils.²⁷

The mechanism by which the cortical MTs control the oriented deposition of callose fibrils must be similar to that involved in the cellulose microfibril deposition. In the latter case, the cortical MTs, acting as molecular rails, directly guide the cellulose synthases as they synthesize cellulose microfibrils,^{54,55} or they serve as passive constraints creating channels that determine the movement of cellulose synthases in the plasmalemma (reviewed in references 56–58). The former hypothesis seems now more probable. In that case, both callose and cellulose synthases must be guided via cortical MTs. In the absence of MTs, shapeless callose masses were deposited in the periclinal GC walls of *A. nidus*. Therefore, the MTs control callose deposition in the form of fibrils as well as their alignment.

Apart from the fern stomata, callose in the developing cell wall thickenings has been found in tracheary elements,^{5,59} transfer cells,⁶⁰ cotton fibers,^{47,61} pollen grains,⁶² moss spores⁶³ and bordered pits.⁶⁴ In these cases, as well as in fern stomata, callose may: (a) create a scaffold or a suitable microenvironment for the deposition of cellulose microfibrils and other cell wall polysaccharides,^{47,48,60,61,64} (b) when degraded provide glucose residues for the synthesis of other cell wall polysaccharides^{43,47} and (c) offer support to plasmalemma to resist to mechanical stresses exerted to it as the wall thickenings grow into the cytoplasm.^{26,60}

The extensive plasmalemma out folding into the apoplastic space of the atypical GC wall thickenings in *A. nidus* stomata affected by callose synthesis inhibitors,²⁶ suggests that the absence of callose leads to destabilization of the mechanism of plasmalemma growth and cycling via endocytosis of the excessive membrane material.

Callose Implication in Stomatal Pore Opening and Closure

In functioning *A. nidus* stomata, callose presents a periodic synthesis/degradation cycle during stomatal pore opening/closure. Callose is definitely absent from the open stomata, but rapidly appears, in the form of fibrils, in the external periclinal cell walls of the closed ones,²⁸ a phenomenon also confirmed in the ferns *Blechnum* sp. and *Nephrolepis exaltata* (our unpublished data). Snap-freezing of *A. nidus* leaf portions in liquid nitrogen, followed by tubulin immunolabeling, as well as MT immunolocalization

after chemical fixation, revealed the existence in both open and closed stomata of well-organized radial MT systems below the periclinal GC walls (Fig. 3B; our unpublished data). This is in contrast to the elliptical stomata of the dicotyledon *Vicia faba*, where the open stomata display highly organized radial MT systems that break down in the closed ones.^{65,66} The persistence of radial MT arrays in *A. nidus* stomata is probably related to the control radial callose deposition in the closing stomata.

In closing stomata of *A. nidus*, callose is mainly deposited in particular external periclinal GC wall regions that bend intensely.²⁸ They are thinner than the rest wall as the result of particular thickening/expansion mode of the periclinal GC walls,^{25,67} a phenomenon commencing in GC mother cells. The central region of the external periclinal wall of these cells is curved, thin and impregnated by callose, probably as a response to local stretching, while its margins appear thickened.⁶⁷

During stomatal closure in *A. nidus* the surrounding epidermal cells seem to exert strong mechanical stresses on the external periclinal GC walls, leading to their local deformation. The plasmalemma lining the deforming regions probably senses this mechanical stress that induces the local increase of Ca²⁺ uptake in the GCs, which in turn triggers local callose synthesis.⁶⁸ Mechanical forces have been shown to alter plasmalemma ion channel permeability, which is associated with Ca²⁺ and other ion fluxes.^{69,70} Immunolabeling of the HDEL ER proteins, after chemical fixation or snap-freezing in liquid nitrogen, revealed that in both opened and closed stomata of *A. nidus* increased quantities of ER line the GC wall thickenings (Fig. 3D; our unpublished data). Among others, they may be involved in the local callose cycling and/or cellulose synthesis.

These stresses are relieved in the opened stomata, the external periclinal walls of which recover. These walls did not display any local deformation as well as callose.²⁸ During recovery, new polysaccharidic material, including cellulose microfibrils might be incorporated in them, a hypothesis that should be investigated. If it is true, during stomatal movement callose synthesis may alternate with that of cellulose microfibrils. Matrix cell wall material and cellulose, but not callose, are synthesized in the opening stomata of the dicotyledon *Vicia faba*.⁷¹

Regardless of the above, in *A. nidus*, GC wall composition definitely changes during stomatal movement, an observation posing the question whether or not the cycling callose that is deposited in response to a signal generated by mechanical stress, is involved in the mechanism of opening and closure of the stomatal pore. A series of experimental data substantiated callose implication in stomatal movement of *A. nidus*²⁸ and in particular:

(1) The worsening of the ability of stomata to open in white light and to close in darkness after the enzymatic degradation of callose by β -1,3-D-glucanase.

(2) The decrease in the ability of stomata to open in white light and close in darkness after inhibition of callose synthesis by 2-DDG. The presence of well-organized radial cellulose microfibrils arrays in 2-DDG-affected stomata supports that this substance disturbs stomatal movement via inhibition of callose synthesis and not by inhibition of cellulose microfibril synthesis.

(3) The improvement of the ability of stomata to open in white light and the prevention of their complete closure in darkness, after induction of callose synthesis by coumarin or dichlobenil. Since coumarin- and dichlobenil-affected functioning stomata possessed well-organized cellulose microfibrils that have been deposited before the onset of the treatment, these drugs probably interfere with stomatal opening and closure by induction of excessive callose synthesis.

Regarding the particular role(s) of callose in the mechanism of stomatal pore opening and closure it can be suggested that this glucan, intercalating between preexisting cell wall polysaccharides may:

(a) Create a proper microenvironment or a scaffold in periclinal GC wall for the deposition of cell wall polysaccharides during stomatal opening, hypothesis previously made for other cell types.^{47,60}

(b) Loosen or disrupt the chemical bonds between the matrix cell wall polysaccharides or even develop chemical bonds with them, in all cases modifying the elastic properties of the periclinal GC walls.²⁸ However, there is not information about the type and the extent of chemical bonds that callose may develop with the matrix cell wall polysaccharides and/or cellulose microfibrils. In angiosperms, the elastic properties of the GC walls seem to change during stomatal movement, probably by compositional changes of the wall matrix polysaccharides. Enzymatic disruption of arabinans, feruloyl esters of pectins and homogalacturonans affects stomatal pore opening and closing.^{72,73}

(c) Facilitate or more probably reinforce the ability of the periclinal GC walls to expand tangentially, promoting stomatal pore opening. In case of shapeless callose deposition, the expansion of the periclinal walls could be deteriorated.

It should be mentioned here that the chemical composition of the primary cell walls of ferns differs from those of other plant divisions.⁷⁴ Our preliminary data derived by examination of GC wall autofluorescence and chemical GC wall composition using JIM5, JIM7, LM6 and LM13 antibodies, showed that the chemical composition of *A. nidus* GC walls differ from those of the

elliptical angiosperm stomata investigated so far. They are rich in phenolic esters of pectins, esterified homogalacturonan and linear arabinans and seem to lack unesterified homogalacturonans and pectic arabinans. Comparison of the chemical composition of the *A. nidus* GC walls to those of elliptical angiosperm stomata^{29,72,73} revealed that the intense presence of the phenolic esters of pectins and the absence of unesterified homogalacturonans and pectic arabinans constitute particular characteristics of the GCs walls of *A. nidus*. Considering the above mentioned, it may be speculated that the implication of callose in the function of the fern stomata is related to their particular GC wall chemical composition, and that it plays a role analogous to that of arabinans in the functioning angiosperm stomata.⁷²

Future Perspectives

Accumulated information on fern stomata revealed that callose participates in stomatal differentiation and function. However, further studies are needed in order to: (a) elucidate the exact function(s) of callose in developing and functioning fern stomata. In this direction, the study of GC wall chemistry as well as the nature and extent of chemical bonds that callose may develop with the other cell wall polysaccharides is essential. (b) Find out whether callose plays any role in stomatal development and function in other plant divisions. (c) Examine the structure of callose fibrils and whether all callose synthases or some of them are able to interact with MTs to create the callose fibrils. Twelve genes encoding putative callose synthases have been found in *Arabidopsis*.¹

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