Running title:  *GSL8* in cytokinesis and cell patterning

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**Research Area:** Cell Biology

**Keywords:** Arabidopsis, callose, callose synthase, cytokinesis, cell plate, *GLUCAN SYNTHASE-LIKE*, stomatal patterning,
The Arabidopsis callose synthase gene, \textit{GSL8}, is required for cytokinesis and cell patterning

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Financial sources: This work was supported by grants from Korea Research Foundation (C00239) and from KOSEF/MEST to the National Research Lab Program (M10600000205-06J0000-20510) and the Environmental Biotechnology National Core Research Center (R15-2003-012-01003-0) and by an NSERC Discovery grant to FDS. XYC, XH, YR, HC, and LL were supported by a scholarship from the BK21 program, the Ministry of Education, Science and Technology, Korea.

Footnotes:

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ABSTRACT

Cytokinesis is the division of the cytoplasm and its separation into two daughter cells. Cell plate growth and cytokinesis appear to require callose, but direct functional evidence is still lacking. To determine the role of callose and its synthesis during cytokinesis, we identified and characterized mutants in many members of the GLUCAN SYNTHASE-LIKE (GSL or CALLOSE SYNTHASE (CalS)) gene family in Arabidopsis. Most gsl mutants (gsl1-gsl7, gsl9, gsl11 and gsl12) exhibited roughly normal seedling growth and development. However, mutations in GSL8, which were previously reported to be gametophytic lethal, were found to produce seedlings with pleiotropic defects during embryogenesis and early vegetative growth. We found cell wall stubs, two nuclei in one cell, and other defects in cell division in homozygous gsl8 insertional alleles. In addition, gsl8 mutants and inducible RNAi lines of GSL8 showed reduced callose deposition at cell plates and/or the new cell walls originated from cell plates. Together these data show that GSL8 gene encodes a putative callose synthase required for cytokinesis and seedling maturation. In addition, gsl8 mutants disrupt cellular and tissue level patterning as shown by the presence of clusters of stomata in direct contact and by islands of excessive cell proliferation in the developing epidermis. Thus, GSL8 is required for patterning as well as cytokinesis during Arabidopsis development.
INTRODUCTION

Cytokinesis divides the cytoplasm of a plant cell by the deposition of plasma membrane and a cell wall during late mitosis. This process requires the phragmoplast, a dynamic, plant-specific cytoskeletal and membranous array, which delivers vesicles containing lipids, proteins, and cell wall components to the division plane to construct the cell plate. Cell plate formation involves several stages, initiation through vesicle fusion, the formation of a tubular-vesicular network (TVN), a transition to a solely tubular phase, and then further fusion to form a fenestrated sheet (Samuels et al., 1995). The outward growth of the cell plate leads to its fusion with the parental cell wall (Jürgens, 2005a, 2005b; Backues et al., 2007).

Key regulators of cytokinesis include KNOLLE, KEULE, KORRIGAN, and HINKEL which when defective induce pleiotropic phenotypes and seedling lethality (Lukowitz et al., 1996; Nicol et al., 1998; Zuo et al., 2000; Assaad et al., 2001; Strompen et al., 2002). KNOLLE, a syntaxin homolog, is required for the fusion of exocytic vesicles via a SNARE/SNAP33 complex (Lukowitz et al., 1996; Heese et al., 2001). KEULE, a homolog of yeast Sec1p, regulates syntaxin function by interacting with KNOLLE (Waizenegger et al., 2000; Assaad et al., 2001). KORRIGAN is an endo-1,4-beta-glucanase required for cell wall biogenesis during cytokinesis (Zuo et al., 2000). And HINKEL is a kinesin-related protein required for the reorganization of phragmoplast microtubules during cytokinesis (Strompen et al., 2002).

Additional regulators include Formin 5, TWO-IN-ONE (TIO), and Arabidopsis dynamin-like proteins (ADLs) (Kang et al., 2001, 2003; Hong et al., 2003; Ingouff et al., 2005; Oh et al., 2005; Collings et al., 2008). Formin 5 localizes to the cell plate and is an actin-organizing protein involved in cytokinesis and cell polarity. TIO, a Ser/Thr protein kinase, functions in cytokinesis in plant meristems and in gametogenesis (Oh et al., 2005). Members of the Arabidopsis DRP family associate with the developing cell plate, whereas DRP1a (ADL1A) locally constricts tubular membranes, interacts with callose synthase, and
may facilitate callose deposition into the lumen.

