Plant Science 183 (2012) 1–8

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

# Expression of *Arabidopsis* callose synthase 5 results in callose accumulation and cell wall permeability alteration

Bo Xie[a], Yunfei Deng[a], Masahiro M. Kanaoka[a,b,1], Kiyotaka Okada[b,2], Zonglie Hong[a,*]

[a] Department of Plant, Soil, and Entomological Sciences and Program of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844, USA
[b] Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

ARTICLE INFO

*Article history:*
Received 16 August 2011
Received in revised form 12 October 2011
Accepted 21 October 2011
Available online 25 October 2011

*Keywords:*
*Arabidopsis thaliana*
Callose
Callose synthase
Cell wall
Tobacco BY-2 cells

ABSTRACT

Callose is the major polysaccharide present in the callose wall of developing microspores and the growing pollen tube wall. It is also an essential component of other specialized cell walls and its synthesis can be induced by pathogen infection, wounding and environmental cues. Among the 12 callose synthase genes (*CalS*) present in the *Arabidopsis* genome, *CalS5* plays the predominant role in the synthesis of the callose wall, callose plugs and pollen tube wall. When expressed as a GFP-tagged protein in cultured tobacco BY-2 cells, CalS5 was found to be present in the plasma membrane and the Golgi-related endomembranes. Unlike the cell plate-specific CalS1 isozyme, CalS5 was not concentrated to the cell plate at cytokinesis. Expression of CalS5 resulted in callose accumulation only in the cell wall of BY-2 cells. The fact that no callose was found in the endomembranes suggests that CalS5 is not functional in that compartment. These cells exhibited a decreased plasmolysis rate in hypotonic solutions and an increased cytolysis rate in hypertonic conditions. This study demonstrates that an artificial callose wall could be synthesized by expressing a callose synthase enzyme.



## 1. Introduction

Callose synthase (CalS; EC 2.4.1.34; UDP-glucose:1,3-β-D-glucosyl transferase) catalyzes the synthesis of callose, or β-1,3-glucan in plants. Compared to cellulose, a linear β-1,4-glucan, callose consists mainly of β-1,3-linked glucose residues associated with a small fraction (approximately 2%) of glucuronic acid [1]. In higher plants, callose is present in specialized cell walls such as the nascent cell plate of dividing cells, sieve plates of phloem elements, the callose wall established during microsporogenesis, and pollen tube wall [1]. Its synthesis and accumulation are also characteristically induced by wounding and physiological stress [2,3]. The Arabidopsis genome contains 12 *Callose Synthase* (*CalS*) genes [2], also known as *Glucan Synthase-Like* (*GSL*) [4]. They are responsible for callose synthesis in different plant tissues and in response to various physiological, developmental and environmental signals [2].

Pollen development requires the formation of the callose wall that consists of callose in addition to minor amounts of other cell wall polysaccharides and lipoproteins. Several CalS isoforms, CalS5 (GSL2), CalS7 (GSL7), CalS9 (GSL10), CalS10 (GSL8), CalS11 (GSL1) and CalS12 (GSL5), have been shown to play different roles during microsporogenesis, microgametogenesis and pollination [5–15]. Among these CalS isoforms, CalS5 has been suggested to be the major CalS involved in the synthesis of the peripheral callose wall, the callose plugs and pollen tube wall during pollen germination [5,6]. The callose wall, located between the plasma membrane and the primary cell wall, is first synthesized by pollen mother cells (PMC) during initiation of meiosis. A massive amount of callose continues to be deposited through meiosis, leading to the formation of a protective enclosure surrounding the tetrad and separating the four microspores within a tetrad. Knockout mutations of *CalS5* do not perturb vegetative growth significantly but result in male sterility that is associated with the complete loss of the peripheral callose wall of tetrads and the callose plugs of growing pollen tubes [5,6]. Overexpression of CalS5 under the CaMV 35S promoter in *Arabidopsis* also causes abnormal callose deposition during microsporogenesis and results in the precocious pollen germination prior to anthesis, suggesting that the expression level and

*Abbreviations:* At, *Arabidopsis thaliana*; CalS, callose synthase; CS5-L, polyclonal antibodies to a peptide at the loop region of CalS5; CS5-N, polyclonal antibodies to an N-terminal peptide of CalS5; G-CS5, GFP-tagged CalS5; GFP, green florescent protein; GSL, glucan synthase-like; HRP, horseradish peroxidase.

* Corresponding author at: University of Idaho, Department of Plant, Soil, and Entomological Sciences, AgBiotech Building, Room 207, Moscow, ID 83844-2339, USA. Tel.: +1 208 885 5464; fax: +1 208 885 7760.
*E-mail address:* zhong@uidaho.edu (Z. Hong).
[1] Present address: Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan.
[2] Present address: Department of Developmental Biology, National Institute for Basic Biology, Nishigonaka 38, Myodaiji, Okazaki, 444-8585 Aichi, Japan.

0168-9452/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2011.10.015

localization pattern of CalS5 are important for pollen development in *Arabidopsis* [11]. Orthologs of the *AtCalS5* gene in rice (*OsGSL5*) [16] and tobacco (*NaGSL1*) [17–19] are also highly expressed in developing anthers and germinating pollen tubes, which is consistent with the proposed role of CalS5 in *Arabidopsis*.

It appears that CalS5 may have evolved as the key CalS isoform responsible for callose synthesis required for pollen development and pollen tube growth in higher plants. However, it is not known if CalS5 is active when expressed in a heterologous system and how callose accumulation affects cell growth and cell physiology at the single cell level. We have previously demonstrated CalS1 is a cell plate-specific isoform during cytokinesis [20]. As CalS5 is mainly involved in pollen development and pollen tube growth [5,6], it would be of interest to compare the subcellular localization and biological function of CalS5 with CalS1. In this report, we expressed *Arabidopsis* CalS5 in cultured tobacco BY-2 cells, and studied its subcellular localization and function. Our results demonstrate that CalS5 is localized to the plasma membrane and endomembrane structures in BY-2 cells, and is distinct from the cell plate localization pattern of CalS1 [20] at cytokinesis. Expression of CalS5 resulted in callose accumulation in the cell wall of BY-2 cells, and the transgenic BY-2 cells exhibited a decreased plasmolysis rate in hypotonic solutions and an increased cytolysis rate in hypertonic conditions.

## 2. Materials and methods

### 2.1. Cell culture

Suspension-cultured tobacco (*Nicotiana tabacum* L. cv BY-2) cells [21] were grown at room temperature (21–22 °C) on a rotary shaker (130 rpm) in the Murashige-Skoog basal medium [22] supplemented with 1.5 mM $KH_2PO_4$, 3 μM thiamine, 0.55 mM inositol, 87 mM sucrose, and 1 μM 2,4 dichlorophenoxy acetic acid. Cells were subcultured every 7 days by transferring 2 ml into 50 ml of fresh medium.

