CalS7 encodes a callose synthase responsible for callose deposition in the phloem

Bo Xie¹, Xiaomin Wang¹, Maosheng Zhu¹,², Zhongming Zhang² and Zonglie Hong¹,*

¹Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844, USA, and
²State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China

Received 1 May 2010; revised 13 August 2010; accepted 31 August 2010; published online 9 November 2010.
*For correspondence (fax +1 208 885 6518; e-mail zhong@uidaho.edu).

SUMMARY

It has been known for more than a century that sieve plates in the phloem in plants contain callose, a β-1,3-glucan. However, the genes responsible for callose deposition in this subcellular location have not been identified. In this paper we examine callose deposition patterns in T-DNA insertion mutants (cs7) of the Callose Synthase 7 (CalS7) gene. We demonstrated here that the CalS7 gene is expressed specifically in the phloem of vascular tissues. Callose deposition in the phloem, especially in the sieve elements, was greatly reduced in cs7 mutants. Ultrastructural analysis of developing sieve elements revealed that callose failed to accumulate in the plasmodesmata of incipient sieve plates at the early perforation stage of phloem development, resulting in the formation of sieve plates with fewer pores. In wild-type Arabidopsis plants, callose is present as a constituent polysaccharide in the phloem of the stem, and its accumulation can also be induced by wounding. Callose accumulation in both conditions was eliminated in mature sieve plates of cs7 mutants. These results demonstrate that CalS7 is a phloem-specific callose synthase gene, and is responsible for callose deposition in developing sieve elements during phloem formation and in mature phloem induced by wounding. The mutant plants exhibited moderate reduction in seedling height and produced aberrant pollen grains and short siliques with aborted embryos, suggesting that CalS7 also plays a role in plant growth and reproduction.

Keywords: Arabidopsis thaliana, callose, wound, vascular tissue, sieve plate.

INTRODUCTION

The vascular system, consisting of xylem and phloem, connects all parts of the plant, from the root system through the stem into leaves and developing seeds. In the stem of Arabidopsis, the xylem and phloem tissues are differentiated from the meristematic procambium cells that form a band sandwiched between the xylem and the phloem. In addition to providing physical support for the plant body, the xylem tissue is responsible for the transport of water and soluble mineral nutrients. The phloem is the ‘food and information superhighway’ of the plant, responsible for long-distance transport of carbohydrates, amino acids, RNA, proteins, fatty acids, hormones and other signaling molecules (Ruiz-Medrano et al., 2001; van Bel et al., 2002; Ye, 2002). Both xylem and phloem tissues contain various cell types, which can vary depending on growth and developmental stage (Carlsbecker and Helariutta, 2005; Evert, 2006). Recent studies have uncovered genes and pathways involved in the patterning and function of the vascular system (Bonke et al., 2003; Fukuda, 2004; Sieburth and Deyholos, 2006; Dettmer et al., 2009). However, the molecular mechanisms underlying the differentiation and functional regulation of the phloem tissue structures are poorly understood.

In angiosperms, the basic components of the phloem are the sieve elements and parenchyma cells. Other morphologically and physiologically specialized cells such as fibers, sclereids, laticifers and resin ducts can also be present in the phloem. The sieve elements, the primary conducting unit of the phloem, comprise longitudinal sieve tube elements and smaller adjacent companion cells, both of which are derived from the same precursor cells of sieve elements (Evert, 2006). The companion cells contain the nucleus with active cellular activities and play a pivotal role in functional regulation of the enucleate sieve tube elements (Scholz, 1997; Oparka and Turgeon, 1999). As they mature, the sieve tube elements undergo profound organelle degeneration including the breakdown of the nucleus, vacuoles, rough endoplasmic reticulum (ER) and Golgi, giving rise to an effective path for transport. At maturity, the sieve tube elements retain a plasma membrane and a reduced number...
of organelles including ER, plastids and mitochondria. The maturation of sieve tube elements also involves perforation of the cell wall, giving rise to the formation of the sieve plate and sieve areas. The sieve tube elements are linked end-to-end by the sieve plate, a unique specialized porous area of the cell wall. The side wall of sieve tube elements can also develop specialized porous zones, known as sieve areas, which are responsible for lateral transport. Sieve pores of the sieve plate and sieve areas are developed from the plasmodesmata. The process of sieve pore development is poorly understood, and the role of callose in this process is controversial.

Callose is a β-1,3-glucan that can be synthesized and degraded in a timely manner during plant development (Verma and Hong, 2001). It is believed that callose is deposited in the form of platelets around the plasmodesmata of the cell wall between two sieve element cells. This newly deposited callose is used to replace the cellulosic wall material surrounding the plasmodesmata (Evert, 2006). Upon dissolution of the callose and removal of the middle lamella cell wall material, a plate structure with widened pores is developed. According to this model, the amount of callose deposition around the sieve pores can also be rapidly induced by mechanical injury, chemical treatments and physiological stress conditions (Sjölund, 1997; Evert, 2006), which has been used as evidence to argue against a role for callose in sieve pore development. According to this theory, the detected presence of callose around the sieve pores is simply an artifact resulting from the response to sampling injury and chemical fixation. Thus, the molecular mechanism underlying callose biosynthesis and regulation in phloem remains an unanswered question in plant cell biology.