Callose, a β-1,3-glucan polymer with β-1,6-branches (Stone and Clarke, 1992), is synthesized in both sporophytic and gametophytic tissues, and appears to play various roles. Callose accumulates at the cell plate during cytokinesis, in plasmodesmata where it regulates cell-to-cell communication, and in dormant phloem where it seals sieve plates after mechanical injury, pathogen attack, and metal toxicity (Stone and Clarke, 1992; Samuels et al., 1995; Lucas and Lee, 2004).

Twelve GLUCAN SYNYHASE-LIKE (GSL) genes (also known as CalS) have been identified in the Arabidopsis genome based on sequence homology (Richmond and Somerville, 2000; Hong et al., 2001; Enns et al., 2005). A GSL that functions in callose deposition after injury and pathogen treatment is GSL5 (Jacobs et al., 2003). Five other members of the Arabidopsis GSL family are required for microgametogenesis. GSL1 and GSL5 act redundantly to produce a callosic wall which prevents microspore degeneration, and both are needed for fertilization (Enns et al., 2005). GSL2 is required for the callosic wall around pollen mother cells, for the patterning of the pollen exine (Dong et al., 2005), and for callose deposition in the wall and plugs of pollen tubes (Nishikawa et al., 2005). GSL8 and GSL10 are independently required for the asymmetric division of microspores and for the entry of microspores into mitosis (Huang et al., 2008; Töller et al., 2008).

Callose is a major component of the cell plate, especially during later plate development (Kakimoto and Shibaoka, 1992; Samuels et al., 1995; Hong et al., 2001). Callose appears to structurally reinforce the developing cell plate after the break down of the phragmoplast microtubule array and during plate consolidation (Samuels and Staehelin, 1996; Rensing et al., 2002). It is likely that callose is synthesized at the cell plate rather than in the endoplasmic reticulum and in the Golgi (Kakimoto and Shibaoka, 1988). GSL6 (CalS1) appears to be involved in callose synthesis at the cell plate since a 35S::GFP-GSL6 fusion in transgenic BY-2 tobacco cells increases callose accumulation and GFP fluorescence was found specifically at the cell plate (Hong et al., 2001). However, functional and genetic data
on the role of any GSL in Arabidopsis sporophytic cytokinesis are still lacking.

Here we report that GSL8 (CalS10) is required for normal cytokinesis. In addition, gsl8 mutants exhibit excessive cell proliferation and abnormal cell patterning, phenotypes not previously reported for cytokinesis-defective mutants.

RESULTS

Isolation of homozygous T-DNA insertion lines of GSL family members

We previously isolated T-DNA insertional mutants in 11 members including GSL1-GSL9, GSL11 and GSL12 of the Arabidopsis thaliana GSL family and screened these primarily for gametophytic phenotypes (Huang et al., 2008). Homozygous T-DNA tagged lines were identified for eleven members of the GSL family except for GSL10 (Table S1) which was gametophytic lethal (Huang et al., 2008; Töller et al., 2008), thus explaining why gsl10 homozygous mutants could not be isolated. To identify the possible functions of different GSL genes in sporophytic callose formation and in cytokinesis we examined gross and microscopic seedling phenotypes in homozygous gsl mutants.

We first examined gsl6 insertion lines because GSL6 has been reported to be involved in cell-plate specific callose synthesis (Hong et al., 2001). Two independent homozygous gsl6 insertion lines showed developmental phenotypes similar to wild-type plants (Fig. S1A-C). These two alleles are likely to be nulls since no GSL6 mRNA could be detected by RT-PCR using a primer set corresponding to the catalytic domain of callose synthase (Fig. S1D). Like Hong and Verma (2007), we were unable to detect any cytokinesis defects in gsl6 insertion lines such as multiple nuclei in one cell and cell wall stubs (data not shown). Thus if GSL6 regulates cytokinesis, its function might be masked by redundancy in the GSL gene family.