### 2.2. Construction of expression vector and transformation

The coding region of *CalS5* was amplified by RT-PCR with RNA isolated from *Arabidopsis* inflorescences using primers 5′-GCA GTC GAC GAG ATG GCA CAG AGT AGT ACA TCT C-3′ and 5′-CGT GTC GAC TTC TTT CTG CTT CTT ACC ACC GGC-3′ (*Sal* I sites underlined). The amplified fragment (5.8 kb) was cloned into pCR2.1 (Invitrogen) and verified by DNA sequencing. The coding region was subcloned into pGFP-MCS [20] at the *Sal*I site, generating pGFP-CS5. The *GFP-CalS5* fragment in pGFP-CS5 was digested with *Not*I and subcloned into binary vector pMON18342 [23] to create pMB-CS5. Plasmids pMB-CS5 was introduced into *Agrobacterium tumefaciens* ABI strain for the transformation to tobacco BY-2 cells. Transgenic BY-2 cells containing pMB-CS5 were selected on BY-2 agar medium [20] supplemented with 200 mg/l kanamycin. All transgenic lines were confirmed by genomic PCR. Expression of GFP alone and GFP-CalS1 (G-CS1) in BY-2 cells was described previously [20].

### 2.3. RNA extraction and RT-PCR

Total RNA isolated using Trizol reagent (Invitrogen) was reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). PCR amplification of the *CalS5* cDNA was performed with primers 5′-AAG ATT TGC AGA GGT CAC TGC AGC-3′ and 5′-CTT CTC GTT CCT CAA CCT CAT C-3′. The amplification condition was 93 °C for 3 min, 20 cycles of 93 °C for 45 s, 58 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. *Actin-2* transcripts were amplified as an internal control for normalization using primers 5′-TGG TGT CAT GGT TGG GAT G-3′ and 5′-CAC CAC TGA GCA CAA TGT TAC-3′. Three independent replicates were performed and the products were analyzed on agarose gels using the Quantity One software (Bio-Rad).

### 2.4. Membrane protein extraction

Flowers (1–2 g fresh weight) of *Arabidopsis* or cell cultures harvested and frozen in liquid $N_2$ were homogenized in 4 ml of extraction buffer containing 50 mM Tris–HCl pH 8.0, 5 mM EDTA, 250 mM sucrose and 1 mM PMSF. The homogenate (total protein) was spun briefly at 3000 × *g* for 4 min. The supernatant was further centrifuged at 10,000 × *g* for 10 min to remove cell debris. The resulting supernatant was then centrifuged at 100,000 × *g* for 2 h. The supernatant was used as the source for soluble proteins, while the pellet containing total membrane proteins was dissolved in 200 μl of the extraction buffer containing 0.25% digitonin.

### 2.5. Western blot analysis

Total membrane proteins (10 μl) were mixed with 5 μl of 10% SDS and 5 μl of 5× PAGE loading buffer. The samples were loaded and run on an 8% acylamide-SDS gel with a running buffer containing 0.4% SDS. The proteins were transferred to PVDF membrane (GE Healthcare) in a transfer buffer containing 0.05% SDS, 25 mM Tris, 192 mM glycine, 20% methanol at 160 mA (or 16 mA/cm) for 24 h. The PVDF membrane was blocked with 5% non-fat milk for 5 h, and incubated for overnight at 4 °C with the rabbit polyclonal antibodies (1:1000) raised against the purified recombinant protein corresponding to the CalS5 peptides. After incubation with the second antibody conjugated with the horseradish peroxidase (HRP), the membrane was submersed in SuperSignal West Pico Chemiluminescent Substrate from Pierce and exposed to X-ray film.

### 2.6. Callose staining and measurement

For staining of callose in BY-2 cells, 5 day-old cell culture was washed with 0.1 M $K_2HPO_4$ (pH 8.5) and stained with 0.01% aniline blue (Sigma–Aldrich) for 10 min. The presence of callose (blue–yellow fluorescence) was detected using a fluorescence microscope with a UV filter. For quantitative measurements of callose content, plant samples were extracted with 1 N NaOH at 80 °C. Supernatants were mixed with aniline blue solution (40 vol of 0.1% aniline blue in water, 21 vol of 1 N HCl, and 59 vol of 1 M glycine/NaOH buffer, pH 9.5). Fluorescence was measured using a fluorospectrometer (Jobin-Yvon/SPEX) as described previously [24].

### 2.7. Subcellular colocalization

For immunofluorescent staining of CalS5, 5-day-old transgenic BY-2 cells were fixed for 30 min in a buffer containing 4% paraformaldehyde, 100 mM PIPES (pH 7.0), 2 mM $MgCl_2$, 5 mM EGTA and 2% glycerol. After a 30 min treatment with a cell wall digest buffer containing 1.5% cellulase, 0.1% macerozyme R-10, 3% mannitol, 20 mM MES (pH 5.7), 20 mM KCl and 10 mM $CaCl_2$, the cells were permeabilized by 0.1% Triton X-100 for 20 min. The cells were blocked by 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h and incubated with CS5-L primary antibody (1:200 dilution in PBS containing 1% BSA) overnight. After washes in PBS, the cells were incubated with Alexa Fluor 594-conjugated chicken anti-rabbit IgG (Molecular Probes) diluted 1:1000 in PBS containing 1% BSA for 3 h and then washed with PBS.

For staining of the Golgi apparatus, BY-2 cells were incubated with 5 μM BODIPY TR ceramide (Molecular Probes) for 30 min in ice and 30 min at 37 °C. They were then washed with BY-2 medium

for 3 times. For staining of brefeldin A (BFA)-treated Golgi apparatus, BY-2 cells were first incubated in growth medium with 10 μg/ml BFA (Sigma–Aldrich) for 1 h, and then with 5 μM BODIPY TR ceramide. The cells were washed with BY-2 medium containing 10 μg/ml BFA. For staining of the endosomes, BY-2 cells supplemented in growth medium with or without 10 μg/ml BFA were incubated with 20 μM FM4-64 (Molecular Probes) for 30 min at room temperature.

### 2.8. Analysis of plasmolysis and cytolysis

For the plasmolysis rate determination, 5 day-old suspensions of BY-2 cells were harvested by centrifugation (3 min at 1000 × *g*), and resuspended in a solution containing 0%, 2%, 4%, 6%, 8% or 10% mannitol. After 30 min, the percentage of plasmolyzed cells having round-shaped cytoplasm retracted from the cell wall was measured under a light microscope. For the cytolysis rate determination, cells were treated in a solution containing 0%, 2% or 4% mannitol for 4 h, and the percentage of the cells showing shrunk and abnormal shaped cytoplasm were measured. 60–80 cells in one field of vision were observed in each measurement, and four independent measurements were performed for each sample.

### 2.9. Z-projection image, confocal and electron microscopy

Fluorescent images of BY-2 cells were taken with a 3 μm vertical interval and Z-projection images were created with ImageJ software (NIH). In confocal microscope detection, BY-2 cells were observed with a Zeiss LSM510 laser scanning confocal microscope with 405 nm excitation wavelength and 420–480 BP emission filters (blue) for aniline blue stained callose, or 488 nm excitation wavelength and 505–530 BP emission filters (green) for GFP. The 543 nm excitation wavelength and 560 LP emission filter (red) was used to detect the staining with Alexa Fluor 594 and BODIPY TR ceramide. The 514 nm excitation wavelength and 650 LP emission filter (red) was used to detect the staining with FM4-64. For colocalization, confocal images were taken from 15 different cells and the quantitation of colocalization of two signals were done as previously described [25].