In Arabidopsis, there are 12 Callose Synthase genes (CalS; EC 2.4.1.34; UDP-glucose:1,3-β-D-glucosyl transferase). Multiple CalS genes appear to have evolved in plants to meet the need to produce callose in different tissues and in response to a range of biotic and abiotic stresses (Verma and Hong, 2001; Dong et al., 2008). CalS1 and CalS10 have been shown to be responsible for cell plate formation (Hong et al., 2001a,b; Thiele et al., 2008; Chen et al., 2009; Guseman et al., 2010). CalS5, CalS9, CalS10, CalS11 and CalS12 play unique roles during the processes of microsporogenesis and pollination (Dong et al., 2005; Enns et al., 2005; Nishikawa et al., 2005; Töller et al., 2008; Huang et al., 2009; Xie et al., 2010). CalS12 can be induced by pathogen infection and wounding (Jacobs et al., 2003; Nishimura et al., 2003; Dong et al., 2008). The physiological roles of the remaining CalS genes in Arabidopsis are still unknown.

The fact that sieve plates are rich in callose has been known for a century (Hartig, 1851; Eschrich, 1956), but the genetic pathway responsible for callose deposition in this location is poorly understood. The callose level in developing vascular tissues is reduced in mutant plants defective in the ALTERED PHLOEM DEVELOPMENT (APL) gene, which encodes a MYB factor required for phloem differentiation in Arabidopsis (Bonke et al., 2003). Callose of the sieve plates is reduced significantly in the double mutant of the phloem-specific Sucrose Synthase (SuSy) 5 and 6 (Barratt et al., 2009). Although these studies may have provided new evidence for metabolically influenced regulation of callose synthesis, the genes responsible for metabolically associated callose biosynthesis are still unknown. In this paper we provide genetic and anatomical evidence for a role for CalS7 in callose deposition in the phloem.

RESULTS
Isolation of CS7 mutants
Four independent mutant lines with T-DNA insertion in the At1g06490 locus (Figure 1a), SALK_048921 (cs7-1), SAIL_114_A01 (cs7-2), SAIL_232_C06 (cs7-3) and SALK_040051 (cs7-4), were obtained from the Arabidopsis Biological Resource Center (ABRC) and grown in greenhouse conditions. Genomic DNA, prepared from individual plants of each line, was used for the identification of homozygous mutant lines by genomic PCR using primers corresponding to the CalS7 gene and to the T-DNA (Figure 1b and S1a in Supporting Information, Table S1). Homozygous mutant lines were characterized with regards to their growth and reproduction phenotypes, ultrastructures of the phloem and callose deposition. The RT-PCR results showed that the cs7 mRNA could not be detected in homozygous mutant plants (Figures 1c and S1b), suggesting that they represent knockout mutants at the CalS7 locus.

Cloning of the CalS7 cDNA
We cloned the CalS7 cDNA (GenBank accession number HM049631) by PCR using RNA isolated from the wild-type plants. The full-length coding sequence (CDS) is 5802 bp and may encode a polypeptide of 1933 amino acid residues. Consistent with the other reported CalS peptides, CalS7 contains the highly conserved putative catalytic domain in the C-terminal half of the polypeptide (underline, Figure S2). Its N-terminal 500 amino acids represent a relatively unique domain in the CalS family. Whereas all studied CalS peptides contain 16 predicted transmembrane domains (TMDs), CalS7 may have 18 predicted TMDs (Figures 1d,e and S2). The two unique TMDs are located in the N-terminal 550 amino acid region. Because this protein is likely to be present in the sieve plate of the phloem, these two unique TMDs may play a role in targeting to this unique subcellular location.
development. When germinated on an agar plate containing MS medium, cs7 mutant seedlings developed much shorter roots than wild-type controls (Figures 2d and S3). This seedling growth defect could be partially rescued by supplementation of 1% glucose to the MS medium (Figures 2d and S3). How this partial rescue of growth defect by glucose was brought about remains to be resolved. These observations demonstrate the important roles of the CalS7 gene in plant development and reproduction.