We then evaluated the rosette-stage phenotypes of mutations in the remaining ten GSL family members. Mutants in nine of the remaining families were indistinguishable from those of wild-type controls (Fig. S1E).
**gsl8 mutations affect plant development resulting in seedling lethality**

Mutations in the remaining locus, GSL8, have been reported to be male gametophytic lethal with aberrant gsl8 pollen failing to enter pollen mitosis I (Töller et al., 2008), leading these authors to speculate that the role of GSL8 during pollen development is independent of its function in callose synthesis. However, as briefly noted in Huang et al. (2008), three homozygous T-DNA insertions, gsl8-1, gsl8-2 and gsl8-3 still allow some sporophytic growth (see below). Similar phenotypes were also found in three newly isolated insertional alleles (Fig. 1A-B, Fig. 2). Previously reported gsl8 seedling viability problems might be attributable to poor germination and/or to the low transmission of mutations (Table S2), since growth on 3% sucrose resulted in the recovery of homozygous mutants at a rate of 9-15% instead of the 25% expected in the case of normal Mendelian segregation. The recovery of homozygous mutants in a medium without sucrose was dramatically reduced to about 2.5%. This reduction was mainly caused by poor germination of homozygous mutants (Table S2). Six independent homozygous mutant alleles (gsl8-1 to gsl8-6) were seedling lethal, and displayed almost identical multiple phenotypes (Fig. 2).

**gsl8** mutants showed a dwarf phenotype and seedlings exhibited abnormally-shaped cotyledons (Fig. 2A). The surface of the gsl8 cotyledon epidermis was undulated, and hypocotyls and roots were thicker and shorter than the wild-type (Fig. 2A-C). Dark-grown, etiolated gsl8 seedlings showed open cotyledons, fat hypocotyls, and reduced height compared to wild-type seedlings; however, hypocotyl length was significantly longer than that of light-grown gsl8 plants (Fig. 2C-E). Because cotyledons and hypocotyls are embryonic organs, we investigated the effect of gsl8 on embryo development. Compared to wild-type controls (Fig. 3A-E), gsl8 disrupted embryonic development with shape defects beginning at the globular-stage (Figure 3F) and increasing in severity over time (Fig. 3G-I). Mature gsl8 embryos showed a range of aberrant phenotypes including asymmetrically-shaped cotyledons and single or triple cotyledon-like organs (Fig. 3I). For example, the
percentage (12.9%) of abnormal *gsl8-3* embryos was similar to that of homozygous mutants observed at the seedling stage (Table S2).

**gsl8 seedlings display pleiotropic phenotypes including patterning defects**

The *gsl8* dwarfing could be due to a disruption in cell division frequency and/or elongation. Normally the shoot apical meristem displays well arranged layers (L1-L3), as well as cells that are small and relatively even (Fig. 4A). By contrast, *gsl8* meristem layers were disrupted, shapes were irregular, and many cells were enlarged (especially in L3 and in young primordia) (Fig. 4B) suggesting a loss of meristematic activity. In addition, three week-old callus induced from *gsl8* seedlings was much smaller than in the wild-type (Fig. S2) again indicating a growth defect.

Cross-sections of cotyledons revealed that *gsl8* mutants not only showed bulging and giant epidermal cells, but also misarranged mesophyll cells with larger intercellular spaces (Fig. 4C, D). Venation patterns were also defective in cotyledons and young leaves (Fig. S3A, B). Compared to the wild-type, *gsl8* hypocotyls showed excess divisions in the pericycle, endodermis and phloem (Fig. 4E-J, K). Similar phenotypes were found in roots with extra cells appearing in the root columella (Fig. 4G-I) and cortex (Fig. 4L, M). Cell layering was also disrupted in *gsl8* roots especially outside the stele and the columella (Fig. 4L, M), and many root cells were abnormally enlarged. *gsl8* roots also exhibited aberrant cell division planes (Fig. S3C). Thus GSL8 is directly or indirectly required for cell number and spatial distribution.

**GSL8 is required for epidermal patterning**

The epidermis of young *gsl8* cotyledons and leaves was highly disorganized (Fig. 5B-E). Non-stomatal cells showed a much wider range in size and in aspect of ratio than in the wild-type e.g. some cells were exceptionally long and narrow. In addition, cell outlines were often less sinuous (Fig. 5B-E).
Stomata also showed extreme variations in size, as well as defects in pore formation and bilateral symmetry (Fig. 5C-E). In all six gsl8 mutant alleles, many gsl8 stomata formed in contact in clusters, whereas normally stomata are spaced at least one cell apart (Fig. 5A; Fig. S4). Clusters in gsl8 varied in the number and arrangement of stomata as well as in overall cluster shape and outline. Strikingly, the gsl8 epidermis showed regions with an excessive number of small cells, many of which divided asymmetrically to produce stomatal precursor cells (Fig. 5C, D). The regions with small cells were often adjacent to groups of larger and more mature epidermal cells (including stomata). By contrast, in the developing epidermis of wild-type leaves, small cells and asymmetric divisions were much more distributed and interspersed with larger cells. Thus GSL8 appeared to be required for correctly distributing asymmetric divisions in the stomatal cell lineage throughout the epidermis.