## 3. Results

### 3.1. Cloning of a CalS5 cDNA and generation of antibody against CalS5 peptides

For protein expression purpose, we cloned the full-length coding region of the CalS5 cDNA by reverse transcription (RT)-PCR using total RNA isolated from *Arabidopsis* inflorescences. DNA sequencing of the cloned CalS5 cDNA confirmed the presence of 39 exons as predicted [5]. Two recombinant peptides corresponding to the N-terminal portion (CS5-N, amino acid residues 98–238) and the loop region (CS5-L, residues 898–1025) of the CalS5 peptide were expressed, purified and used to raise polyclonal antibodies in rabbits. Antibodies to both peptides were used for assessing the CalS5 protein expression levels in transgenic BY-2 cells and *Arabidopsis* tissues.

### 3.2. Expression of CalS5 in cultured tobacco BY-2 cells

CalS5 was expressed as a fusion protein with the green fluorescent protein tag (GFP) at the N-terminus (G-CS5; Fig. 1A) in cultured tobacco BY-2 cells. A total of 30 independent transgenic tobacco cell lines with stable kanamycin resistance were obtained and confirmed by genomic PCR. Four transgenic lines (#28, 22, 17 and 10) were randomly chosen for analysis of the expression of the *CalS5* mRNA and protein. Both *CalS5* mRNA and protein were readily detected in these transgenic lines (Fig. 1B and C), indicating CalS5 was stably expressed in tobacco BY-2 cells. Line #10 with a moderate expression level of G-CS5 was chosen for further cell biology studies on the subcellular localization of G-CS5 and callose deposition in BY-2 cells.

The polypeptide encoded by the *CalS5* gene was predicted to be a membrane protein with 16 transmembrane domains [5]. In the western blot analysis, a band of approximately 220 kDa in the total membrane fraction of wild type plants could be detected by the CS5-L or CS5-N antibodies (Fig. 1D and E). The band was not present in the membranes of *cals5* plants, or in the soluble fractions of both genotypes. To verify the membrane association of CalS5 protein in tobacco BY-2 cells, we prepared membrane and soluble fractions from cultured BY-2 cells expressing G-CS5. The antibodies CS5-N did not react with proteins from the BY-2 cells and GFP-expressing control (Fig. 1C and E lanes 3, 5). A protein band was detected in the membranes of BY-2 cells expressing G-CS5 (Fig. 1E lane 2). With the GFP tag (26 kDa), the band appeared slightly larger in apparent molecular weight than the one from *Arabidopsis* plants on SDS-PAGE (Fig. 1E lane 1).

### 3.3. Subcellular localization of CalS5 in tobacco BY-2 cells

In non-dividing cells, while GFP alone was found in the cytoplasm and the nucleoplasm (Fig. 2A), G-CS5 was found at the plasma membrane and in endomembrane structures inside the cell (Fig. 2B). Such a localization pattern was also detected in most of the other transgenic cell lines expressing G-CS5 (Supplementary Fig. S1A). We also compared the subcellular localization of CalS5 in tobacco BY-2 cells with the cell plate-specific CalS1 [20] during cell division. While GFP alone was not present in the center of the cell plate in dividing cells (arrowhead in Fig. 2E), G-CS1 was specifically localized to the cell plate (Fig. 2F). However, G-CS5 was found uniformly in the plasma membrane and in the cell plate vicinity, but was not present as a concentrated band at the cell plate (Fig. 2C and D). These data clearly demonstrate that different isoforms of CalS characteristically localize to different subcellular compartments [2].

### 3.4. Colocalization of CalS5 with the Golgi apparatus and endosomes

As most of plasma membrane proteins are targeted through the Golgi apparatus, and recycled by endocytosis through the endosomes, we further examined the colocalization of the endomembrane-localized G-CalS5 with Golgi apparatus marker BODIPY TR ceramide and endosome marker FM4-64. Colocalization was performed by treatment with brefeldin A (BFA), a fungal toxin that blocks the secretory pathway in eukaryotic cells between the ER and the Golgi. In BY-2 cells, brefeldin A is known to induce the formation of a Golgi-ER hybrid compartment [26,27].

Before the colocalization tests, BY-2 cells expressing G-CS5 were reacted with CS5-L primary antibody to verify the subcellular localization pattern of G-CS5. Consistent with the above results, most of the immunofluorescent G-CS5 signals were targeted to the plasma membrane and endomembrane structures. These immunofluorescent signals were well colocalized with the green fluorescence of G-CS5 (Fig. 3A–C). In the control BY-2 cells expressing GFP alone, no immunofluorescent signal was detected (Fig. 3D), suggesting that the CS5-L primary antibody was specific.

In the colocalization tests (Fig. 3E–G), a majority of the endomembrane structures of G-CalS5 (65.3 ± 13.7%) was co-localized with the BODIPY TR-stained Golgi apparatus, while the remaining structures were in close vicinity of the Golgi apparatus. When treated with BFA, most of the endomembrane structures

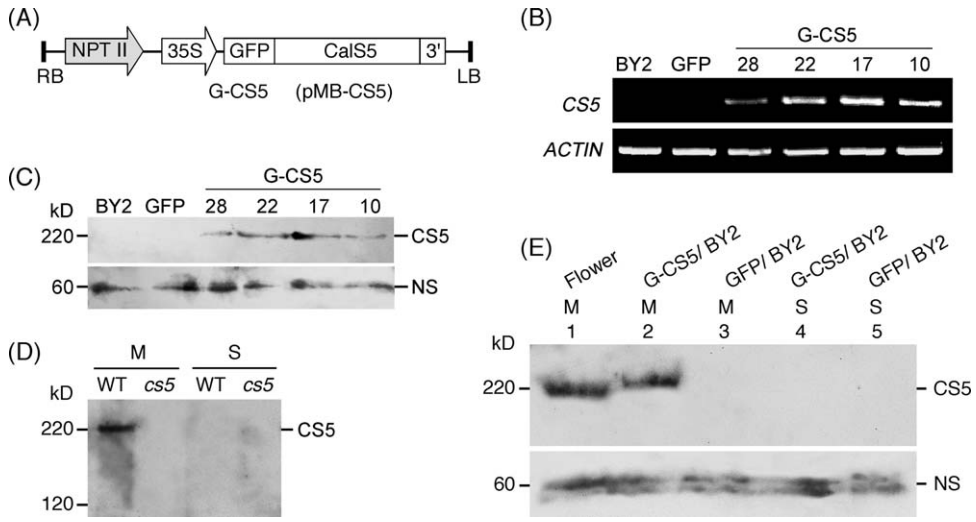


**Fig. 1.** Expression of CalS5 in transgenic tobacco BY-2 cells. (A) Map of CalS5 expression vector pMB-CS5. The N-terminus of CalS5 was fused with GFP (G-CS5) and expressed under the control of the CaMV 35S promoter. RB and LB, right and left borders of the T-DNA; *NPT* II neomycin phosphotransferase (kanamycin resistance). (B) RT-PCR analysis of *CalS5* gene expression in transgenic tobacco BY-2 cell lines 28, 22, 17 and 10, and in control BY-2 cells and BY-2 cells expression GFP. *CS5*, amplified PCR product of the *CalS5* mRNA. *ACTIN*, amplified *Actin* PCR product used as an internal control. Three replications were conducted and one representative result is shown. (C) Western blot of total proteins of tobacco BY-2 cells as detected by the polyclonal antibodies against CalS5N. NS, non-specifically reacting protein bands that served as an internal control. Shown is one representative Western blot from three replications conducted. (D) Western blot of total membranes (M) and soluble (S) fractions of *Arabidopsis* flower buds as detected by polyclonal antibodies against CalS5L. (E) Western blot of BY-2 cells expressing GFP (lanes 3, 5) or G-CS5 (lanes 2, 4) as detected by the CalS5N antibodies. The membrane fraction (M) of wild type *Arabidopsis* flower buds (lane 1) was used as a positive control. NS, non-specifically reacting protein bands that served as an internal loading control. M, total membrane fraction; S, soluble fraction.