Phloem-specific expression of CalS7

We performed a quick survey of the expression pattern of CalS7 by RT-PCR, which showed that CalS7 is expressed widely in all plant organs including the stem, roots, leaves, flowers and siliques (Figure 3a). To further understand the tissue specificity of CalS7 gene expression, we examined transgenic Arabidopsis plants expressing a β-glucuronidase reporter under the control of the CalS7 gene promoter, CalS7:GUS (Figure 3b). GUS activity was detected specifically in the vascular system of all plant organs examined (Figure 3c–k). Further analysis of the stem and leaf sections revealed that CalS7 is a phloem-specific gene and is not expressed in the xylem tissue (Figure 3c–f). Strong GUS activity could be detected in both sieve elements and companion cells (Figure 3d). Analysis of in situ hybridization using a labeled CalS7 mRNA probe also confirmed the presence of CalS7 gene expression in the phloem cells of the vascular tissue (Figure 3b). This tissue specificity of gene expression implicates that CalS7 may be involved in callose biosynthesis in the phloem.

Lack of callose deposition in the vascular system of cs7 mutants

In order to test if callose deposition in various plant tissues would be altered in cs7 mutants, we used aniline blue dye to stain leaves, stem, roots and flowers of 4–5-week-old plants prepared with the routine sampling and fixation method described in Experimental Procedures. We were able to detect callose in the pollen mother cells, microspore tetrads, pollen tubes, root hair tips, megaspores and cell plates of dividing root cells in cs7 mutants as in the control plants (Figures S5). However, the levels of callose deposition in the vascular system of leaves, roots, stems and flowers were significantly reduced (Figure 4). Callose deposition in the stem and peduncle was mainly detected in the phloem of vascular tissues in wild-type plants, but was absent in cs7 mutants (Figures 4e,f and S1d). In order to determine if this lack of phloem-callose was unique to cs7, we tested for the presence of callose in the phloem tissue of available mutants defective in other CalS genes, including homozygous mutants of cs1, cs5, cs10 and cs12, as well as heterozygous cs9/+ that produces no homozygous mutant progeny due to the male gametophytic lethality (Xie et al., 2010). We found that callose accumulation in the phloem of leaves and stem
was not affected in cs1, cs5, cs10, cs9+/ and cs12 (Figure S6). CalS12 is known to be responsible for callose accumulation in leaf mesophyll cells around the wounding sites generated by mechanical damage and pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003; Dong et al., 2008). Our result shows that while CalS12 is indeed responsible for wound-induced callose accumulation in the mesophyll cells, it plays no role in wound-induced callose deposition in the phloem (Figure S6a). Thus, CalS7 is specifically required for callose biosynthesis in the phloem.

We also applied the modified pseudo-Schiff propidium iodide (mPS-PI) staining technique for the visualization of internal tissue structures in Arabidopsis (Truernit et al., 2008), coupled with aniline blue staining, to investigate callose deposition in the sieve elements of phloem under a confocal microscope. In the wild-type plant, callose deposits were found largely associated with the sieve plate (Figure 5a–c). In a longitudinal section of the stem in the control plants, callose is deposited at the sieve plate in a plug-like pattern that covered the tooth-like structure of sieve pores (Figure 5d,e). Callose was also detected in the side cell wall of the sieve elements, possibly at the sites of the sieve areas (Figure 5b,c, arrowheads) and plasmodesmata (Figure 5b,c, punctate light-blue spots), which serve as communication channels between the sieve element and its companion cells. In cs7 mutants, however, callose was rarely detected in the sieve plate and the side cell wall of the sieve elements (Figure 5f–j). The plug-like pattern of callose was absent from the sieve plate of the mutant plants (Figure 5i,j). Coincidently, the tooth-like structure of sieve pores was also poorly formed (Figure 5i,j), suggesting that the CalS7 gene is required for the deposition of callose at the sieve pores as well as for the development of the pore structure of sieve elements.

**Aberrant pore structures of callose-less sieve plates**

In the wild-type plants, sieve plates in a transverse view contained easily recognizable pores at a density of $2.7 \pm 0.5 \mu m^{-2}$ ($n = 10$), which were covered with plenty of callose deposits (Figure 6a–c, Type I). Callose-positive sieve plates (Figure 6a–c, Types I and II) were rarely detected in cs7 mutants. Some sieve plates of the cs7 mutants might develop seemingly normal pores, but not as many ($1.6 \pm 0.3 \mu m^{-2}$, $n = 10$) and with only remnant
callose deposits (Type II). Most sieve plates of the cs7 mutants were callose-less, and had either only a few tiny pore-like structures (Type III), or a strong reaction with PI dye but no recognizable pores at all (Type IV). In wild-type plants, Type IV structures accounted for approximately 10% of the sieve plates, while no Types II and III were detected (Figure 6a,c). In contrast, no Type I sieve plates could be found in cs7 mutants, suggesting that mutations at the CalS7 gene not only affected callose deposition to the sieve plates but also disturbed the formation of the sieve pores.