Each asymmetric division in Arabidopsis stomatal development generates a meristemoid, a precursor cell that ultimately produces a stoma. Mutants of gsl8 appeared to produce many more meristemoids in contact than wild-type plants (Fig. 5B). Normally, meristemoids themselves divide asymmetrically, divisions which can restore a one-celled spacing pattern between stomatal precursor cells in wild-type plants (Fig. S5). However, gsl8 meristemoids seemed to divide fewer times than in the wild-type; this defect, along with the abundance of meristemoids in islands of small cells, appeared to induce pattern violations. Because stomatal patterning might involve cell-cell signaling via the apoplast, we asked whether spacing defects were only found in cells whose apoplast was abnormal. As in other gsl8 epidermal cells (see below), those in the stomatal lineage often showed gaps in their walls. However, many stomata and meristemoids in contact had apparently intact cell walls throughout their cell depth (data not shown), suggesting that local cytokinesis defects were not directly responsible for stomatal pattern violations.
**GSL8 Is widely expressed**

Data from the AtGenExpress expression atlas (Schmid et al., 2005) show that GSL8 is widely expressed in most organs throughout plant development. To determine the organ-level expression pattern of GSL8, we performed RT-PCR using RNA extracted from various organs. GSL8 transcripts were detected in all organs and regions tested (Fig. S6). GSL8 expression was relatively higher in actively dividing cells such as in root tips and floral buds than in fully expanded or expanding tissues such as rosette and cauline leaves, cotyledons, and flowers. Expression was also detected in mature organs such as rosette leaves of 6-week-old plants.

**gsl8 mutants exhibit cell wall stubs and incomplete cytokinesis**

GSL8 encodes a putative callose synthase, and callose is the major component of developing cell plates during plant cytokinesis. We thus examined whether gsl8 mutants disrupt cytokinesis as evidenced by defects such as cell wall stubs and two or multiple nuclei in one cell that have previously been described for other genes (Söllner et al., 2002). We first examined the epidermal cells of gsl8 mutant cotyledons. As shown in Fig. 6A-C, cell wall stubs were observed throughout the depth of cotyledon epidermal cells stained with propidium iodide. Transmission electron microscopy (TEM) analysis confirmed the presence of binucleated cells with or without cell wall stubs in gsl8 cotyledons and roots (Fig. 6D, E). To determine whether embryo abnormality in gsl8 mutants might result in part from defective cytokinesis, we used TEM to examine abnormal embryos at various developmental stages from siliques produced by heterozygous gsl8 plants. Perturbations similar to those found in the seedling stage were found in embryos that presumably were homozygous for gsl8. These defects included disorganized cell files, cell wall stubs, and binucleated embryo cells (Fig. 6F). Together, these results show that gsl8 mutations disrupt cell division and that GSL8 is required for Arabidopsis cytokinesis starting in the early embryo.
Reduced callose deposition at cell plates and/or the new cell walls originated from cell plates in *gsl8*

The callose localization by aniline blue staining or immunolocalization in root tips was frequently used to localize cell plates and/or the new cell walls (young daughter cell walls) originated from cell plates (Samuels et al., 1995; Frantzios et al., 2001). To test whether the loss of GSL8 callose synthesis activity in *gsl8* mutants correlated with reduced callose deposition at cell plates, root tips were stained with aniline blue. In wild-type roots we detected prominent aniline blue staining at positions to mark a cell plate but not longitudinal cell walls (Fig. 7A-C). Callose is also assumed to deposit at the new cell walls originated from cell plates, since we sometimes observed callose bands in two opposite sides of cells suggesting that at least one of them is new cell wall (Fig. 7B, C). In *gsl8* mutants, we failed to find clear callose bands at these positions, although we sometimes found ectopic callose deposition (Fig. 7D-F). This failure to detect callose might be caused by the low frequency of cell divisions in some root tip tissues such as the epidermis and cortex. To produce plants with a range of cytokinesis defects, we constructed inducible RNAi lines for *GSL8*. A dexamethasone-induced reduction in *GSL8* mRNA levels produced seedlings that resembled *gsl8* homozygotes in their dwarfism and defective cell patterning (Fig. S7). These lines also resembled severe *gsl8* alleles in the absence of callose staining at root cell walls. Some induced plants showed relatively mild developmental phenotypes and the presence of callose at cell plates and/or the new cell walls, but their frequency and intensities were less and very weak compared to those of non-induced lines, relatively (Fig. 7G-I). Taken together, these observations suggest that *GSL8* is involved in for the deposition of callose at cell plates during cytokinesis.
DISCUSSION