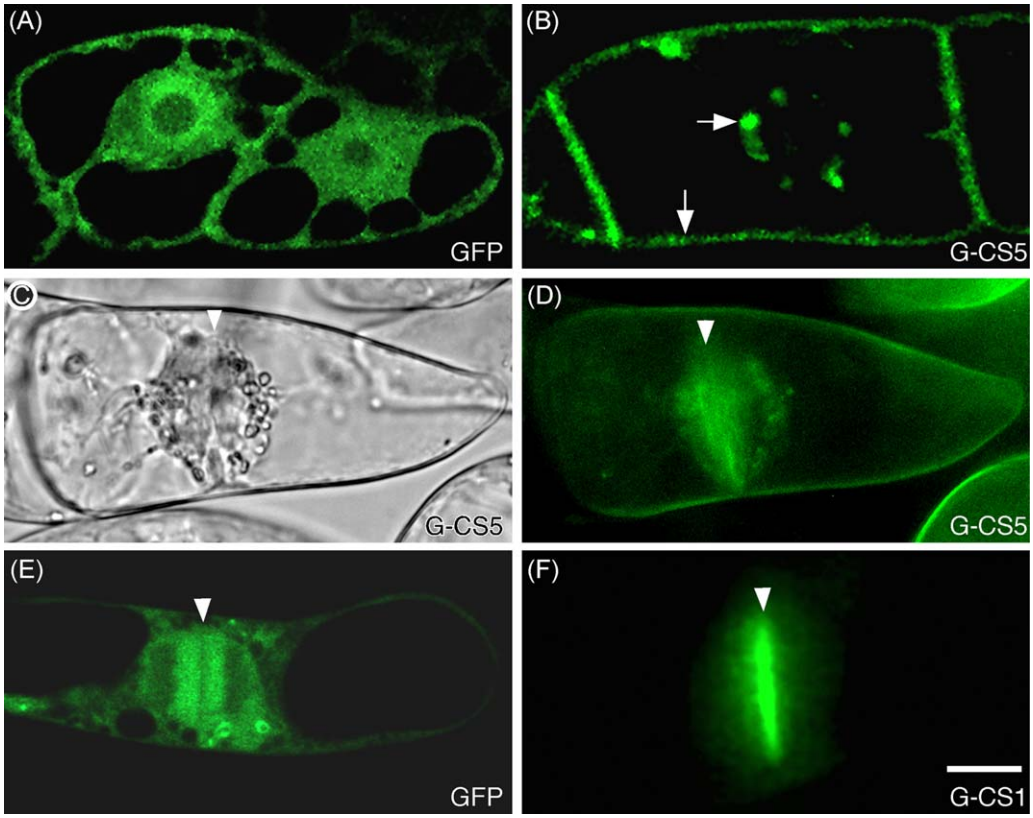


**Fig. 2.** Subcellular localization of CalS5 in BY-2 cells expressing G-CS5. (A and B) Confocal image of non-dividing BY-2 cells expressing GFP (A) or G-CS5 (B). (C) Transmission image of a cytokinetic tobacco BY-2 cell expressing G-CS5. (D) Fluorescence view of (C). CalS5 was localized to the plasma membrane, endomembrane structures and cell plate vicinity at cytokinesis. (E and F) Confocal images of the dividing BY-2 cells expressing GFP alone (E) or GFP tagged CalS1 (F). Free GFP was excluded from the cell plate (arrowhead), whereas GFP-tagged CalS1 was concentrated to the cell plate at cytokinesis. Bars, 10 μm.