**Aberrant sieve plate structure in the cs7 mutant**

Callose is believed to play a role in the differentiation of sieve pores from plasmodesmata during the development of sieve elements (Esau and Thorsch, 1985). We examined the ultrastructure of sieve plates at different developmental stages by transmission electron microscopy (TEM). In the shoot apical region immediately below the apical meristem, where sieve elements were still in their early development stages, the plasmodesmata of the incipient sieve plates at the perforation stage were surrounded with thick layers of callose in the control plants (Figure 7a). In cs7 mutants, however, the plasmodesmata of perforating sieve plates (Figure 7b) or of the newly developed sieve plates (Figure 7c) contained no callose. Without callose present, the differentiation of the plasmodesmata to sieve pores appeared to be hampered in cs7 mutants, resulting in the formation of sieve plates with fewer, abnormally developed pores (see below). In mature sieve plates in the control plants, the cell wall consisted of three distinct layers distinguishable on the
basis of electron density of the cell wall material. In the middle of the sieve plate cell wall lies the electron-dense middle lamella (Ml), a pectin-rich layer cementing the two adjacent sieve elements. Primary cell walls (Pw), the thick electron-light layers comprising primarily cellulose and hemicelluloses, are deposited on each side of the middle lamella. Callose forms a distinctive electron-rare layer (Ca) that surrounds the islands of the sieve plate cell wall. In the control plants, sieve plates contained well-developed sieve pores (diameter 380 ± 47 nm, n = 12), which were surrounded by the electron-sucking P-protein (Pp) (Figure 7d,g). By contrast, the cell wall of mature sieve plates in cs7 did not have the callose layer and had thin primary cell walls (Figure 7e,f,h). Most of the sieve pores of cs7 mutants were incompletely open (Figure 7e,h) or were very narrow in diameter (Figure 7f,i; diameter 190 ± 32 nm, n = 12).

In addition, we found that many of the sieve pores in cs7 were fully occluded with P-protein-like structure aggregates (Figure 7c,i). The elevated level of P-protein-like structure aggregates in cs7 mutants might compensate for the function of the lost callose deposition in plugging the sieve pores. In the cs7-2 line, which was a slightly weaker allele and occasionally produced plants with slightly weaker phenotypes, some of the sieve plates were found to contain a residual layer of callose (Figure 7i). Although this residual callose deposition failed to cover the whole sieve plate, it was sufficient to support the development of thicker layers of the primary cell wall (Figure 7i). These data clearly show

Figure 4. Lack of callose deposition in the vascular tissues of cs7 mutants.

Tissues of the leaf (a), root (b), stem (c–e) and peduncle (f) were fixed and stained with aniline blue (a–d, f) or aniline blue and propidium iodide (Pl) (e). Fluorescence images (a, b, c right, d right, f) were generated by superimposing fluorescence images of callose (light blue) using a UV filter and autofluorescence background (red) taken using a TRITC filter. The characteristic spiral thickenings of the tracheids on the bright-field images of the stem (c, d left) serve as indicators of the xylem. Confocal images of stem cross-sections (e) were generated by merging the images for callose (light blue) with a filter set of 405 nm excitation and 480–520 nm emission, and the images for PI staining (red) taken using 515 nm excitation and 560–660 nm emission.

Ph, phloem; Xy, xylem. Scale bars: 40 μm (a, e), 30 μm (b), 100 μm (c, d), 80 μm (f).
that CalS7 is required for the deposition of the callose layer of the sieve plate cell wall and for the development of sieve pores.

**Lack of wound-induced callose in the phloem in cs7**

It has long been known that injury and treatment with chemicals can induce callose deposition in plant tissues (Stone and Clarke, 1992; Verma and Hong, 2001). Sampling and chemical fixation might inevitably induce callose deposition in the phloem (Davis and Evert, 1970; Eschrich, 1975). In order to examine the potential role of CalS7 in wound-induced callose deposition in phloem, we prepared samples that were subjected to different wounding conditions. To reduce the time of exposure to wounding, we immersed the whole intact Arabidopsis plants directly in the fixation solution, or froze the stem samples in liquid nitrogen immediately after excision. To enhance wounding stress, we incubated excised stem samples (0.5 cm) in water-saturated papers for 1–10 min before chemical fixation.

As shown in Figure 8, in the control plants, callose deposition in the vascular tissues, most of which was in the sieve plate, was greatly increased by wounding treatments (Figure 8a). The number of callose-positive sieve plates, as indicated by the fluorescent dots in the low-magnification images (Figure 8), increased from 50–70 mm$^{-1}$ of longitudinal phloem in the control samples (liquid N$_2$ or intact plant fixation) to 120–150 in the wound-treated samples (1–10 min incubation). The induction of callose deposition in the phloem was a rapid process, because even 1 min of incubation of the excised stems was sufficient to significantly induce callose deposition in the sieve plates in the control plants, which was consistent with a recent report (Mullendore et al., 2010). In cs7 mutants, however, no significant callose deposition could be detected.