To obtain genetic data on the role of callose in cytokinesis, we identified T-DNA insertional mutants for most GSL callose synthase genes in Arabidopsis. We have shown that *GSL8* is required for cytokinesis as evidenced by the presence of incomplete cell walls and two nuclei in a single cell in seedling tissues, by the reduced callose levels at cell plates and/or the new cell walls originated from cell plates of *gsl8* mutants or inducible RNAi lines of *GSL8*. In addition, *GSL8* was found to play a novel and pervasive role in cell and tissue patterning.

**GSL8 is a critical for cytokinesis in the GSL gene family**

Callose deposition has been extensively studied in plants. Green algae, bryophytes, ferns and seed plants are all capable of producing wound-induced callose, and all plant taxa investigated, from multicellular green algae to land plants, accumulate callose during cell division (Scherp et al., 2001). At least some callose deposition at the cell plate is not wound-induced since rapidly frozen cells assayed using callose-specific antibodies show an increase in staining during cell plate development (Samuels et al., 1995). Callose deposition can be indirectly inhibited by caffeine which destabilizes the developing cell plate and generates cytokinesis defects (Samuels and Staehelin, 1996; Yasuhara, 2005). However, while many mutants have been described that affect cytokinesis, no mutant in a callose synthase gene has been shown to control cytokinesis (Hong and Verma, 2007). Our findings that several *gsl8* mutant alleles or knock-down lines display severe cell division defects and reduction of callose deposition at cell plates and/or the new cell walls originated from cell plates provides direct functional genetic evidence for the importance of callose synthesis in cytokinesis by GSL members. Although these *gsl8* alleles are nulls and induce cytokinesis defects, cell divisions still continue in these mutants raising the possibility that other *GSL* genes might be functionally redundant. Two candidates are *GSL10* and *GSL6*. *GSL10* encodes a callose
synthase that is most similar to that of GSL8. gsl10 mutants are gametophytic lethal, and they perturb the symmetry of microspore division and induce irregular callose deposition during microgametogenesis (Huang et al., 2008; Töller et al., 2008). In addition, the silencing of GSL10 in transgenic RNAi lines resulted in a dwarfed growth habit, suggesting additional functions for GSL10 in normal plant growth (Töller et al., 2008).

Although it remains to be seen if GSL6 localizes to the cell plate in Arabidopsis, it was previously shown that a GFP-GSL6 fusion protein expressed under the control of 35S promoter localizes to the cell plate in tobacco BY-2 cells (Hong et al., 2001). However, GSL6 does not appear to be essential for cytokinesis since our null gls6 mutants did not exhibit seedling-level phenotypes found in gsl8 (Fig. S1B), and since cytokinesis defects appear to be absent from gsl6 alleles (this study; Hong and Verma, 2007). Similarly, gsl6 does not appear to disrupt callose accumulation at the cell plate (Hong and Verma, 2007). These data suggest that GSL6 is either not required for cytokinesis or that it is redundant with GSL8.

**Tissue organization in gsl8**

Cytokinesis mutants often display defects in tissue organization, in the range of cell sizes, and in division plane placement (Lukowitz et al., 1996; Nicol et al., 1998; Zuo et al., 2000; Assaad et al., 2001; Strompen et al., 2002). Such defects were common in gsl8 mutants e.g. cells and intercellular spaces in the shoot apex and in cotyledons were enlarged abnormally. However, gsl8 also exhibits abnormal cell overproliferation such as an increased number of cells in specific tissues and layers in the rootcap columella, in the hypocotyl cortex and endodermis, and in the phloem. This overproliferation phenotype is especially severe in the epidermis (see below). Since an overproliferation phenotype has not been reported for any cytokinesis mutants (Lukowitz et al., 1996; Nicol et al., 1998; Zuo et al., 2000; Assaad et al., 2001; Strompen et al., 2002), these cytokinesis and division restriction functions of GSL8 might be due to different underlying processes. GSL8 was expressed in all the organs tested (Fig. S6). Its expression was not restricted to actively dividing tissues, and was also detected
in mature rosette leaves that were no longer dividing. This expression study raises the possibility that GSL8 is involved in cellular processes in addition to cytokinesis.