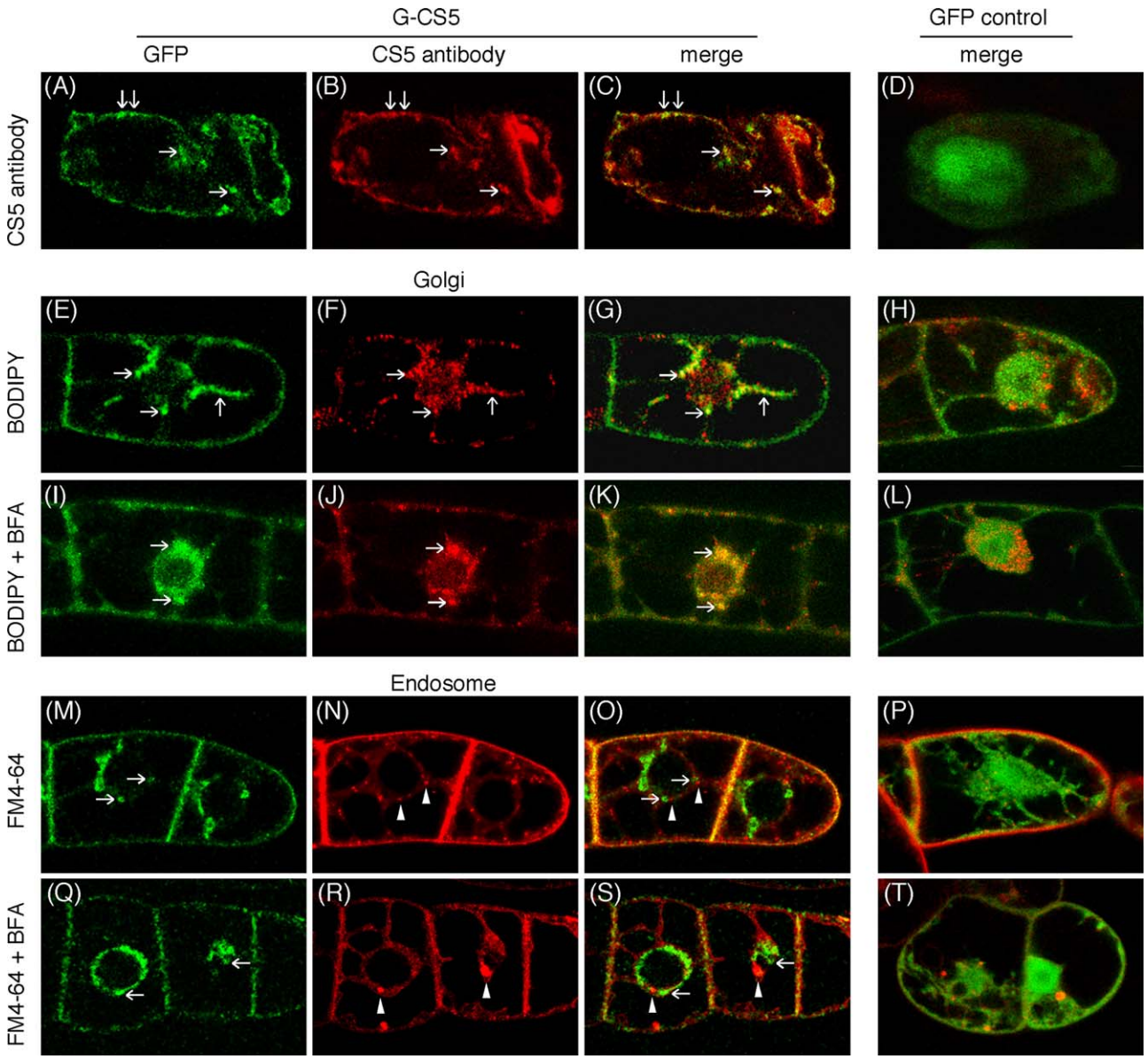


**Fig. 3.** Immunofluorescent staining and colocalization of GFP-CalS5 (G-CS5) in BY-2 cells. Cells expressing G-CS5 were imaged using the GFP-fluorescent tag (A, E, I, M and Q), CS5-L antibody (B), BODIPY TR ceramide (BODIPY) marker (F and J), or FM4-64 (N and R). They were superimposed to generate the overlaid images shown in C, G, K, O, and S, respectively. Control cells expressing GFP alone were shown only in the corresponding overlaid images (D, H, L, P and T). (A–D) Immunofluorescent staining of CalS5 in BY-2 cells. Arrows indicate the colocalization of G-CS5 (green) and CS5-L antibody-immunofluorescent signals (red). (E–L) Colocalization of G-CS5 (green) with the Golgi apparatus (red) as stained with BODIPY TR ceramide without BFA (E–G) or with BFA treatment (I–K). Arrows indicate the colocalization of G-CS5 (green) with the Golgi marker signals (red). (M–T) Colocalization of G-CS5 (green) with the endosomes as stained with FM4-64 (red) without BFA (M–O) or with BFA treatment (Q–S). Arrows and arrowheads indicate the different localizations of G-CS5 (arrows) and endosome marker signals (arrowheads). Bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(83.0 ± 3.9%) was redistributed to the perinuclear membrane structures and well co-localized with the BFA-induced Golgi-ER hybrid compartment (Fig. 3I–K). However, only 9.0 ± 1.0% of G-CalS5 was colocalized with FM4-64 stained endosomes, and 23.4 ± 3.4% of G-CalS5 was colocalized with the BFA-induced endosome compartments (Fig. 3M–O, Q–S). In control cells expressing GFP alone (Fig. 3H, L, P and T), only 8.1 ± 3.7%, 13.3 ± 3.7%, 4.1 ± 0.1% and 5.8 ± 2.8% of the GFP signals were colocalized with the Golgi apparatus and endosomes in the conditions (with and without BFA) as indicated in Fig. 3, respectively. These results suggest that the endomembrane-localized G-CalS5 is mainly associated with the Golgi apparatus.

### 3.5. Callose accumulation in BY-2 cells expressing CalS5

In control BY-2 cells, callose was present at high concentrations in the forming cell plate at cytokinesis (Fig. 4A and B). Callose also remained detectable in the newly formed cell wall, *i.e.* in the adjacent area of two newly formed daughter cells (Fig. 4C and D). However, in BY-2 cells expressing G-CS5, callose deposition was detected on the whole cell wall (Fig. 4E and F). Under a confocal microscope, callose could be detected only in the cell wall (Fig. 4H–J), but not inside the cell (Fig. 4H), although G-CS5 was clearly present in the plasma membrane as well as in the endomembrane structures (Fig. 4G and I, arrows). This suggests

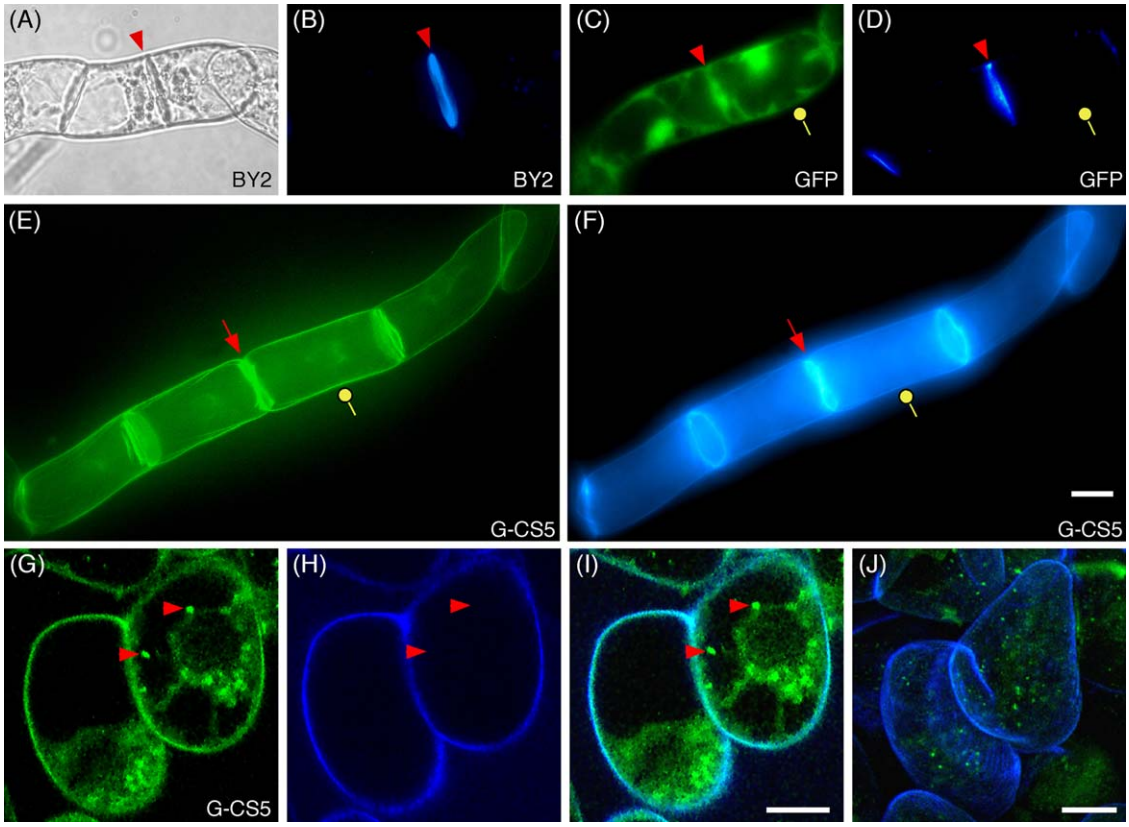


**Fig. 4.** Accumulation of callose in BY-2 cells expression GFP-CalS5 (G-CS5). (A and B) Transmission image (A) and the corresponding fluorescence view (B) of a cytokinetic BY-2 cell stained with aniline blue. Note that callose was detected at the cell plate (arrowhead) but not on the cell surface. (C–F) Z-projected epifluorescence images of BY-2 cells expressing GFP (C and D) or G-CS5 (E and F). Callose was deposited at the adjacent cell wall of newly divided cells (red arrows) in GFP and G-CS5 cells (D and F), and only in the cell wall of G-CS5 cells (F, yellow circles). (G) Single layer confocal images of the GFP signals of G-CS5 in BY-2 cells. (H) Callose detection of the corresponding layer of (G). (I) Overlaid image of (G) and (H). (J) Projection of confocal images of the same cells in (I). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

that CalS5 might be functional only in the plasma membrane. Such callose deposition pattern could also be detected in other BY-2 cell lines expressing G-CS5 (Supplementary Fig. S1A). Callose content in the extract of the transgenic G-CS5 cells was also significantly higher than that in the control cells in the quantitative measure (Supplementary Fig. S1C).