![Figure 5. Lack of callose deposition in the sieve plate.](image-url)
in the phloem either under the control conditions or after wound treatment, indicating that CalS7 is required for the wound-induced callose deposition in the phloem.

We further tested whether the wound-induced callose deposition in the phloem was accompanied by an elevated level of CalS7 gene transcription. We examined the expression level of the GUS reporter under the control of the CalS7 gene promoter. Transgenic plants harboring the CalS7PRO::GUS construct were subjected to different wound treatments. No significant differences in GUS reporter activity between wound treatments were detected (Figure 8b), suggesting that the transcription of the CalS7 gene was not induced by wounding. We also verified this result by RT-PCR analysis. No difference in expression level of the CalS7 mRNA between wounding treatments could be detected. Taken together, these data suggest that the rapid response of callose deposition in the sieve plate under wounding conditions is not due to transcriptional activation of the CalS7 gene. It remains to be tested if this rapid callose response is a result of activation at the protein/enzyme level.

We also tested whether CalS7 is required for wound-induced callose deposition in other plant tissues. We stained wound-treated leaves with aniline blue and could detect as much callose deposition around the wounding site in cs7 mutants as in the wild-type control (Figure S5o,p), suggesting that CalS7 is not responsible for the wound-induced callose response in leaf epidermal cells. Previously, CalS12 has been shown to be required for the callose response to mechanical wounding and to pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003; Dong et al., 2008). We therefore wounded cs12 mutant plants and examined the resulting callose production. We confirmed that leaf epidermal cells of cs12 mutant plants failed to accumulate callose around the wounding site. However, callose deposition in the sieve plate was not affected by the cs12 mutation (Figure S6). Thus, we conclude that CalS7 is responsible for wound-induced callose deposition in the

![Figure 6. Types of sieve plates and callose deposition.](image)

(a) Confocal images of the sieve plates stained with aniline blue (light blue) and propidium iodide (PI) (red). Type I: normal sieve plates with well-distributed sieve pores and accumulated callose deposits. Type II: sieve plates with a reduced number of pores and remnant callose deposits. Type III: sieve plates with a few pore-like structures and no detectable callose. Type IV: sieve plates with enhanced PI staining, no visible pores and no callose. Scale bar: 2 μm.

(b, c) Percentages of sieve plate showing detectable callose deposition (b) and percentages of different sieve plate types (c) in wild type and cs7 plants.
phloem, whereas CalS12 is involved in the callose response in leaf epidermal cells treated with physical wounds or pathogens. These results clearly demonstrate that members of the CalS family have unique biological functions and are regulated in a tissue-specific manner as well as in response to various environmental cues.

DISCUSSION

Callose can be easily detected in plant tissues with an epifluorescence microscope, thanks to its characteristic reaction with the fluorescent dye aniline blue. Its presence in the vascular tissues of the stem was observed more than a century ago (Hartig, 1851; Eschrich, 1956; Stone and Clarke, 1992). Wound-induced callose deposition in the phloem has also been examined extensively (Evert and Derr, 1964; Eschrich, 1975). Callose plays a pivotal role in sieve plate development, and in controlling the phloem transport in response to wounding and other endogenous and exogenous signals. In this study we characterized four homozygous T-DNA insertion mutant lines of cs7. The cs7 mRNA could not be detected in the mutant plants (Figures 1c and S1b), suggesting that the T-DNA insertions may have resulted in the formation of unstable or size-altered cs7 mRNA. We examined the growth and reproduction phenotypes of cs7 mutant plants and observed changes in callose deposition in association with the cs7 mutation. Our genetic and cell biology evidence clearly demonstrates that CalS7 is a phloem-specific CalS isoform and is responsible for callose biosynthesis in the sieve elements during phloem development and for the induced callose accumulation in response to wounding signals in the mature vascular tissues of the stem. These data shed new light on the function of a callose synthase gene in phloem development and provide a new molecular tool and target for future studies of stem development and transport in the vascular tissues.
A family of highly tissue specific CalS genes

Arabidopsis contains 12 CalS genes that were named on the basis of their sequence similarity with CalS1, the first CalS identified in plants (Hong et al., 2001a). Multiple CalS genes appear to have evolved in plants to meet the need for callose synthesis in different tissues and in response to a range of biotic or abiotic stresses (Verma and Hong, 2001). Previous studies performed by our group and other laboratories have suggested that CalS1 is responsible for the synthesis of the nascent callose present at the cell plate during cytokinesis (Hong et al., 2001a,b). CalS5 is highly expressed during microsporogenesis, microgametogenesis and pollination, and is responsible for the biosynthesis of the callose wall, pollen tube wall and callose plugs of growing pollen tubes (Dong et al., 2005, 2008; Nishikawa et al., 2005; Xie et al., 2010). CalS9 and CalS10 are highly expressed during pollen formation, but are not responsible for callose wall biosynthesis. CalS9 is required for both symmetric and asymmetric mitosis during male gamete development (Töllner et al., 2008; Xie et al., 2010). CalS10 is required for the asymmetric mitosis during microgametogenesis in the another (Thiele et al., 2008; Töllner et al., 2008) and during stomatal formation in leaves (Chen et al., 2009; Guseman et al., 2010). The callose wall formed during microsporogenesis can be differentiated into the peripheral wall that surrounds the four microspores in a tetrad and the interstitial callose wall that separates the four microspores. Interestingly, the peripheral