**GSL8 in stomatal and epidermal development**

To our knowledge, *GSL8* is the only locus identified to date that is strongly involved in both cytokinesis and stomatal patterning. Other mutants that disrupt cytokinesis show a more normal stomatal distribution (Söllner et al., 2002; Falbel et al., 2003) suggesting that patterning and cytokinesis are mostly independent processes.

Normally stomata follow the “one-cell-spacing” rule, in which two stomata are separated by at least one intervening non-stomatal cell (Geisler et al., 2000; Bergmann and Sack, 2007). In Arabidopsis, this spacing pattern is primarily established when an asymmetric division places a new stomatal precursor (a meristemoid) away from a pre-existing stoma, and only secondarily when the meristemoids in contact divide away from each other (Figure S5) (Geisler et al., 2000; Lucas et al., 2006). Several genes control these events, including those encoding likely receptors and ligands such as *TOO MANY MOUTHS (TMM)*, *ERECTA (ER)*, and *EPIDERMAL PATTERNING FACTOR 1 (EPF1)*. Mutations in these genes induce the formation of clusters of stomata in contact (Nadeau and Sack, 2002; Shpak et al., 2005; Bergmann and Sack, 2007; Hara et al., 2007). These clusters tend to be distributed similarly throughout the epidermis, and non-stomatal epidermal cells mostly resemble those in wild-type plants. However, unlike the above mutants, *gsl8* clusters vary greatly in the arrangement and size of stomata. In addition, the *gsl8* epidermis displays global disruptions in the distribution of epidermal cells such as islands of small cells that harbor excess asymmetric divisions and meristemoids in contact. Perhaps this dense packing of small cells inhibits division and the correction of pattern violations.

Clearly *GSL8* is also required for properly organizing the distribution, planes, and number of other tissues and cell types consistent with a required and ubiquitous role in cytokinesis throughout the plant. However, it remains to be determined how likely defects in callose deposition might induce these abnormal morphologies.
In summary, *GSL8* encoding a putative callose synthase required for cell plate formation during sporophytic cytokinesis. In addition to typical cytokinesis-defective phenotypes, the loss-of-function of *GSL8* induces or results in novel developmental phenotypes including defects in epidermal planarity and patterning, stomatal spacing, and cell type overproliferation.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

All Arabidopsis mutant lines and wild-type plants used in this study are in Columbia (Col) background. Unless otherwise indicated, seeds were surface sterilized (20% commercial bleach and 0.1% Triton X-100) for 5 min, washed three times in sterile water, and stored at 4°C for 3 d. The seeds were sprinkled on 0.6% agar-solidified media containing 1 x MS salts and 0-3% sucrose (pH 5.8). Growth conditions were at 25°C with a 16-h-light/8-h-dark cycle, either in soil or on MS plates. *gsl8-1* (SALK_111094), *gsl8-2* (GABI_851C04), *gsl8-3* (SALK_057120), *gsl8-4* (SALK_109342), *gsl8-5* (SAIL_21_B02), *gsl8-6* (SAIL_679_H10) were obtained from ABRC or NASC. To determine the site of T-DNA insertion in all of these alleles, we sequenced the PCR products amplified using the appropriate left border primer and gene specific primer. Primer sequences for all PCR-based genotyping are listed in Table S3 and S4. The homozygosity of all mutant alleles of *GSL8* was verified by PCR genotyping and RT-PCR. Calli were induced from cotyledons excised from one-week-old wild-type and *gsl8* seedlings, and were grown on media previously described (Encina et al., 2001).
RNA Extraction and RT-PCR

Plant tissues were harvested and total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. A total of 1μg DNA-free RNA was reverse transcribed using an oligo(dT) primer and reverse transcriptase (M-MLV RTase, SolGent). The PCR amplification protocol was 96°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s. The ACTIN2 transcript was used as an internal control. The primer sequences were: AtGSL8-d1 5’-CCCTCTGGATTTGAATGGCA-3’ and AtGSL8-r1 5’-GGCTAAAGAGAGTAGAGCCC-3’ for GSL8, and ACTIN2-d1 5’-TCAATCATGAAGTGTGATGTGG-3’ and ACTIN2-r1 5’-TTAGAAACATTTTCTGTGAACGAT-3’ for ACTIN2.