### 3.6. *Altered plasmolysis and cytolysis in transgenic BY-2 cells*

On the basis of the observation that callose was highly accumulated in the cell wall of BY-2 cells expressing G-CS5, we speculated water movement through the cell wall might be affected by the accumulated callose. We measured the plasmolysis or cytolysis rates of BY-2 cells treated with mannitol solutions. When treated in the solutions containing different concentrations of mannitol for 30 min, the G-CS5 cells showed a lower plasmolysis rate than the control BY-2 cells and GFP-expressing cells (Fig. 5A), indicating that CalS5-expressing cells were more tolerant to osmotic stress treatments.

When cells are treated in a hypotonic solution, the cytoplasm absorbs more water and exhibits turgor, which keeps the plasma membrane closely attach to the cell wall and could lead to cytolysis if the cell wall is significantly altered. In the control BY-2 cells and GFP-expressing cells treated with the hypotonic solutions, burst or cytolyzed cytoplasm was rarely found (Fig. 5J and M). However, a significant number of the G-CS5 cells, when suspended in water for 4 h, were found to contain shrunk cytoplasm that was detached from the cell wall (Fig. 5K). This type of cells was referred to as “cytolyzed cells” in our work. The percentage of cytolyzed G-CS5 cells decreased as the mannitol concentrations increased to 4% (Fig. 5J). All these results suggested water permeability was altered significantly in the G-CS5 cells.

We also examined callose deposition in BY-2 cells under the hypertonic or hypotonic conditions. A thick layer of callose deposition could be detected in the compartment between the primary cell wall and plasma membrane in the plasmolyzed G-CS5 cells (Fig. 5B–E). This layer of callose appeared to be separable from the native cell wall (Fig. 5B). In plasmolyzed G-CS5 cells, most of the fluorescence signals were retracted with the plasma membrane, confirming its localization in the plasma membrane instead of the cell wall (Fig. 5C). When G-CS5 cells were suspended in water for 4 h, abnormal callose deposition was detected around the shrunk plasma membrane in the cytolyzed-like G-CS5 cells (Fig. 5K and L). A much lower level of callose deposition could also be detected in the primary cell wall of the control cells expressing GFP (Fig. 5H and N), probably due to a stress response. However, no significant callose deposition was detected in the compartment induced by plasmolysis (Fig. 5H and I) or in the intracellular space caused by the hypotonic treatment (Fig. 5N).

## 4. Discussion

Twelve callose synthases have been identified in the *Arabidopsis* genome based on the sequence homology with the β-1,3-glucan synthase catalytic subunit FKS1 of yeast [20]. These callose synthases are expressed in tissue- and developmental stage-specific manners as well as in response to different physiological and environmental inducers [2–15,20,28–30]. However, the subcellular

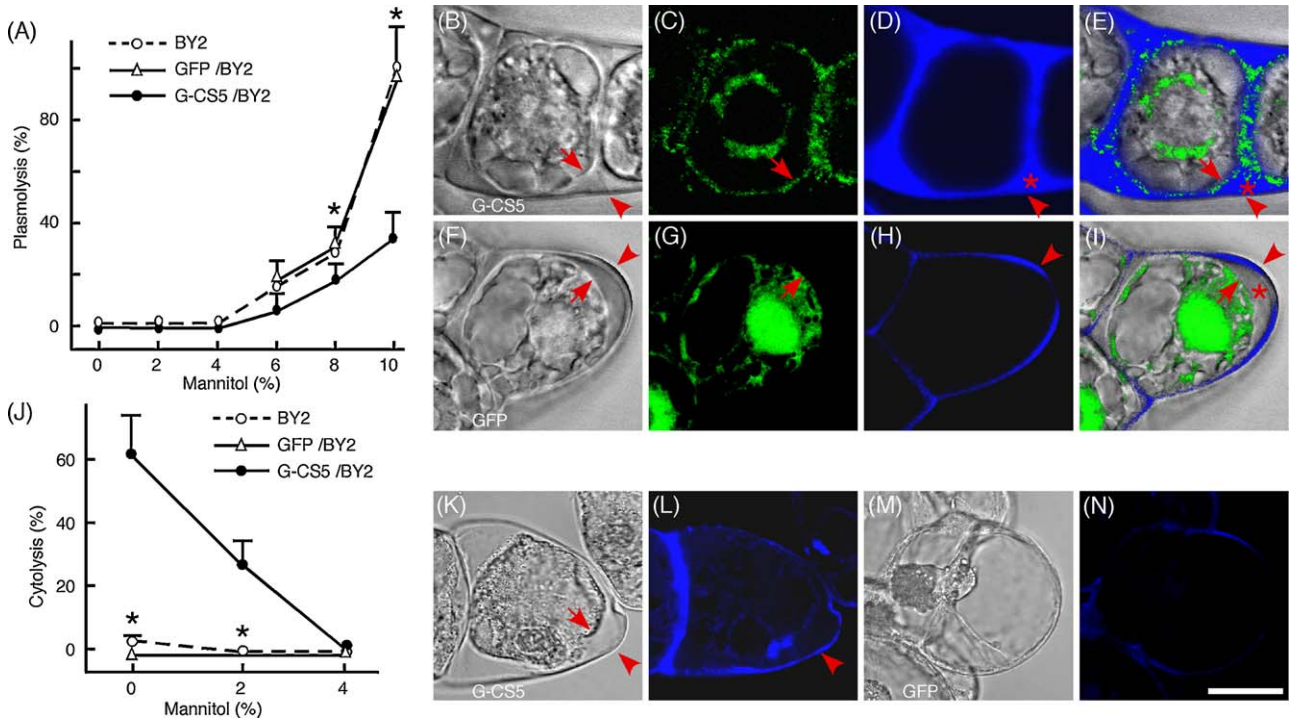


**Fig. 5.** Altered water permeability in tobacco BY-2 cells expressing G-CS5. (A) Percentages of plasmolyzed tobacco BY-2 cells (BY2), GFP-expressing cell line (GFP/BY2) and transgenic line 10 (G-CS5/BY2) treated with different concentrations of mannitol for 30 min. (B–I) Single layer confocal images of the plasmolyzed cells expressing G-CS5 (B–E) or GFP control (F–I) treated with an 8% mannitol solution for 30 min. Images of the same cells with transmission light (B and F), green fluorescence (C and G) and UV fluorescence (D and H) showing aniline blue-stained callose, were superimposed to generate overlaid images (E and I). (J) Percentages of cytolyzed tobacco BY2, GFP/BY2 and transgenic line 10 (G-CS5/BY2) treated with different concentrations of mannitol for 4 h. (K–N) Single layer confocal images of cytolyzed cells expressing G-CS5 (K and L) or GFP control (M and N) after suspension in water for 4 h. The cells were imaged using transmission light (K and M) and UV light for callose (L and N). Stars (*) in (B, D and I) indicate the compartment between the primary cell wall (arrowheads) and plasma membrane (arrows). Bar, 10 μm in (B–I) and (K–N). Error bars in (A and J) were the means + standard deviations from four independent measurements. Stars (*) in (A and J) indicate a significant difference between the values of the G-CS5 transgenic and control cells (two sample *t*-test, $p \leq 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

localizations and functions of the callose synthases are less studied, except the cell plate specific CalS1 and CalS7. CalS1 may form a complex with a UDP-glucose transferase and is localized at the cell plate of cytokinetic cells [20,31], whereas CalS7 is required for callose deposition at the sieve plate [7,32]. In this report, we expressed GFP tagged CalS5, a key *Arabidopsis* CalS isoform responsible for callose synthesis during pollen development and pollen tube growth, in tobacco BY-2 cells to examine its subcellular localization and function. We identified that CalS5 was localized to the plasma membrane and Golgi-associated endomembrane structures, and was contrast to the cell plate-specific localization of CalS1 during cytokinesis. The data demonstrate that different CalS isoforms may localize to different subcellular compartments, and suggest that each CalS may play a special cellular function in response to different development requirements.