Figure 8. Wound-induced callose deposition in vascular tissues of the stem.
(a) Callose deposition in the vascular tissue induced by wound treatments. The control samples were prepared by freezing with liquid N₂ immediately after stem excision (N₂) or by fixing intact plants directly in fixation buffer (intact plant fixation). For wound treatments, excised fragments (0.5 cm) of the stem were placed on water-saturated paper for 1 min and 10 min. The frozen specimens were then kept in the ECA fixation buffer and stained with aniline blue. Overlaid fluorescence images were generated by merging the images of callose staining (light blue) with autofluorescence background (red). Scale bars: 50 μm.
(b) Expression levels of the GUS reporter in the stem of transgenic plants harboring CalS7PRO:GUS treated with wounding. Scale bar: 400 μm.
(c) A RT-PCR analysis of the expression levels of the CalS7 mRNA in the stem samples treated with different wound conditions.
callose wall is synthesized by CalS5 (Dong et al., 2005, 2008; Nishikawa et al., 2005; Xie et al., 2010), while the biosynthesis of the interstitial wall is dependent on a joint function of CalS11 and CalS12 (Enns et al., 2005). In this study, we demonstrate that CalS7 is expressed specifically in the phloem of various plant tissues (Figure 3). Loss of the CalS7 gene resulted in the failure of callose deposition in the sieve elements. The fact that pollen grains of cs7 mutants clamped together in a tetrad-like manner (Figures 2d and S2c) suggests that CalS7 might also have a minor role in the formation of the callose wall during pollen formation or the release of microspores from tetrads. This function of CalS7 is beyond the scope of this work and remains to be exploited in more detail in future work. We also examined callose deposition patterns in the mutant plants of CalS1, CalS5, CalS9, CalS10 and CalS12, and detected no significant alteration in phloem-associated callose deposition (Figure S6). Taken together, these data suggest that CalS7 is the key isoform of the callose synthases responsible for callose biosynthesis in the phloem.

A role for callose in sieve plate development

During phloem development, the incipient sieve plate is penetrated by densely distributed plasmodesmata. A membranous cylinder, known as the plasmotubule, runs within the plasmodesmal pore and is linked to the cisternae of the ER on both sides of the wall. A thick layer of callose is deposited beneath the plasma membrane around each plasmodesma on both sides of the sieve plate wall. The paired callose deposits, or callose platelets, form a collar that encloses the plasmodesma. The presence of the callose platelets precludes further deposition of cellulose near the plasmodesma. As nuclear degeneration takes place in sieve elements, the cell wall surrounding the plasmodesma begins to be hydrolyzed, resulting in the formation of sieve pores. This perforation process starts with the degradation of the middle lamella followed by the simultaneous removal of the cellulose primary cell wall and the callose platelets (Deshpande, 1975; Esau and Thorsch, 1985). At the completion of perforation, residual callose remains in the sieve pore area (Ehlers et al., 2000). The ER cisternae may transverse the desmotubule of a single plasmadesma, or remain closely associated with the callose platelets throughout pore development. After the perforation is complete, the ER disappears from the pore areas. It has been proposed that callose may not play a role in the perforation of sieve plates. This view was supported by two lines of evidence obtained from observations of sieve plates in the roots of common duckweed (Lemna minor) and sieve areas of gymnosperms. No callose was detected during pore development in these sieve organs (Walsh and Melaragno, 1976). The fact that callose could be induced by wounding during specimen preparation (see below) also complicates the debate on the role of callose. In this study, we took advantage of the cs7 mutants that could not produce callose during the perforation of sieve plates (Figure 7a–c). Sieve plates formed in cs7 mutants contained a significantly reduced number of pores per plate (Figure 6) and pore-like structures that did not penetrate through the sieve plates (Figure 7). These lines of genetic and cytological evidence argue strongly for an important role for callose in sieve pore formation.