Microscopy

For phenotypic analysis of the mutant embryos, developing seeds were collected from siliques at different developmental stages from wild-type and heterozygous gsl8 plants. They were cleared with a drop of Hoyer’s solution (50 g of chloral hydrate, 3.75 g of Gum Arabic, and 2.5 ml of 100% glycerol in 15 ml of distilled water) for at least 30 min at room temperature. Cleared seeds were examined with an Olympus FV1000 confocal microscope equipped with Nomarski DIC optics.

For histological and TEM examination, intact tissue samples were fixed in 2% glutaraldehyde in 50 mM PBS (pH 6.8) at 4°C for 12-24 h, followed by postfixation in 1% osmium tetraoxide (OsO4) buffer at 4°C overnight. Samples were then sequentially dehydrated through an ethanol series, embedded in Epon-812 resin, and polymerized at 60°C for 48 h. Both semithin (1 μm) and ultrathin (70 nm) sections were cut using an ultracutmicrotome (EM UC6, Leica). Semithin sections stained with toluidine blue O were examined under a light microscope (Axioplan 2, Zeiss), and photographs were taken with a digital camera (AxioCam HRc, Zeiss). Ultrathin sections were mounted on slots and then stained with uranyl acetate followed by lead citrate for imaging with a Philips TECNAI 12.
transmission electron microscope.

Propidium iodide staining was performed as described previously (Lucas et al., 2006) using a Nikon C1 Plus confocal laser scanning microscope. Root tips were stained with callose staining buffer for 3-5 min, which is a mixture of 0.1% aniline blue in autoclaved triple-distilled water and 1M glycine, pH 9.5, at a volume ratio of 2 : 3. Aniline blue was examined by using a fluorescence microscope (Axioplan 2, Zeiss). For co-staining, root tips were stained with the dye FM4-64 (Molecular Probes) according to a previous study (Fujimoto et al., 2008) before aniline blue staining. Aniline blue and FM4-64 fluorescence was examined with 405 nm and 523 nm lasers by using a fluorescence confocal microscope (LSM5, Zeiss).

**Constructs and Plant Transformation**

To generate the dexamethasone-inducible *GSL8* RNAi construct, the entire *GSL8* RNAi cassette comprising sense and antisense arms of *GSL8* interspersed by two introns (PDK and CAT) was amplified from AGRIKOLA *GSL8*-specific hairpin RNA expression plasmids (CATMA 2a35110) using the primers dsGSL8-SalI-d1: 5'-GGGC GTCGAC CGCAAGACCCTTCCTCTATA T-3' (SalI site was introduced) and dsGSL8-SpeI-r1: 5'-CCCG ACTAGT CGCATATCTCATTAACGAGGA-3' (SpeI site was introduced). Sequence-confirmed PCR product was digested with SalI and SpeI and cloned into a dexamethasone-inducible binary transformation vector pTA7002 to produce pTA7002-GSL8-RNAi. Dexamethasone treatment was carried out according to Binarová et al. (2006).

The pTA7002-GSL8-RNAi vector were transferred into *Agrobacterium tumefaciens* strain GV3101 and used to transform Arabidopsis ecotype Col-0 by dipping. Seeds harvested from mature transformed plants were sterilized, stratified for three days and sown on MS agar plates containing hygromycin (50 mg/L). Positive lines were selected after growing for 10 days on hygromycin plates. Putative transgenic plants were transplanted onto soil and grown in a growth chamber.
ACKNOWLEDGMENTS

Fluorescence imaging was partially supported by the Live Molecular Imaging Center of KRIBB, Korea.

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Figure Legends

Figure 1. Characterization of GSL8 T-DNA insertion lines. (A) The GSL8 gene contains 45 exons in the coding region. Insertion sites are numbered for T-DNA lines: Salk_111094 (gsl8-1), SAIL_679_H10 (gsl8-6), GABI_851C04 (gsl8-2), SALK_057120 (gsl8-3), SALK_109342 (gsl8-4), and SAIL_21_B02 (gsl8-5). (B) RT-PCR of total RNA showing the presence of a 500 bp PCR product against 3’ coding region from WT plants, but none from the T-DNA lines. ACTIN2 is used as an internal control.