There has not been a study on the use of callose synthase for the purpose of plant cell wall modification. Our data show that an artificial callose wall could be synthesized in tobacco BY-2 cells by the expression of a callose synthase isoform. This offers a new opportunity for genetic engineering of plants with improved cell wall polysaccharides. The observed results are also consistent with the proposed function of CalS5 in the formation of the callose wall during microsporogenesis [5,6], and suggest that cultured tobacco cells contain all necessary factors required for the CalS5 activity. Previous reports show that CalS enzyme activity is regulated by a series of intracellular and extracellular factors, including $Ca^{2+}$, $Mg^{2+}$ and β-glucoside [33], calmodulin [34], annexin [35] and CalS associated proteins [20,28]. The enzyme activity in *Nicotiana alata* pollen tube extracts can be increased by treatments with detergents and trypsin [17–19,36,37], suggesting that an activation process of the protein from an inactive form, zymogen, may exist *in vivo*. In our results, although GFP-tagged CalS5 was present in both the plasma membrane and the endomembrane structures, a massive amount of callose was detected only in the cell wall. This suggests that the plasma membrane-associated CalS5 appears to be the 'active' form of the enzyme. The fact that callose was not detected in the endomembranes of the transgenic cells (Fig. 5) indicates that the endomembrane-localized CalS5 may be inactive. This is consistent with a previous report on the callose synthase (NaGSL1) from *N. alata* pollen tube, which is only activated in the plasma membrane in spite of its multiple subcellular localizations in the ER, Golgi, intracellular vesicles and plasma membrane [19]. However, previous report also showed that callose can be detected in the Golgi vesicles in the pollen tubes of *Camellia japonica* by immunogold labeling [38]. It is not clear what contributed to the inactivity of the CalS5 enzyme in this intracellular location. Future research in this direction may eventually reveal a mechanism for CalS5 posttranslational processing and activation.

Callose has long been considered as a permeability barrier or leak sealant in plant cells [1]. During seed development in muskmelon, callose is synthesized and deposited into a thick layer surrounding the endosperm envelope cells. This callose layer is required for the osmotic distention of muskmelon seeds and responsible for the apoplastic semipermeability, which allows the movement of water but not of solutes into endosperm cells [39]. Consistent with this, callose extracted from developing seeds has been shown to have a high water holding capacity [40]. In contrast, a previous report on callose deposited on the clover seed coat has suggested that the callose layer is impermeable to water [41].

In this study, we took advantage of the availability of BY-2 cells expression G-CS5 and examined the relationship between callose accumulation and water permeability in plant cells. The G-CS5 cells showed a lower plasmolysis rate than the control cells in the hypertonic solutions (Fig. 5), suggesting that the G-CS5 cells may have a reduced rate of water movement from the cytoplasm to the medium, or may have an unidentified mechanism by which an equilibrium of mannitol concentration between the cytoplasm and the surrounding medium is achieved at a faster rate. When treated with hypotonic solutions, the G-CS5 cells had a higher percentage of cytolyzed cells, implying that the accumulated callose may render the cell wall less rigid and more sensitive to the cytoplasmic turgor. These altered cell wall properties of BY-2 cells by expressing CalS5 are likely resulted directly from the accumulated callose in the cell wall. This conclusion is consistent with a proposed role of the thick callose wall present in developing microspores in plants, *i.e.* allowing fast absorption of water and nutrition from the tapetum layer [1,4]. However, the expression of G-CS5 may also induce other cellular changes, including the intracellular osmotic potential, adjustment to nutrient stress, and physical properties of the plasma membrane, which can contribute together to the abnormal plasmolysis and cytolysis. All these alternations remain to be determined in future work, which may shed new light into the biological function of callose in cell structure, water retention and cell protection in plants.

## Acknowledgements

We thank Dr. Allan Caplan for critical comments on the manuscript and Ms. Shizhen Gu and Ms. Duanning Wang for technical assistance. We also thank ABRC (Ohio State University) for seeds of *Arabidopsis* T-DNA insertional lines. This work was supported by grants from the National Science Foundation (Grant Numbers MCB-0548525 and IOB-0543923). M.M.K. was supported by a grant from the Kyoto University Foundation.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2011.10.015.