Wound-induced callose deposition

Mature sieve elements are very responsive to a variety of stimulus cues. Such rapid responses are needed for proper control of mass flow through the phloem, for preventing leakage of the precious translocates and for defense against pathogen infections (Evert, 1982; Verma and Hong, 2001). While the presence of callose in the sieve pores of undisturbed plants remains a topic of debate, wounding, sampling cuts and fixative chemicals such as glutaraldehyde and Ca²⁺ ions can certainly cause significant deposition of callose in mature sieve elements (Hughes and Gunning, 1980; Evert, 1982; Radford et al., 1998; Mullendore et al., 2010). In this study, our data clearly show that callose deposition was eradicated in the phloem of the stem in cs7 mutants, both before and after wounding treatments (Figures 4–6 and 8). Thus, CalS7 should be responsible for callose biosynthesis in both developing and mature sieve elements, as well as in mature phloem in response to wound treatments. Wound-induced callose deposition is known to occur within a very short time period (Mullendore et al., 2010). However, how the sieve elements achieve this rapid response in callose accumulation, either at the transcriptional level of CalS7 expression or by post-translational activation of CalS7 enzyme, remains to be studied in future.

In response to injury, the phloem-specific P-proteins also form filamentous aggregates that rapidly occlude the sieve pores (Ehlers et al., 2000). So long as plants are uninjured, P-proteins are present parieta]
Callose biosynthesis in sieve elements

Recent biochemical data (Li et al., 1999; Brownfield et al., 2007) and genetic evidence (Dong et al., 2005; Enns et al., 2005; Nishikawa et al., 2005; Töller et al., 2008; Huang et al., 2009; Guseman et al., 2010) have clearly demonstrated that CalS genes encode enzymes that catalyze callose biosynthesis in plants. The CalS proteins containing multiple transmembrane domains are targeted to specific subcellular locations, where their enzyme activities are regulated via interactions with various regulatory proteins and ions (Ca^{2+}) and in response to different developmental and environment signals. We have previously proposed a model of callose biosynthesis at the cell plate (Verma and Hong, 2001), in which phragmoplastin, UDP-glucose transferase (UGT1) and SuSy are proposed to be associated with the cell plate-specific CalS1, (Hong et al., 2001a,b). For callose biosynthesis at the sieve elements, CalS7 is not likely to function alone, but may form a complex with other protein components, such as APL and SuSy5/6, which appear to be involved in callose biosynthesis in phloem (Bonke et al., 2003; Barratt et al., 2009). Additionally, where CalS7 protein is synthesized, how CalS7 is targeted to the sieve plate and how the enzyme activity is regulated by the wound signal, remain to be examined in future work.

A possible role for CalS7 in the development of non-vascular tissues

Previous studies have shown that the loss of certain callose synthases may result in the formation of multiple defects in plant development and in response to environmental signals. In the cs12 mutant plants, the resistance to pathogen infection is surprisingly enhanced in a salicylic acid-dependent manner (Nishimura et al., 2003). In the csS+ heterozygous mutants, pollen cell division and pollen germination are altered (Töller et al., 2008; Huang et al., 2009; Xie et al., 2010). CalS10 is found to play multiple roles in pollen development and in stomatal formation (Chen et al., 2009; Guseman et al., 2010). Our observations on cs7 show that in addition to the lack of phloem callose, other growth and reproduction phenotypes are also associated with the cs7 mutation, suggesting that CalS7 also plays important roles in plant growth and development. Because the phloem is the main channel for the long-distance transportation of organic nutrients and signal molecules (Ruiz-Medrano et al., 2001; van Bel et al., 2002; Ye, 2002), the loss of callose in sieve elements and the aberrant sieve plate structure observed in cs7 plants may directly affect transportation in the phloem, which may subsequently cause collateral damage to plant tissue development. How transportation though the phloem is altered in cs7 remains an interesting topic for future investigations.

Taken together, this study has clearly identified CalS7 as the key CalS enzyme responsible for callose biosynthesis in the phloem, and has provided a very important molecular tool and target for future research in the field of phloem cell development, stress response and vascular transport.

EXPERIMENTAL PROCEDURES

Plant materials

Arabidopsis thaliana ecotype Columbia seeds were germinated on the surface of vermiculite in small pots. Seedlings were transplanted to Metro-Mix 360 (Hummert, http://www.hummert.com/) 14 days post-germination and grown at 21–23°C in a greenhouse.

Genomic DNA extraction and genotyping

Genomic DNA was extracted from the leaves and inflorescence using the cetyl trimethyl ammonium bromide (CTAB) method (Allen et al., 2006). Genotyping of plants was performed by PCR using CalS7 gene-specific primers and the T-DNA left-border primers (Table S1).

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, http://www.invitrogen.com/) and reverse-transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Expression levels of the CalS7 mRNA and the control Actin2 were assessed by PCR using the primers shown in Table S1. For PCR amplification, the reaction mix was pre-heated at 94°C for 2 min, followed by 20 cycles of amplification at 94°C, 40 sec, 58°C, 30 sec, and 72°C, 30 sec.

GUS staining

Plant tissues were incubated with GUS staining solution (50 mM sodium phosphate buffer, pH 7.2, 0.05% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 2 mM 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (Sigma, http://www.sigma-aldrich.com/). Samples were incubated at 37°C for defined times and then cleaned with 70% ethanol.