Figure 2. Phenotypes of gsl8 mutant seedlings and roots. (A) 10-day-old light-grown seedlings on MS plates containing 3% sucrose. Mutants are seedling lethal and display short, wide, and serrated roots and irregular cotyledon margins. Numbers in (A, C) denote gsl8 mutant alleles. (B) Higher magnification of gsl8 mutant cotyledons. (C) gsl8 mutant seedlings grown in the dark (7 days) have short, wide, and deformed hypocotyls and roots. (D and E) Higher magnifications of dark-grown cotyledons from WT (D) and gsl8 mutant (E). Scale bars: 5 mm in (A, C), 1 mm in (B, D and E).

Figure 3. Embryo development in wild-type and gsl8 mutants. Seeds were harvested from wild-type (A-D) and gsl8-4 homozygous mutant (E-I) plants at different developmental stages and then cleared and viewed using Nomarski optics. Abnormal phenotypes are detectible at globular (F) and heart (H) stages, as well as in mature embryos (I). Other five gsl8 mutant alleles showed similar results to those of gsl8-4. Embryos in (E, I) were cleared from the same gsl8-4 heterozygous siliques. Embryos in (H, J) were dissected away from surrounding seed tissues. Scale bars: 50 µm in (A-D, F, G) and 100 µm in (E, H, I), respectively.
**Figure 4.** Tissue phenotypes in *gsl8* mutant seedlings. (A to D) Light micrographs of sections of shoot apical meristems (A, B) and cotyledons (C, D). Compared to the WT (A, C), *gsl8-4* meristem (B) and mesophyll (D) tissues are disorganized. Large intercellular space (arrow) in (D). (E to H) Cross sections of hypocotyls (E, F) and longitudinal section of roots (G, H) from WT (E, G) and *gsl8-4* mutant (F, H). (I) TEM of columella cell layers in *gsl8-4*. Arrowhead indicates quiescent center. (J to K) TEMs of hypocotyls in cross section of WT (J) and *gsl8-4* (K) with endodermal cells numbered. (L and M) TEM of the root tip in WT (L) and *gsl8-4* (M). Co: columella cells, CR: cortex, CRi: internal layer of cortex, CRO: outer layer of cortex, EN: endodermis, EP: epidermis, PE: pericycle, QC: quiescent center, S: sieve elements, T: tracheary elements. Similar patterns were observed from *gsl8-1* and *gsl8-2* mutants. Scale bars: 20 μm in (A-D, I), 30 μm in (E-H) and 10 μm in (J-M).

**Figure 5.** GSL8 is required for stomatal and epidermal patterning. (A) Developing wild-type epidermis showing distributed asymmetric divisions and relative uniformity in cell size. (B to E) Developing *gsl8* epidermis of leaves and cotyledons. Stars indicate clusters of developing or mature stomata or of three adjacent meristemoids in lower part of (B). Arrows in (D-E) indicate regions containing abnormally high numbers of small cells. 10-day-old plants. All images show cell walls stained with propidium iodide and visualized by confocal microscopy. All PI images have inverted grayscale. Scale bars = 25 μm.

**Figure 6.** Aberrant cell walls in *gsl8-4* mutants. (A to C) Cell wall stubs in epidermal cells visualized using propidium iodide staining and confocal microscopy. Optical sections from outer (A), middle (B), and bottom parts of same cells (C). All PI images were inverted. Other five *gsl8* mutant alleles showed similar results to those of *gsl8-4*. (D to F) TEMs of cotyledons (D) and root tips (E) from 3-day-old *gsl8-4* mutant, and TEM of *gsl8-4* embryo (F). Arrows indicate cell wall stubs. N: nucleus. Scale bars: 10 μm in (A-C) and 2 μm in (D-F).
Figure 7. Callose deposition at cell plates and/or the new cell walls originated from cell plates. (A) Comparison of images of same roots using fluorescence optics from aniline blue staining of wild-type (bottom root) and a dexamethasone-treated GSL8 RNAi line (top root). Scale bar: 30 μm. (B to I) Confocal images following aniline blue and FM4-64 staining from root tips of 1-week-old plants of WT (B, C), gsl8 mutant (D-F) and Dex-inducible GSL8 RNAi line (G-I). Merged images of aniline blue (blue) and FM4-64 fluorescence (red) (B, E, H). Grayscale images of blue channel (C, F, I). Bright field images (D, G). Arrowheads indicate representative aniline blue stainings. Rectangular boxes indicate callose bands in two opposite sides of a cell, suggesting that at least one of them is new cell wall originated from cell plate. Scale bars: 20 μm.