## References

[1] B.A. Stone, A.E. Clarke, Chemistry and physiology of higher plant 1,3-β-glucans (callose), in: Chemistry and Biology of (1,3)-β-Glucans, La Trobe University Press, Victoria, Australia, 1992, pp. 365–429.
[2] D.P.S. Verma, Z. Hong, Plant callose synthase complexes, Plant Mol. Biol. 47 (2001) 693–701.
[3] X. Dong, Z. Hong, J. Chatterjee, S. Kim, D.P.S. Verma, Expression of callose synthase genes and its connection with *Npr1* signaling pathway during pathogen infection, Planta 229 (2008) 87–98.
[4] T.A. Richmond, C.R. Somerville, Integrative approach to determining Csl function, Plant Mol. Biol. 47 (2001) 131–143.
[5] X. Dong, Z. Hong, M. Sivaramakrishnan, M. Mahfouz, D.P.S. Verma, Callose synthase (CalS5) is required for exine formation during microgametogenesis and for pollen viability in *Arabidopsis*, Plant J. 42 (2005) 315–328.
[6] S. Nishikawa, G.M. Zinkl, R.J. Swanson, D. Maruyama, D. Preuss, Callose (beta-1,3 glucan) is essential for *Arabidopsis* pollen wall patterning, but not tube growth, BMC Plant Biol. 5 (2005) 22.
[7] B. Xie, X. Wang, M. Zhu, Z. Zhang, Z. Hong, CalS7 encodes a callose synthase responsible for callose deposition in the phloem, Plant J. 65 (2011) 1–14.
[8] D.H. Barratt, K. Koelling, A. Graf, M. Pike, G. Calder, K. Findlay, S.C. Zeeman, A.M. Smith, Callose synthase GSL7 is necessary for normal phloem transport and inflorescence growth in *Arabidopsis*, Plant Physiol. 155 (2011) 328–341.
[9] A. Toller, L. Brownfield, C. Neu, D. Twell, P. Schulze-Lefert, Dual function of *Arabidopsis glucan synthase-like* genes *GSL8* and *GSL10* in male gametophyte development and plant growth, Plant J. 54 (2008) 911–923.
[10] L. Huang, X.Y. Chen, Y. Rim, X. Han, W.Y. Cho, S.W. Kim, J.Y. Kim, Arabidopsis Glucan Synthase-Like 10 functions in male gametogenesis, J. Plant Physiol. 166 (2009) 344–352.
[11] B. Xie, X. Wang, Z. Hong, Precocious pollen germination in *Arabidopsis* plants with altered callose deposition during microsporogenesis, Planta 231 (2010) 809–823.
[12] J.M. Guseman1, J.S. Lee1, N.L. Bogenschutz, K.M. Peterson1, R.E. Virata, B. Xie, M.M. Kanaoka, Z. Hong, K.U. Torii, Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in *Arabidopsis CHORUS* (*GLUCAN SYNTHASE-LIKE 8*), Development 137 (2010) 1731–1741.
[13] K. Thiele, G. Wanner, V. Kindzierski, G. Jürgens, U. Mayer, F. Pachl, F.F. Assaad, The timely deposition of callose is essential for cytokinesis in *Arabidopsis*, Plant J. 58 (2009) 13–26.
[14] X.Y. Chen, L. Liu, E. Lee, X. Han, Y. Rim, H. Chu, S.W. Kim, F. Sack, J.Y. Kim, The *Arabidopsis* callose synthase gene *GSL8* is required for cytokinesis and cell patterning, Plant Physiol. 150 (2009) 105–113.
[15] L.C. Enns, M.M. Kanaoka, K.U. Torii, L. Comai, K. Okada, R.E. Cleland, Two callose synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen development and in fertility, Plant Mol. Biol. 58 (2005) 333–349.
[16] T. Yamaguchi, T. Hayashi, K. Nakayama, S. Koike, Expression analysis of genes for callose synthases and Rho-type small GTP-binding proteins that are related to callose synthesis in rice anther, Biosci. Biotechnol. Biochem. 70 (2006) 639–645.
[17] M.S. Doblin, L. De Melis, E. Newbigin, A. Bacic, S.M. Read, Pollen tubes of *Nicotiana alata* express two genes from different β-glucan synthase families, Plant Physiol. 125 (2001) 2040–2052.
[18] L. Brownfield, K. Ford, M.S. Doblin, E. Newbigin, S. Read, A. Bacic, Proteomic and biochemical evidence links the callose synthase in *Nicotiana alata* pollen tubes to the product of the *NaGSL1* gene, Plant J. 52 (2007) 147–156.
[19] L. Brownfield, S. Wilson, E. Newbigin, A. Bacic, S. Read, Molecular control of the glucan synthase-like protein NaGSL1 and callose synthesis during growth of *Nicotiana alata* pollen tubes, Biochem. J. 414 (2008) 43–52.
[20] Z. Hong, A.J. Delauney, D.P.S. Verma, A cell-plate specific callose synthase and its interaction with phragmoplastin, Plant Cell 13 (2001) 755–768.
[21] K. Kato, T. Matsumoto, S. Koiwai, S. Mizusaki, K. Nishida, M. Nogushi, E. Tamaki, Liquid suspension culture of tobacco cells, in: G. Terui (Ed.), Fermentation Technology Today, Japan, Osaka, Soc. Ferment. Technol., 1972, pp. 689–695.
[22] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497.
[23] S.Z. Pang, D.L. DeBoer, Y. Wan, G. Ye, J.G. Layton, M.K. Neher, C.L. Armstrong, J.E. Fry, M.A. Hinchee, M.E. Fromm, An improved green fluorescent protein gene as a vital marker in plants, Plant Physiol. 112 (1996) 893–900.
[24] E. Shedletzky, C. Unger, D.P. Delmer, A microtiter-based fluorescence assay for (1,3)-beta-glucan synthases, Anal. Biochem. 249 (1997) 88–93.
[25] L. Jiang, J.C. Rogers, Integral membrane protein sorting to vacuoles in plant cells: evidence for two pathways, J. Cell Biol. 143 (1998) 1183–1199.
[26] C. Ritzenthaler, A. Nebenführ, A. Movafeghi, C. Stussi-Garaud, L. Behnia, P. Pimpl, L.A. Staehelin, D.G. Robinson, Reevaluation of the effects of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera, Plant Cell 14 (2002) 237–261.
[27] N. Emans, S. Zimmermann, R. Fischer, Uptake of a fluorescent marker in plant cells is sensitive to brefeldin A and wortmannin, Plant Cell 14 (2002) 71–86.
[28] L. Ostergaard, M. Petersen, O. Mattsson, J. Mundy, An *Arabidopsis* callose synthase, Plant Mol. Biol. 49 (2002) 559–566.
[29] A.K. Jacobs, V. Lipka, R.A. Burton, R. Panstruga, N. Strizhov, P. Schulze-Lefert, G.B. Fincher, An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation, Plant Cell 15 (2003) 2503–2513.
[30] M.T. Nishimura, M. Stein, B.H. Hou, J.P. Vogel, H. Edwards, S.C. Somerville, Loss of a callose synthase results in salicylic acid-dependent disease resistance, Science 301 (2003) 969–972.
[31] Z. Hong, Z. Zhang, J.M. Olson, D.P.S. Verma, A novel UDP-glucose transferase is part of the callose synthase complex and interacts with phragmoplastin at the forming cell plate, Plant Cell 13 (2001) 769–779.
[32] B. Xie, Z. Hong, Unplugging the callose plug from sieve pores, Plant Signal. Behav. 6 (2011) 491–493.
[33] T. Hayashi, S.M. Read, J. Bussell, M. Thelen, F.C. Lin, R.M.J. Brown, D.P. Delmer, UDP-glucose: (1 → 3)-beta-glucan synthases from mung bean and cotton: Differential effects of $Ca^{2+}$ and $Mg^{2+}$ on enzyme properties and on macromolecular structure of the glucan product, Plant Physiol. 83 (1987) 1054–1062.
[34] X. Cui, H. Shin, C. Song, W. Laosinchai, Y. Amano, R.M. Brown, A putative plant homolog of the yeast beta-1,3-glucan synthase subunit FKS1 from cotton (*Gossypium hirsutum* L.) fibers, Planta 213 (2001) 223–230.
[35] A. Andrawis, M. Solomon, D.P. Delmer, Cotton fiber annexins: a potential role in the regulation of callose synthase, Plant J. 3 (1993) 763–772.
[36] H. Li, A. Bacic, S.M. Read, Activation of pollen tube callose synthase by detergents. Evidence for different mechanisms of action, Plant Physiol. 114 (1997) 1255–1265.
[37] H. Li, A. Bacic, S.M. Read, Role of a callose synthase zymogen in regulating wall deposition in pollen tubes of *Nicotiana alata*, Planta 208 (1999) 528–538.
[38] Y. Hasegawa, S. Nakamura, N. Nakamura, Immunocytochemical localization of callose in the germinated pollen of *Camellia japonica*, Protoplasma 194 (1996) 133–139.
[39] K.O. Yim, K.J. Bradford, Callose deposition is responsible for apoplastic semipermeability of the endosperm envelope of muskmelon seeds, Plant Physiol. 118 (1998) 83–90.
[40] H.I. Vithanage, P.A. Gleeson, A.E. Clarke, The nature of callose produced during self-pollination in *Secale cereale*, Planta 148 (1980) 498–509.
[41] P.L. Bhalla, H.D. Slattery, Callose deposits make clover seeds impermeable to water, Ann. Bot. 53 (1984) 125–128.