In situ RNA hybridization

A cDNA fragment of 232 bp corresponding to the mRNA sequence flanking the first ATG of CalS7 was used to prepare RNA probes labeled with digoxigenin as described elsewhere (Goodrich et al., 1997). The CalS7 cDNA fragment was amplified by PCR using primers CS7UTR-98F and CS7133R (see Table S1) and cloned into pCRII (Invitrogen). The plasmid was linearized with Apal and transcribed with SP6 RNA polymerase (Roche, http://www.roche.com/) to produce antisense RNA probe. For the preparation of negative control with the CalS7 sense RNA probe, the plasmid was linearized with BamH1 and transcribed with T7 RNA polymerase (Roche).

Sample preparation and wounding treatments

Plant samples were fixed in ECA fixation buffer (60% ethanol, 30% chloroform, 10% acetic acid), MA buffer (50% methanol, 10% acetic acid) or TEM fixation buffer (2% paraformaldehyde, 2% glutaraldehyde in 50 mM PIPES buffer, pH 7.0). For routine preparations of plant specimens, fresh plant stems were excised in 0.5-cm fragments and immersed in the fixation buffer, which lasted <40 sec from cutting to chemical fixation. For the preparations of the less wounded samples, fresh plant stems were directly frozen in liquid N₂ and excised in 0.5-cm fragments in liquid N₂. The fragments were fixed in the fixation buffer pre-cooled at ~20°C. For the preparation of uncut plants, whole seedlings were fixed directly in the fixation buffer for 24 h. The middle fragments of the stem were then excised.
for further analysis. For the preparation of wound-treated samples, excised stems in 0.5-cm fragments were placed on water-saturated paper for 1 min or 10 min, and frozen in liquid N2 to stop the wound treatment.

Callose staining

For the routine detection of callose in plant tissues, samples were fixed in ECA solution (60% ethanol, 30% chloroform, 10% acetic acid) for at least 2 h, and treated with 2 N NaOH for 5–10 min. After washing with 0.1 M K2HPO4, pH 8.5, the specimens were incubated with 0.05% aniline blue (Sigma). For confocal detection of callose deposition, plant samples were fixed in MA solution (50% methanol, 10% acetic acid), and treated with hot ethanol and periodic acid. After staining with PI as described elsewhere (Truernit et al., 2008), the specimens were cleaned with a chloral hydrate solution (4 g chloral hydrate, 1 ml glycerol, 2 ml water) and then stained with 0.05% aniline blue for 10 min.

Microscopy

For epifluorescence microscopy, a UV filter was used to detect the presence of aniline blue-stained callose (blue-yellow fluorescence). Autofluorescence of the sample background (red fluorescence) was used with a TRITC filter. Superimposed images of the blue and red fluorescence were created using PHOTOSHOP software (Adobe, http://www.adobe.com). For confocal microscopy of callose detection, we used an Olympus laser scanning microscope FV1000 (Leeds, http://www.leedsmicro.com) with an excitation filter of 405 nm and emission filter of 485–515 nm. The cell boundary stained with PI was imaged using a set of filters with excitation at 514 nm and emission at 560–660 nm (red fluorescence).

For transmission electron microscopy (TEM), samples were fixed in 2% paraformaldehyde, 2% glutaraldehyde in 50 mM PIPES buffer, 660 nm (red fluorescence). The specimens were cut into sections and observed using a Jeol 1200 EX (www.reichertms.com/). Following uranyl acetate and lead citrate treatment (1 day in 1:3, 1:1 and 3:1, respectively). The specimens were cut into 60 nm sections and observed using a Jeol 1200 EX transmission electron microscope (http://www.jeol.com/).

ACKNOWLEDGEMENT

We thank Dr A. Caplan for critical comments on the manuscript, Ms Shizhen Gu for technical assistance and the Franceschi Microscopy and Imaging Center, Washington State University for assistance in transmission electron microscopy. We also thank the ABRC (Ohio State University) for seeds of Arabidopsis T-DNA insertional lines. This work was supported by National Science Foundation grants (MCB-0654825 and IOB-0543923) to ZH, and an National Natural Science Foundation of China grant (NSFC-30670188) to ZZ. MZ was supported by fellowship from the China Scholarship Council (CSC).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

- Figure S1. Consistent phenotypes exhibited in cs7 alleles.
- Figure S2. The CalS7 gene and its deduced peptide.
- Figure S3. Growth and reproduction defects in cs7 alleles.
- Figure S4. In situ hybridization of CalS7 gene expression.
- Figure S5. Normal callose deposition in non-vascular tissues of cs7 mutant plants.
- Figure S6. Callose deposition in the phloem of other cs mutants.
- Table S1. Primers used in this study.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES


