Maize *CslD1* affects cell division

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Cell Biology
Cellulose Synthase-Like D1 is integral to normal cell division, expansion, and leaf development in maize

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ABSTRACT

The Cellulose Synthase-Like D (CslD) genes have important, though still poorly defined roles in cell wall formation. Here we show an unexpected involvement of CslD1 from Zea Mays in cell division. Both division and expansion were altered in the narrow-organ and warty phenotypes of the csld1 mutants. Leaf width was reduced by 35% due mainly to a 47% drop in number of cell files across the blade. Width of other organs was also proportionally reduced. In leaf epidermis, the deficiency in lateral divisions was only partially compensated by a modest, uniform increase in cell width. Localized clusters of mis-divided epidermal cells also led to formation of warty lesions, with cell clusters bulging from the epidermal layer, and some cells expanding to volumes 75-fold greater than normal. The decreased cell divisions and localized epidermal expansions were not associated with detectable changes in cell-wall composition of csld1 leaf blades or epidermal peels, yet a greater abundance of thin, dense walls was indicated by high-resolution X-ray tomography of stems. Cell-level defects leading to wart formation were traced to sites of active cell division and expansion at the bases of leaf blades, where cytokinesis and cross-wall formation were disrupted. Flow cytometry confirmed a greater frequency of polyploid cells in basal zones of leaf blades, consistent with disruption of cytokinesis and/or the cell cycle in csld1 mutants. Collectively, these data indicate a previously unrecognized role for CSLD activity in plant cell division, especially during early phases of cross-wall formation.
INTRODUCTION

The ancient, highly-conserved family of Cellulose Synthase-Like D (CSLD) proteins are required for cell growth and development, yet their biochemical and cellular functions are only now emerging (Richmond and Somerville, 2000; 2001; Favery et al., 2001; Wang et al., 2001; Bernal et al., 2007; 2008; Yin et al., 2009; Park et al., 2011; Yin et al., 2011). CSLDs belong to one of ten distinct subfamilies in the Cellulose Synthase superfamily, defined by amino acid sequence similarity to Cellulose Synthase (CESA) (Richmond and Somerville, 2000; Hazen et al., 2002; Farrokhi et al., 2006; Penning et al., 2009; Fincher, 2009). All members of this superfamily share predicted function based on sequence identity as membrane-bound, processive glycosyltransferases that synthesize beta-linked glycan polymers, such as those of cell wall polysaccharides (Richmond and Somerville, 2000; 2001). Known products range from cellulose to hemicellulose backbones, and may include additional beta-linked glycan chains (Arioli et al., 1998; Dhugga et al., 2004; Liepman et al., 2005; Burton et al., 2006; Cocuron et al., 2007; Doblin et al., 2009). The CSLDs remain poorly understood despite their importance in cell development and evidence for their evolution in plant lineages extending back to non-vascular land plants and possibly before (Roberts and Roberts, 2007).

Of the cellulose synthase-like genes, CslDs are the most closely related to the CesAs themselves, leading to early suggestions that CSLDs may also function as cellulose synthases (Doblin et al., 2001). The CSLD proteins also share the greatest amino acid sequence identity (40-50%) with CESAs and are similar or slightly larger in size (Richmond and Somerville, 2001). Other than CESAs, CSLDs are the only members of the superfamily that have the amino-terminal, Zn-finger-like domain thought to function in protein-protein interactions, possibly mediating formation of complexes, or protein turnover (Richmond and Somerville, 2000; Kurek et al., 2002; Gamsjaeger et al., 2007). Also, recent evidence suggests cooperative action between CSLD proteins in Arabidopsis (Yin et al., 2011), possibly in a similar fashion as cellulose synthases (Taylor et al., 2003; Persson et al., 2007). To date, the strongest evidence that CSLDs function as cellulose synthases comes from data for (1→4)-β-glucan synthase activity from a CSLD protein, and successful complementation of a csld3 mutant in Arabidopsis using a chimeric CSLD3 protein with a CESA catalytic domain (Park et al., 2011).

The suggestion that the CSLD subfamily may be ancestral to the entire Cellulose Synthase superfamily is consistent with the locations and sizes of introns in CslD genes.
The CslD genes are also present in all plant genomes examined thus far, including mosses (Richmond and Somerville, 2000; 2001; Roberts and Bushoven, 2007; Yin et al., 2009). In contrast, many of the other CSL subfamilies appear only in specific taxa (Farrokhi et al., 2006; Keegstra and Walton, 2006; Vogel, 2008; Penning et al., 2009; Fincher, 2009). Of the five CSL subfamilies yet to be assigned a specific polysaccharide synthase role, only CslD and CslE subfamilies are found in both dicot and monocot genomes (unlike CslB, CslG, and CslJ). The broad distribution of the CslD genes across taxa implies a highly-conserved function (Richmond and Somerville, 2000; 2001; Roberts and Bushoven, 2007; Yin et al., 2009).

Clues to the biological roles of the CSLDs have been sought by defining their biochemical activity and/or sub-cellular localization, but interpretation of this work has not yet been conclusive. Heterologous expression studies demonstrated that CSLA (Liepman et al., 2005), CSLF (Burton et al., 2006), CSLC (Cocuron et al., 2007), and CSLH (Doblin et al., 2009) proteins catalyze synthesis of hemicellulose polysaccharide backbones, and consequently, the CslD genes were also hypothesized to encode hemicellulose synthases (Sandhu et al., 2009). However, similar approaches have thus far been unsuccessful with CSLDs. Other important lines of evidence have led to alternate interpretations. Analysis of cell-wall polysaccharides, for example, from key cell types, cell-culture treatments, or genetic perturbations suggest CSLDs could function in either production of cellulose (Manfield et al., 2004; Li et al., 2009) or hemicellulose backbones (Bernal et al., 2007; Li et al., 2009; Yin et al., 2011). However, interpreting differences in cell wall composition is complicated by broad changes in multiple wall constituents that often occur in response to genetic perturbation (Orfila et al., 2005; Persson et al., 2007; Bernal et al., 2007; Li et al., 2009).

Localization studies, which would indicate where CSLD functions within a cell, have also been inconclusive to date. Targeting studies show CSLD proteins appear to localize in the Golgi, where they could aid hemicellulose biosynthesis (Favery et al., 2001; Bernal et al., 2007, 2008; Zeng and Keegstra, 2008; Li et al., 2009). However, these observations are also consistent with transit of CSLD proteins through Golgi enroute to the plasma membrane, as has been observed for cellulose synthases (Kimura et al., 1999; Crowell et al., 2009; Gutierrez et al., 2009). Recent studies also demonstrate that CSLD proteins localize to the plasma membrane in rice suspension culture cells (Natera et al., 2008) and in Arabidopsis root hair cells (Park et al.,
Polarized plasma membrane localization of CSLD3 supports a role in polysaccharide deposition at the growing tip of Arabidopsis root hair cells (Park et al., 2011). Thus far, the majority of genetic evidence has implicated CSLDs in polarized cell expansion associated with polar tip growth typical of pollen tubes and root hairs (Bernal et al., 2008; Favery et al., 2001; Wang et al., 2001; Kim et al., 2007; Bernal et al., 2008; Penning et al., 2009), and the intrusive tip growth typical of xylem and other sclerenchyma fibers (Samuga and Joshi, 2004). In the moss, Physcomitrella patens, where tip growth predominates in caulonema cells, CslD genes comprise 46% of all expressed sequence tags from the CESA superfamily, including all CesAs and CSLs (Roberts and Bushoven, 2007). In vascular plants, root hairs and pollen tubes provide classic models for cellular tip growth (Hepler et al., 2001; Cole and Fowler, 2006), but xylem and sclerenchyma fibers also elongate by a form of intrusive tip growth (Mellerowicz et al., 2001; Samuga and Joshi, 2004). Expression of CslD2 in developing xylem of Populus is consistent with its proposed influence on xylem fiber length (Samuga and Joshi, 2004). However, the function of CSLDs in other aspects of cell growth, such as cell division, non-polar cell expansion, or differentiation, has not been explicitly studied to date. Some mutant phenotypes cannot be readily explained by polar growth defects (Bernal et al., 2007; Li et al., 2009; Yin et al., 2011), suggesting an expanded role for CSLDs in other cellular processes.

Here we present evidence for an unexpected, but integral role for CSLDs in plant cell division. In cslD1 mutants of maize, defects in cell division were identified as the underlying basis for the narrow-organ morphology, as well as characteristic epidermal warts. Using reverse genetics tools in maize, we identified several mutants whose phenotype is caused by transposon insertion in CslD1. The cslD1 phenotype was uniquely informative for dissecting the relationship between division and expansion. Through a detailed cellular analysis, we demonstrate that altered cell division in cslD1 null mutants reduces cell number and is an underlying cause of the narrow-organ morphology. In-depth analysis of wall positioning, cell shape, nuclear size, cell ploidy, wall architecture, and alterations in cell number indicated that defective cell division was an early consequence of a CSLD1 deficiency. These data provide new insight into the function of CSLD proteins in plant growth, extending our understanding of their roles from tip-growth, to include cell division in most or all organs.
RESULTS
Phylogenetic Analyses Indicate Early Evolution and Conservation of Divergent Roles for CSLD Proteins

Phylogenetic analyses (Fig. 1A) identified three distinct clades in the CSLD subfamily and found these to represent three phenotypic classes of csld mutants. These three major phenotypic groups include, for Arabidopsis, pollen tube defects in csld1 and csld4 mutants, (Bernal et al., 2008), root hair defects in csld2 and csld3 mutants (Favery et al., 2001; Wang et al., 2001; Bernal et al., 2008), and reduced plant size in csld5 mutants (Bernal et al., 2007). Thus far, mutants in related clades of CslD genes in rice and maize have yielded phenotypes similar to those of Arabidopsis. Mutants of rice CslD1 (Kim et al., 2007), for example, and its maize homolog, CslD5 (Penning et al., 2009), result in root hair-deficient phenotypes. Additionally, alterations in rice CslD4 (closest homolog of Zm-CslD1 and At-CslD5), confer a narrow leaf and dwarf1 (nd1) phenotype (Li et al., 2009; Hu et al., 2010; Wu et al., 2010). Similar functional roles are indicated by the reduced-growth phenotypes common to all three of these mutants (Fig. 1A, Bernal et al., 2007; Li et al., 2009; Hu et al., 2010; Wu et al., 2010). Collectively, these data suggest conservation of specific developmental roles for individual CSLD proteins in plants, and indicate that these arose early in plant evolution.

An Allelic Series of csld1 Mutants in Maize Enabled Functional Analysis

Seven independent loss-of-function mutants for the maize CslD1 gene were identified in reverse genetic screens, including two from the UniformMu maize population (University of Florida) and five from the Trait Utilities for Screening of Corn (TUSC) lines (Meeley and Briggs, 1995; McCarty and Meeley, 2009) (Pioneer Hi-Bred Int.) (Fig. 1B). The two UniformMu alleles, csld1-1 and csld1-2, were examined in the greatest depth because of their uniform genetic background (McCarty et al., 2005). Both of these were null for detectable expression of CslD1 mRNA by qPCR (data not shown). Phenotypes of csld1-1 and csld1-2 homozygous mutants, as well as offspring from their reciprocal F1 hybrids, were indistinguishable, thus demonstrating a causal role for the dysfunctional CslD1 gene. Mutant plants showed overall reduced growth, narrow leaves, and had a rough leaf texture caused by
warty protrusions from the mature leaf epidermis. Genotypic analysis of over 200 individuals from segregating families showed a 100% correspondence between this phenotype and homozygosity for the csld1-1 mutation (data not shown). Mendelian segregation ratios were typical of a recessive mutation. The five other transposon insertions in CslD1 (csld1-3 through csld1-7, courtesy of Pioneer Hi-Bred Int.) also resulted in mutant phenotypes that included narrower organs and wart-like clusters of epidermal cells, regardless of heterologous genetic background.

**Phenotype of csld1 Mutants: Narrow Organs and Wart-Like Cell Clusters**

Null mutants of csld1 showed a striking phenotype that included narrow leaves, reduced stature, and highly textured leaf blades (Figs. 2-3). Light microscopy showed that the visibly rough texture of csld1 mutant leaves was due to irregular swelling by groups of epidermal cells that formed wart-like cell clusters (Fig. 2). Some epidermal cells expanded 75-fold in volume, and were generally arranged in linear profiles along the longitudinal axis of the leaf midrib and blade (Fig. 2A), but not on leaf sheaths or stalks (data not shown). The Groups of cells that formed so-called warts were interspersed with normal-appearing regions of leaf epidermis along the entire length of the leaf blade. Swollen cells remained filled with fluid until the onset of leaf senescence (Fig. 2C). Warts were present on both surfaces of mutant leaf blades, but were larger and more abundant on the abaxial face (Fig. 2D). These malformed cells consistently lacked chloroplasts (Fig. 2C, E), indicating an epidermal origin, as confirmed in serial cross sections of mutant leaves (Fig. 2F, G). These ballooned epidermal cells in csld1 mutants often had diameters well over 100 µm, at least five-, and sometimes 20-fold greater than epidermal pavement cells of non-mutant plants (Fig. 2G). Both SEM and optical microscopy of fresh, intact leaves showed that lesions continued to expand throughout leaf maturation and that the largest clusters included swollen cells that had collapsed. In other instances cells remained intact, even in lesions greater than 300 µm across (Fig. 2C).

Potentially-analogous, wart-like epidermal swellings were described by Burton et al., (2000) in a VIGS gene silencing experiment in tobacco. Although a CesA gene was targeted, the highly-similar CslD genes may also have been silenced. The transgenic tobacco had wart-like lesions on the abaxial side of leaves and an overall phenotype strikingly similar to the maize csld1 mutants (Burton et al., 2000). This commonality would be consistent with some degree of
repression of the Zm-CsiD1 ortholog in the tobacco experiment. Alternatively, if observed lesions did result from down-regulation of CesA genes alone, then this would support a role for CSLD proteins in cellulose biosynthesis (Doblin et al., 2001; Park et al., 2011).

**Plant Dry Weight and Organ Width are Reduced in csld1 Mutants**

Total growth and organ size were reduced in homozygous csld1 mutants (Fig. 3) even though overall plant architecture, leaf number, and flowering time, were similar for csld1 mutant and non-mutant siblings under field and greenhouse conditions (data not shown). At maturity, the mean height of mutant plants (to auricle of the uppermost leaf) was only 11% less (p < 0.001), whereas dry weight decreased by a more pronounced 44% (p < 0.0025) (Fig. 3A). Proportional reductions in dry weight were also evident for all organs examined, including ears, tassels, stalks, roots, and leaves (data not shown). Organ width decreased 35% (p < 0.0003) for mature leaf blades of csld1 mutant plants, but length was only 10% less (p < 0.0003) (Fig. 3B). This narrow-leaf phenotype was proportional in all leaves examined, indicating a consistent defect in lateral development rather than an ontological effect at specific leaf positions (Fig. 3B).

Organ width was also reduced in stalks from csld1 mutants (Supplemental Figure 2). The cross-sectional areas of csld1 stalks were an average of 24% less (p < 0.025). The narrow-organ phenotype extended to cobs and tassels as well (data not shown).

**Cell numbers are Reduced in Mutants and are More Pronounced than Changes in Cell Size.**

Both cell division and cell expansion contribute to the final shape of an organ; yet it has been traditionally difficult to separate causative factors during maize leaf morphogenesis. Given the reduced leaf width in csld1 mutants, we asked whether the mutant leaf morphology was due to decreased cell number, indicating CSLD1 acts directly or indirectly on cell division, or whether the primary cause of narrower leaves was the altered cell width. To answer this question, we quantified cell numbers and sizes in mutant and non-mutant leaf blade epidermis (Fig. 4). Examination of impressions from non-warty areas of mutant and non-mutant leaves showed that the length of epidermal cells was not detectably different, and the width of cells from the csld1 mutant was 17% greater (p < 0.0003) (Fig. 4). Interestingly, the most pronounced difference between mutant and non-mutant was that of total cell numbers, and thus divisions,
across the leaf blade, which was 47% less for csld1 mutants (Fig. 4) based on calculations from cell-level (Fig. 4) and leaf-level analyses (Fig. 3).

Leaf cross sections were examined to ascertain whether similar abnormalities were present in non-epidermal cells of csld1 mutant leaves. Leaf thickness was consistently increased in mutant leaf blades, and vascular bundles were closer together (Supplemental Figure 3A). Fully-expanded csld1 mutant leaf blades were 40% (p < 0.004) thicker than non-mutant leaf blades (Supplemental Figure 3B). The extent of non-epidermal contributions to total leaf thickness was 43% (p < 0.002) greater for csld1 mutant leaf blades, and thus proportional to those of epidermis (Supplemental Figure 3B). Increases in cell size were variable but most often apparent in mesophyll-sheath cells. Overall organization of the mesophyll sheaths was less regular. The csd1 phenotype was therefore not limited to the epidermis alone. Additionally, mutant leaves showed a 12% (p < 0.03) increase in vascular-bundle density (Supplemental Figure 3C).

**Levels of CslD1 mRNA are Greatest in Regions of Active Cell Division**

In order to view the phenotypes of csld1 mutants in the context of where the wildtype gene is expressed, levels of CslD1 mRNA were measured via quantitative RT-PCR across diverse tissues and stages of development (Fig. 5). The CslD1 transcript levels were greatest in young, pre-emergent leaves (inside the whorl) and still relatively abundant in young primary root tips and bases of more mature leaves (Fig. 5A). These mRNAs were also detected in basal zones of leaves by other recent studies of maize leaf development (Li et al., 2010). To more clearly define the pattern of transcript accumulation during leaf development, staged samples of young to mature leaves were analyzed. The CslD1 mRNA levels were highest in tissues with actively dividing cells, and maximal in shoots 6 days after germination (Fig. 5B). Later in development, single-leaf analyses showed that levels of CslD1 mRNA were greatest in basal portions of blades from expanding leaves, 15-to-25 cm long. In non-dividing, fully-expanded leaves, CslD1 mRNA had dropped below detectable levels and remained so in the fully-differentiated portions of leaves (Fig. 5B). Notably, wart formation and expansion continues in leaves through maturity, even within areas where CslD1 is no longer expressed.
Cell Wall Composition Was Similar, but Thin, Dense Walls Were More Abundant in *csld1* Mutants.  

To further characterize differences between mutant and non-mutant tissues, high-resolution X-ray micro computed tomography (micro-CT) was used to analyze hand-cut sections of stalks, since this tissue was found amenable to the X-ray approach. Three-dimensional reconstructions revealed a shift in average wall thickness towards thinner walls in the *csld1* mutant (Fig. 6). Additionally, this method allowed a comparison of overall wall density, which was greater for stalks from mutant plants, regardless of whether the pith and vascular-rich rind were examined together or separately (Fig. 6).

To determine whether these changes were associated with alterations to cell wall polysaccharide composition, we examined cell walls from mature leaf blades as well as isolated leaf epidermis. No significant differences in alcohol-insoluble cell wall composition were detected between *csld1* mutants and non-mutants for either cellulose or sugar subunits of non-cellulosic constituents (Table 1). Cell walls from epidermal cells of both plant types revealed distinctive composition relative to samples from whole-leaf blades. Specifically, epidermal cell walls had less glucose, rhamnose, galactose, and galacturonic acid, but more xylose, compared to whole-leaf blades (Table 1).

Multiple Cell Division Defects are Evident in *csld1* Epidermis

Dark-field images of epidermal peels from mature leaf blades showed that the normal ordering of epidermal cells was disrupted in *csld1* mutants, and revealed frequent anomalies in recently-formed cross walls. The latter included stubs of incomplete cell walls, particularly along the longitudinal axis of leaves (Supplemental Figure 3). Other alterations of cell shape and misaligned cell walls were also consistent with causal defects in cell division, and these persisted in mature leaves.

To determine the developmental stage at which these cell abnormalities could first be detected, 5-10 cm immature leaves (sites of maximal *CslD1* expression) were sampled from *csld1*-mutant and non-mutant plants, stained with propidium iodide, and examined by confocal microscopy (Fig. 7). Disrupted cell files and misshapen cells were evident in images of abaxial epidermal cells from the pre- and post-differentiation zones (as defined by Mitkovski and Sylvester [2003] and determined by the absence and presence of stomata, respectively) (Fig. 7A,
B). Cell wall stubs were frequently observed in epidermal cells of both pre- and post-differentiation zones (Fig. 7). Again, these were typically oriented along the longitudinal axis (Fig. 7). While many epidermal cells at these stages were approximately twice the normal width (as if they had failed to undergo a single longitudinal division), nearly all cells of csld1 mutants were at least some degree wider than those of non-mutants (Fig. 7 A, B).

A greater cell width also remained evident later in development, even for otherwise normal-looking cells outside wart-like lesions (refer back to Fig. 4). Also, in multiple instances, files of atypically large epidermal cells were bounded at proximal and distal ends by cells that appeared to have mis-divided. Cell wall stubs were visible in each, and faced the large files. These files of wide cells were located at positions normally occupied by two distinct files of smaller cells (Fig. 7C). This pattern indicated either failure of consecutive neighboring cells to divide, or more likely, a clonal file derived from a single cell that failed to complete division. In addition, three-dimensional imaging with confocal microscopy identified a large number of gaps and holes in cell walls that would otherwise have appeared to be complete when viewed by standard imaging techniques (Fig. 7D). The actual number of incomplete walls may thus be underestimated when non-confocal approaches are used.

Because cells with large or multiple nuclei are commonly observed in cell-division-defective mutants (Smith et al., 1996; Lukowitz et al., 1996; Spitzer et al., 2006), propidium-iodide stained nuclei were examined in basal regions of immature leaf blades from csld1 mutants (Fig. 8A). Compared to non-mutant epidermis of the same stage (pre-differentiation zone of 5-10 cm leaves), csld1 mutant cells generally had larger nuclei (Fig. 8A). While significant variation in nuclear size was observed even in non-mutant tissue, the range in csld1 mutants was much greater, with cells containing nuclei ranging from one-, to approximately four-fold the two-dimensional area of normal nuclei (Fig. 8A). Large nuclear size was consistently correlated with large cell size (Fig. 8A). Flow cytometry of nuclei from basal regions of immature leaves showed significantly more (p < 0.05) 4N nuclei in csld1 mutants compared to non-mutants (Fig. 8B).

DISCUSSION
CSLDs Acquired Divergent Roles Early in Plant Evolution
Integration of mutant phenotypes from maize, rice, and Arabidopsis with the CSLD phylogeny reveals ancient functional divergence and highly-conserved developmental roles for sub-groups within the CSLD family (Fig. 1A). Although double- and triple-mutant analyses have demonstrated some overlapping function for Csld genes in Arabidopsis (Yin et al., 2011), each single-gene mutation described thus far has a distinctive phenotype (except for a putative pseudogene At-Csld6) (Favery et al., 2001; Wang et al., 2001; Bernal et al., 2007, 2008; Kim et al., 2007; Li et al., 2009; Penning et al., 2009; Hu et al., 2010; Wu et al., 2010). When we overlay data on mutant phenotypes onto a phylogenetic tree, CSLD clades correspond to distinct classes of phenotypes; (i) root-hair-defective, (ii) male-transmission-defective, or (iii) reduced-growth (Fig. 1A).

There are several notable aspects of this pattern. First, the observation that each Csld mutant has a visible phenotype indicates limited functional redundancy within the gene family. Second, the associations shown in Figure 1A persist despite fundamental structural differences between the Type I primary cell wall of Arabidopsis and the Type II cell wall of rice and maize (Harris and Hartley, 1980; Carpita and Gibeaut, 1993; Carpita, 1996; Carpita et al., 2001). Roles of Csld genes thus transcend the major differences in non-cellulosic cell wall constituents of diverse plant species, and the primary developmental functions of individual Csld genes appear to have been maintained. Third, previous results suggested specific contributions by CSLD proteins in tip-growing cells (Bernal et al., 2008), however, disruptions in At-Csld5, Os-Csld4, and Zm-Csld1 led to reduced overall plant growth without visibly altering classic tip-growing cells (Fig. 1A, Bernal et al., 2007; Li et al., 2009; Hu et al., 2010; Wu et al., 2010; Yin et al., 2011). Broader developmental functions are thus indicated for these genes. Among the reduced-growth phenotype clade, that of maize csld1 is unique in its production of wart-like lesions. This difference might lie in greater growth rate and expansion of maize leaves. In other respects, however, commonalities between the reduced-growth phenotypes suggest a shared function for this sub-group of CSLDs.

**Reduced Cell Division Is a Central Effect of the csld1 Mutation**

The combination of reduced leaf width, together with overly expanded cells is intriguing and points to the possibility that analysis of the cellular effects of the mutation could provide information about the controls of cell division and expansion. We show here that cell division...
defects are early effects of the mutation (Figs. 2, 3, and 4) and that the observed reduction in cell number contributes to the narrow-leaf phenotype (Figs. 3 and 4). Although cell size increases dramatically in epidermal cells that balloon out of the leaf plane in the csld1 mutant (Fig. 2), most epidermal cells expand by only a modest 17%, and do so only along their lateral axis (Fig. 4). Collectively, decreased cell number and fewer cell divisions that affect leaf width (35% narrower) and plant dry weight (44% less) (Fig. 3), is only partially compensated by enhanced cell expansion. Estimated numbers of cells across leaves is reduced by 47%. This drop in cell number can be attributed to reduced cell divisions in the lateral dimension, indicating that this specific decrease in divisions is a central effect of csld1 mutations. Although examination of cross-sections suggested that leaf vascular bundle number and density was altered in mutant leaves, the size and shape of vascular bundles were generally unchanged. Instead, the greater thickness of csld1 leaf blades typically corresponded most closely with irregular increases in size by cells of the mesophyll sheath (Supplemental Figure 3), indicating a broader role for CSLD1 than epidermal development alone.

A cell-division role for CslD1 early in leaf development is also supported by its maximal expression in zones of wildtype leaves where cells are most actively dividing (Fig. 5), and with the occurrence of anomalous divisions within this zone in the csld1 mutant (Fig. 7). These disrupted divisions result in formation of abnormal cell clusters implicated in later wart development, and potentially affect determination of cell-file numbers across leaf blades. Expansion of the epidermal warts appears to be largely a secondary effect of the csld1 mutation, because these cells continue to expand in leaves after CslD1 mRNAs would have dropped below detectable levels (Fig. 5).

While results are compatible with a central defect in rate or total number of cell divisions, diverse, indirect effects may also contribute to the observed phenotype. Narrow leaves of csld1 mutants, for example (Fig. 3), could exacerbate reductions in plant dry weight by decreasing total-plant photosynthetic capacity. The smaller csld1 root system (Fig. 3) could further reduce growth. Also, the epidermis may have a prominent physical role in organ expansion and meristem geometry (Green, 1980; Moulia, 2000) providing additional potential for secondary or tertiary effects of the csld1 mutations.

Cell expansion is clearly also altered by direct and/or indirect effects of the mutation. Much of the cellular over-expansion is likely secondary, since compensatory expansion is a
common response to decreases in cell number (Reynolds et al., 1998; Mitkovski and Sylvester, 2003; Beemster et al., 2003; Horiguchi and Tsukaya, 2011). However, the csld1 mutations could theoretically affect more than cross-wall deposition during division and subsequent, compensatory expansion. If properties of mutant walls are altered, then these too could contribute to expansion-based aspects of the phenotypes (discussed further below).

Cell Wall Thickness, but Not Composition Are Altered in the csld1 Mutant

Although the overall quantity of cell wall material is reduced by 45% in the csld1 mutant plants, cell wall composition is unaffected in either whole organs or epidermis alone (Table 1). Several possibilities could account for the lack of detectable difference. First, the CSLD1 polysaccharide product may normally be present in only small amounts, particularly if synthesis is restricted to a defined period during cytokinesis or new cell wall formation. If so, then cell wall contributions from CSLD1 could be masked by more abundant polymers. As noted above, an early developmental role for small amounts of CSLD product would be consistent with the maximal expression of CslD1 in very young leaves and basal portions of fast-growing blades (Fig. 5), since these are zones of active cell division (Sylvester et al., 1990; Freeling, 1992; Sylvester, 2000). One permutation of this suggestion is that CSLD1 might aid a specialized, directed deposition of early-arriving polymers to the growing cell plate during division. The CSLD1 product itself could thus be limited to a narrow point in time.

Another possibility is that the CSLD1 enzyme might synthesize a limiting constituent of cell walls, such that a drop in levels of the CSLD1 product would result in similar decreases for other cell-wall polymers. In this way, csld1 mutants could produce less total cell wall without changing relative proportions of individual cell-wall components. This suggestion is consistent with the reduction in total dry matter. Still another, compatible scenario, is that the polysaccharide product of CSLD1 may not differ in a way immediately detectable by current analyses, but may nonetheless have functionally altered properties and/or capacities for interaction that affect later cell-wall behavior. All three of the above possibilities remain consistent with a temporally-limited contribution of a known wall component.

Cell wall analysis by high-resolution, X-ray, micro CT (Fig. 6) provides detailed structural information on internal regions of intact tissue, including the relative density of cell wall material, based on X-ray beam attenuation (Steppe et al., 2004; Dhondt et al., 2010). Stems
of csld1 mutants (tissues found most amenable to the X-ray, micro CT approach) show slight, but consistent increases in wall density and a greater overall abundance of thin-walled areas, regardless of position in rind or pith (Fig. 6). These findings indicate an altered cell-wall or cellular architecture that is apparently independent of changes in wall composition. One, albeit speculative possibility is that the changes in cell wall density may reflect a relative increase in cellulose crystallinity, with less amorphous cellulose being present in the cell walls of the csld1 mutant.

**Role of CslD1 in division and expansion**

Cell-level effects of csld1 mutations highlight differences between global and local responses that lead to narrow leaves and overly expanded, warty clusters, respectively (Fig. 9). In whole leaves, cell divisions are reduced markedly (almost 50%), while expansion is increased slightly (about 20%) (see also Figs. 3, 4). In clusters of abnormal cells, however, division anomalies are more frequent and expansion effects more extreme. One reason for this distinction is likely a contrast in mechanisms by which cell expansion responds to altered cell number (Horiguchi and Tsukaya, 2011). At the whole-leaf level, classical compensatory expansion is widely recognized as a means of adjusting organ shape via cell enlargement (Reynolds et al., 1998; Mitkovski and Sylvester, 2003; Beemster et al., 2003; Tsukaya, 2008; Horiguchi and Tsukaya, 2011), whereas local responses to changes in cell number may differ and mechanisms remain unclear (Horiguchi and Tsukaya, 2011). Primary effects of CslD1 disruption clearly include cell division, and although many aspects of expansion may be secondary, some could also be direct.

**Warts Are Distinctive, Informative Features of the csld1 Mutant**

In warts, swelling epidermal cells (Fig. 2) trace to cell-division defects early in leaf development (Fig. 7). Assuming CSLD1 is a wall-synthesizing enzyme, these cellular observations indicate that loss of CSLD1 activity disrupts the synthesis of early cell wall components, and this in turn alters division. The narrow organs result from a decreased number of long-axis cell divisions throughout the plant (discussed above). Wart formation also derives from disrupted divisions, but the locally-extreme cell size may involve additional effects of csld1 on wall expansion. Two possible scenarios for epidermal wart formation are presented below.
Hypothesis 1 is based on a role for CSLD1 in new cell wall formation during cytokinesis, and posits that epidermal warts would be initiated when rapid elongation by leaves outpaces capacity of dividing cells to form normal cross-walls. The first set of disrupted cross-walls would lead to misplacement of the next, and resulting clusters of irregular cells would be prone to anomalous expansion. Extent and irregularity of expansion would be exacerbated by asymmetric support from surrounding cells. Hypothesis 2 includes basic tenets of Hypothesis 1, but also suggests that csld1 mutants may have broader alterations to cell wall properties that reduce control of expansion. This in turn could contribute to both incomplete and misplaced cross-wall formation. Altered wall properties could reduce control of cell expansion at each step in development of irregular cell clusters and epidermal warts, and could act in a slow, uniform manner, and/or add a threshold component to the ballooning of epidermal cells. Under either hypothesis, the excessive expansion of cells in wart formation can be considered a secondary effect of the CslD1 deficiency, resulting from a cascade of interactions between altered cell division and expansion.

The sequence of events in hypothesis 1 begins with a central role for the CSLD1 polysaccharide product (likely cellulose) in formation of new cross-walls. In csld1 mutants, this cross-wall formation is implicated in reducing the number of long-axis cell divisions and thus organ width. In leaf blades especially, a CSLD1-based limitation to new wall formation could have prominent effects when the demands of rapid growth outpace the compromised capacity for cross-wall production. Resulting anomalies in csld1 epidermis include instances of non-divided cells and defective cross-walls. The latter could arise from either partial formation of these walls and/or their expansion before full completion. Resulting cells have non-existent or defective cross walls with central holes or cell wall stubs (as observed). A single cell division altered at the base of a growing leaf blade could lead to other disruptions by altering the timing and placement of the subsequent cross-walls. The abnormal divisions would then lead to the uneven and disrupted cell files, as well as large and misshapen cells (Fig. 7A, B). These cells would lack the support otherwise afforded a normal, highly-ordered, brick-like pattern of epidermal cells, and would be more prone to excessive expansion during turgor-driven growth. In addition, hypothesis 1 predicts that the warty-ness of csld1 phenotypes will be more extreme under conditions where growth is most rapid and cross walls cannot form correctly. Consistent with
this prediction, phenotypes vary in plants grown under field- versus greenhouse-conditions (data not shown).

Another possibility is that *csld1* alters cell-wall properties that affect expansion and these in turn cause cell division defects that lead to wart formation (Hypothesis 2). Such changes could have functional significance even if generated by trace amounts of materials contributed only during a brief period early in cell development. Although altered cell walls (missing a CSLD1 product) could be “weaker,” they might also be more responsive to effectors of cell expansion such as endogenous signals or physical aspects of turgor pressure. Larger or irregularly-shaped cells could result, and this could compromise their capacity to form accurately-positioned or complete cross walls. The resulting series of anomalous, failed or disrupted divisions would produce warty cell clusters. The suggestion that *csldl* cells expand more readily would be consistent with the somewhat larger size of epidermal cells overall. Wall properties might be altered in subtle ways throughout development or impart a threshold component to ballooning cells of warts.

**The CslD1 Gene Is Essential for Specific Aspects of Cell Division**

Primary effects of the *csldl* mutation on cell division can be clearly discerned in the narrow leaves and fine stems of the *csldl* phenotype. Although the overly-expanded warty lesions exhibit cascades of anomalous secondary events, the majority of cells in leaves and stems are well-ordered. Reduced cell numbers and cell divisions in these organs are consistent with a global response to the *csldl* mutation. In particular, long-axis divisions are decreased, and proportional to reductions in leaf width, other organ diameters, and plant dry weight. Although narrowness of leaf blades could theoretically be exacerbated by secondary effects of warts, the same cannot be said of the other narrow organs of *csldl* plants. As a cell-wall biosynthetic gene, *CslD1* can have direct and indirect effects. The latter can include compensatory expansion (Horiguchi and Tsukaya, 2011) as well as wall-based signaling (Hématy and Höfte, 2008; Seifert and Blaukopf, 2010; Ringli, 2010; Boisson-Dernier et al., 2011). However, changes in cell number are seldom secondary consequences of altered expansion at an organ level (Wang et al., 2003; Cnops et al., 2004; Hu et al., 2006; Szécsi et al., 2006; Horiguchi et al., 2011; Horiguchi and Tsukaya, 2011), and a primary role is suggested here for *CslD1* in cell division.
Deposition and integrity of new cross-walls are clearly impaired in dividing cells of csld1 mutants. Long-axis divisions are most markedly affected, with new walls having large central openings or appearing as incomplete stubs. Collective data reveal a new dimension to functions of the CslD gene sub-family, since these were previously considered largely related to tip growth (Favery et al., 2001; Wang et al., 2001; Kim et al., 2007; Bernal et al., 2008; Penning et al., 2009; Park et al., 2011). However, both tip growth and cross-wall formation during division share a common, directional aspect to the deposition of new wall material. Bednarek and coworkers (Bednarek and Flabel, 2002) previously suggested that such mechanisms could involve similar components. Work here is consistent with that possibility, as well as contributions by CslD genes to targeted wall formation in tip-growing and dividing cells.

The csld1 mutation also disrupts cytokinesis and/or the cell cycle. Larger nuclei were observed by confocal imaging in cells of the pre-differentiation zone of immature csld1 leaves (Fig. 8A). Similar, large-nucleate cells have been reported in studies of cell-division-defective mutants (Lukowitz et al., 1996). Here, flow cytometry also showed a small, but significant increase in endoreduplication (Fig. 8B). Even with 50% of epidermal cells undergoing endoreduplication, these would comprise a relatively small portion of nuclei from whole-leaf tissue. Whether the increase in endoreduplication and larger-sized nuclei reflects a response to larger cell size or the arrest of the cell cycle after DNA replication, but before nuclear division, remains unclear. A more rapid entry of dividing cells into an endoreduplicative state is typically observed where cell size is increasing as a compensatory response to limited cell number in a developing organ (Beemster et al., 2005; Ferjani et al., 2007; Horiguchi and Tsukaya, 2011). The delay of cells at the G2 phase implies a disruption of the normal cell cycle due to a lack of CSLD1 activity.

CONCLUSION

The significance of collective findings is threefold. 1) A previously-unrecognized role in cell division is demonstrated here for a cell-wall biosynthetic gene. 2) CslD1 is also found here to be central for effective formation and integrity of new cross-walls during cell division. 3) Collective roles for the CslD gene subfamily are shown to include not only tip-growth by cells, but also probable contributions to other aspects of directional wall deposition. Additionally, csld1 maize mutants, besides helping our understanding of the specific functions of a clearly
important cell-wall synthesizing enzyme, will provide valuable tools for dissecting the complex and interconnected processes of cell division and cell expansion. Protein localization efforts are underway and should further clarify the specific role of Cellulose Synthase-Like D1 in early cell wall formation.

MATERIALS AND METHODS

Phylogenetic Analyses

Protein sequences predicted from full length cDNAs for each of the CslD genes from rice, maize, and Arabidopsis were used to create a neighbor-joining tree using Mega 4.0 (Tamura et al., 2007; megasoftware.net), with 2,000 bootstrap repetitions using the pairwise deletion option. Alignments used to create the tree can be accessed in Supplemental Figure 1 online. Nomenclature for proteins encoded by the CslD gene family in maize was assigned as per Van Erp and Walton (2009). Protein domains were identified using SMART (smart.embl-heidelberg.de) and TMHMM (cbs.dtu.dk/services/TMHMM).

Identification of cslD1 Mutants

The UniformMu population was screened using PCR-based assays to identify Mu transposon inserts in Zm-CslD1 as per Penning et al., (2009). Close to 15,000 UniformMu lines were screened using a series of pooled DNA samples, which were forerunners of the sequence-indexed materials currently available at MaizeGDB (maizegdb.org; UniformMu.UF-genome.org). For PCR screening, CslD1-specific primers (AGTTCGTGCACTACACCCTGCACATCC and TGCTACCTGTAAAGGAATGGGATGGAATG) were used along with the Mu-specific primer TIR6 (AGAGAAGCCACCGCAGCACTCYATTTTCGTC). Resulting products were separated on 1% agarose gels, blotted onto nylon membranes, and probed with a CslD1-specific PCR product. Positive probe-binding samples were identified at X45:Y4 of the UniformMu Reverse Genetics Grid 6 (of 8 total). Seeds from the UniformMu family corresponding to these coordinates (04S-1130-27) were grown, and PCR-genotyped to identify individuals homozygous for an insertion in CslD1. The cslD1-1 allele was identified from this family, and a cslD1-1 line was established after three generations of successive backcrosses to the W22 inbred.
A second mutant allele, *csld1*-2, was identified during a visual screen of field-grown UniformMu lines. Its phenotype was indistinguishable from that of *csld1*-1. PCR primers (ACCAGATCCTCTCTCTCCGTCGTTTGC, ACCTTGTTCCCTGAGGAAGTCCCTCTC, GTGGTGATCAGCTGGCATCATTCC, AGGAGGGCTGATGTAGACCCACAG,) were designed to cover nearly the entire length of the *CslD1* gene and identified a Mu insertion in the third exon of *CslD1*. Homozygous recessive mutants of this allele were obtained from segregating progeny of this family, and the *csld1*-2 line was established after two successive backcrosses into the W22 inbred.

Additional Mu-insert alleles of *csld1* were identified from the Trait Utilities for Screening of Corn (TUSC) population of Pioneer Hi-bred as per McCarty and Meeley, (2009). Primers used were: ACCAGATCCTCTCTCTCCGTCGTTTGC, ACCTTGTTCCCTGAGGAAGTCCCTCTC, GTGGTGATCAGCTGGCATCATTCC, AGGAGGGCTGATGTAGACCCACAG, which identified five additional mutant alleles designated *csld1*-3 through *csld1*-7.

**Overall Phenotypic Analyses and Size Measurements**

Plant height was measured from soil level to auricles at the base of the uppermost leaf blades for 55 field-grown, mature plants (25 mutant, 30 non-mutant). These same 55 plants were used to measure width (at the widest point) and length of leaves at positions three, four, and five (relative to the apex). For dry weight measurements, whole-plant samples (including released root mass) were collected 3 days after ear harvest and did not include mature ears. Samples were weighed after drying for 4 weeks at 38°C. Below-and above-ground dry weights were determined by separating root masses from the aerial portions of these plants.

**Tissue Fixation and Sectioning**

One-cm squares were excised from leaves of greenhouse-grown plants and fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH). Samples were vacuum infiltrated overnight at 4°C, then shaken at 4°C during a dehydration series using ethanol in PBS (60 min each, progressing from 1x PBS with 30% EtOH to 40%, 50%, 60%, 70%, 85%, and finally 95% EtOH). Samples were stained overnight with eosin in 95% EtOH, followed by four, 1-hr incubations in 100% EtOH and eosin at 25°C. Wax imbedding was initiated by introducing CitriSolv (Fisher Cat # 22-143975) into samples using a series of 1-hr incubations (while shaking) in ethanol with increasing CitriSolv/EtOH content (25/75, 50/50, 75/25,100/0). Paraplast wax chips (Fisher Cat # 23-021-399) (1 g wax/mL CitriSolv) were added to the 100%
CitriSolv and incubated overnight at 25°C. Additional wax was added, followed by a 2-hr incubation at 42°C. Samples were transferred to 60°C where wax was poured-off and replaced eight times over 4 days before samples were allowed to harden in molds. Sections (10 µm, cut with a Leitz 1512 microtome) were de-waxed with three, 5-min incubations in xylene (Fisher Lot # 083423), then washed twice in 100% EtOH (5 min each), and once in 95% EtOH (3 min). Slides were dried and examined under a Olympus BH2 light microscope.

**Cell Volume Estimates**

The extent of maximal expansion was estimated for ballooning epidermal cells of the csld1 mutant by comparing their volume to standard epidermal pavement cells of non-mutant plants. Cells were considered to be roughly cylindrical ($V=\pi r^2 \cdot m$), with non-mutant pavement cell dimensions approximately 40 µm (diameter) x 200 µm (length) (Figs. 4, 5). Ballooned epidermal cells of blades from csld1 mutant plants were often as large as 200 µm (diameter) x 600 µm (length) (Figs. 4, 5).

**Epidermal Impressions and Non-warty Cell Size Determination**

Fresh samples from mature leaves of greenhouse-grown plants were cut into 1-cm² pieces and firmly pressed into Superglue on glass slides. Glue was allowed to dry completely before leaf tissue was removed, leaving detailed epidermal impressions. These were imaged under a light microscope (Olympus BH2) with an RT SPOT camera (Diagnostic Instruments). Average cell length and width were determined by quantifying the total number of cells in a given area (1.88 x 1.40 mm). Longitudinal and lateral transects were used that did not include warty clusters.

**Real Time Quantitative RT-PCR**

For each sample, RNA was extracted from approximately 200 mg of tissue, initially frozen in liquid nitrogen, then homogenized in 1.0 mL Trizol (Invitrogen Cat # 15596-018) using a Q-BIOgene FastPrep 120 with Lysing Matrix D (MP Biomedicals Cat # 116913). Samples were incubated 5 min at 25°C, with frequent vortexing. Chloroform (200 µL) was added and samples were vortexed 15 sec before, and after, a 1-min incubation at 25°C. Phases were separated by centrifuging 10 min at 15,000 x g, and 200 µL of the aqueous layers were transferred to 700 µL of Qiagen RLT buffer (from RNase Plant Mini kit, Qiagen Cat # 74904). Ethanol was added (500 µL, 100% EtOH) and samples were vortexed. Half of this volume was
used to clean and elute total RNA as per the RNeasy Plant Mini kit (Qiagen Cat # 74904). Resulting RNA was treated with DNase-1 (Ambion Cat # AM1906), and quantified using a BioRad SmartSpec 3000. The cDNA was synthesized using SuperScript One-Step kit and protocol (Invitrogen Cat # 10928-042).

Levels of *CslD1* mRNA were quantified in diverse maize tissues and in leaf blades at a range of developmental stages via Real-time RT-PCR using a Step One Plus Real-Time PCR System (ABI). At least three biological replicates were analyzed for each tissue or time point, and for each of these replicates, reactions were performed in duplicate. A given reaction included 10 µL Fast SYBR Green Master Mix (ABI Lot # 1003024), 5.0 µL of cDNA sample (diluted 10x from cDNA reaction), and 100 nM of each gene-specific primer (Fwd: GCCGCTCACGTCAATGG, Rev: CGCCATCTCAGGGTGT) in a final volume of 20 µL. The relative abundance of transcripts was normalized with 18S rRNA controls (Taqman Ribosomal RNA Control Reagents, ABI Lot # 0804133) as in Eveland et al., 2008. Primer pairs for *CslD1* were designed using Primer Express 3.0 (ABI).

**High-resolution X-ray Computerized Tomography Analysis**

Field-grown plants were sampled three days after harvesting ears and dried at 38°C for three weeks. Sections (~0.5 cm) from the middle of the second internode of the conditioned stems (~9% moisture content) were cut using a small band saw and scanned using a Scanco Medical Ag uCT35 instrument (Brüttisellen, Switzerland). Initial measurements were taken on whole-stem sections at 10-micron resolution. Regions including pith and rind (3 x 4 mm) were hand-cut from the edge of these sections and scanned at 3.5-µM resolution over a 0.88 mm high region to quantify cell wall and air space sizes. The 232 slices from each scan were reconstructed into three dimensional images and contoured over whole stems for volumetric analyses. All scans were conducted with integration times of 600 microseconds and averaging two times. Both a fixed, common threshold and an adaptive threshold were used to segment cell wall from airspace and volumetric analyses were calculated with an algorithm developed for trabecular bone (Hildebrand and Ruegsegger, 1997). For rind-only analyses, hand-drawn contours were used to isolate the vascular bundle-rich region along the edge of the stem prior to 3D reconstruction.

**Cell Wall Composition Analysis**
Samples from leaves and epidermal peels were ground in liquid nitrogen along with 200 µL of extraction buffer (50mM Tris-Cl with 1% SDS at pH 7.2). Homogenate was transferred to 14-mL polypropylene, round-bottom tubes (Falcon product # 352059) along with 9 mL extraction buffer, incubated for 15 minutes at 80°C, and centrifuged at 3500 rpm for 5 min (~2,000 x g) in a swinging-bucket rotor centrifuge (ThermoForma 1LGP). Supernatant was removed with an aspirator, and pellets (water-insoluble cell-wall fraction) were washed, re-suspended, and re-pelleted three times in about 10 mL 80°C water. The same process was repeated three times with 50% EtOH at 80°C, followed by three washes with 80°C water. Samples were transferred to 1.5-mL Eppendorf tubes, alcohol-insoluble cell wall fractions were pelleted and dried, and non-cellulosic sugar composition was analyzed by the Complex Carbohydrate Research Center (University of Georgia; Athens, GA).

For cellulose content, the alcohol-insoluble cell wall fractions from whole-leaf samples were isolated in the same way, dried for 30 h at 60°C, transferred to 14-mL polypropylene, round-bottom tubes, and weighed. For each sample, approximately 50 mg of cell wall isolate was used, to which 3 mL 80% aqueous acetic acid and 300 µL 70% nitric acid were added. Tubes were incubated in an oil bath at 110°C and 120°C for 20 min each, to hydrolyze hemicellulose and lignin (from Sun et al., 2004). Samples were cooled, 1.8 mL distilled water was added, tubes were centrifuged for 5 min (~2,000 x g), and supernatant was removed with an aspirator. Celluose was rinsed thoroughly with water (3 times) and 95% EtOH (3 times), and dried for 30 h at 60°C. Samples were weighed and compared for cellulose content as a fraction of alcohol-insoluble cell wall isolate.

Propidium Iodide Staining

Immature leaves (10 to 15 cm) were dissected from whorls of csld1 mutant and non-mutant plants. The basal portions (2 cm) of these leaves were immediately submerged in a solution of 0.1 mg/mL propidium iodide, and allowed to absorb the dye for 5 min at 25°C. Samples were then rinsed thoroughly in water to remove excess stain and flattened on a glass slide. The abaxial epidermis was imaged using a Zeiss confocal microscope. For visualization of nuclei, the same process was followed, but leaf samples were first fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH), before staining with propidium iodide.

Flow Cytometry
The basal 1 cm of immature leaves (2-3 cm) was dissected and finely sliced (~0.5 mm) with a razor blade in ice-cold chopping buffer (4% MOPS [0.5 M, pH 7.2], 9% MgCl₂ [0.5 M], 6% Na₃Citrate [0.5 M], 0.1 % Triton X-100 [Sigma, Lot # MKBD6639V], 1 mg RNase [Thermo Scientific, Cat # AB-0549], in water). Homogenate was filtered through 50 micron nylon mesh followed by 20 micron nylon mesh, then transferred to a 1.5 mL microcentrifuge tube. Nuclei were pelleted at 1,000 rpm for 3 min and supernatant was discarded. Pellets were resuspended in staining buffer (chopping buffer plus 1% propidium iodide [5 mg/mL]), and incubated at room temperature for 5 min. Nuclei were re-pelleted at 1,000 rpm for 3 min and supernatant was discarded. Pellets were resuspended in 300 µl of staining buffer and analyzed on a LSR-II cytometer (BD Biosciences, San Jose, CA). Nuclei were excited using a solid state laser emitting 100 milliwatts at 488 nm. Forward light scatter and orange fluorescence (575 +/- 13 nm) were collected on up to 5000 particles per sample. Small particles of debris were gated out using a fluorescence vs forward light scatter dot plot. Peaks were identified on a fluorescence histogram plotted on logarithmic scale and the geometric and median fluorescence values for each peak were calculated. Software used was Diva 6.1.2 (BD Biosciences).

**Accession Numbers**

Accession numbers for each of the gene sequences referred to in this work are: At-CslD1:AT2G33100.1, At-CslD2:AT5G16910.1, At-CslD3:AT3G03050.1, At-CslD4:AT4G38190.1, At-CslD5:AT1G02730, At-CslD6:AT1G32180.1, Os-CslD1:AC027037.6, Os-CslD2:Os06g0111800, Os-CslD3:AC091687.1, Os-CslD4:AK242601.1, Os-CslD5:Os06g0336500, Zm-CslD1:GRMZM2G015886, Zm-CslD2:GRMZM2G052149, Zm-CslD3:GRMZM2G061764, Zm-CslD4:GRMZM2G044269, Zm-CslD5:GRMZM2G436299.

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FIGURE LEGENDS

Figure 1. Comparison of mutant phenotypes to CSLD protein phylogeny and diagram of the CSLD1 protein and mutant genes in maize. A, Neighbor-joining tree of predicted protein sequences encoded by *Csld* genes in maize, rice, and *Arabidopsis*. Reported phenotypes for null alleles are shown in red (Zm-CSLD5 [Penning et al., 2009], Os-CSLD1 [Kim et al., 2007], At-CSLD2 [Bernal et al., 2008], At-CSLD3 [Favery et al., 2001; Wang et al., 2001], At-CSLD5 [Bernal et al., 2007], Os-CSLD4 [Li et al., 2009], At-CSLD1 and At-CSLD4 [Bernal et al., 2008]). At- *Csld6* and Zm- *csld3* are predicted to be pseudogenes. The Zm-CSLD1 protein is starred. Members of distinct clades are grouped by shapes, with dashed lines including proteins for which no genetic evidence exists. The tree was created using Mega 4.0 (megasoftware.net/mega.html; Tamura et al., 2007), with 2,000 bootstrap repetitions and pairwise deletion option. Units are in amino acid substitutions per site. Supplemental Figure 1 online shows the ClustalW alignments used in this phylogenetic analysis. B, Diagram of the maize *Csld1* gene and protein. Sites of Mu transposon insertion (triangles) are shown on the *Csld1* gene. Lengths of exons and introns are indicated below each region (in bp). Both the *csld1-1* and *csld1-2* mutants were obtained from the UniformMu maize population at the University of Florida (uniformmu.uf-genome.org [McCarty et al., 2005; Settles et al., 2007]). Smaller triangles represent Pioneer-TUSC alleles (McCarty and Meeley, 2009). The predicted protein includes 1218-amino acids, an N-terminal RING-type Zn-finger-like domain, eight transmembrane domains, and the conserved residues of an inverting, processive glycosyltransferase. The protein diagram is modeled after Richmond, (2000), and protein domains were identified using SMART (smart.embl-heidelberg.de) and TMHMM (cbs.dtu.dk/services/TMHMM).

Figure 2. Epidermal lesions on the leaf blades of *csld1* mutants. A-C, Fresh, intact leaves. Epidermal warts were distributed across the entire blade and midrib of mature-fully expanded leaves. D-E, Fresh-sectioned leaf blades. Swollen lesions were most abundant on the abaxial leaf blade surface. F – G, Cross-sections of fixed, imbedded leaves, with F a composite of overlapping images. Epidermal cells were more visibly affected than cells of the leaf-blade interior. Some epidermal cells had two-dimensional, cross-sectional areas up to 25-fold greater (*r* = 100 µm for swollen cell in G).
Figure 3. The narrow-leaf phenotype of csld1 mutant plants. A, Plant height and dry weight (above- and below-ground) were quantified for field-grown csld1 mutant and non-mutant plants. Plant height was 90.9 cm (SE, 2.8; N = 20) for mutants and 102.2 cm (SE, 1.7; N = 35) for non-mutants. For dry weight measurements, whole plants were sampled three days after ear maturity (40 days post pollination), and did not include mature ears or fine roots. Total plant dry weight was 41.9 g (SE, 4.0; N = 4) for mutants and 76.5 g (SE, 3.2; N = 4) for non-mutants. Below-ground dry weight was 8.7 g (SE, 0.2; N = 4) for mutants and 17.1 g (SE, 1.1; N = 4) for non-mutants. Above- and below-ground dry weights were reduced by similar amounts (av. 44% and 49%, respectively). Standard errors are indicated with vertical bars, and are marked by * wherever values for mutant plants differed significantly from those of non-mutants (p < 0.05). B, Leaf blade length and width (at widest point) were quantified for leaf positions 3 through 5 (as diagramed) for field-grown mutant and non-mutant plants. Non-mutant plants included both wildtype and heterozygous individuals from segregating progeny after 3 back-crosses into the W22 inbred. Imaged blades were from leaves in position #3 from the apex. The left-most portion of each graph shows combined data from all leaf positions measured. The leaf blade width-to-length ratio was 27% less for mutant plants. Blade width and length were reduced 35% and 10% respectively, relative to those of non-mutant plants. Data were similar for csld1-2 (not shown), and visual appraisals of the five other csld1 mutants. Note: Leaf photos were from greenhouse-grown plants, whereas quantifications were from field-grown plants. Significantly different from non-mutant (p < 0.05) indicated by *.

Figure 4. Estimated dimensions of non-lesion epidermal cells, and cell number along lateral and longitudinal axes of leaf blades from csld1 mutant and non-mutant plants. Epidermal impressions from abaxial surfaces of fully-expanded leaf blades from mature, greenhouse-grown plants were used to estimate cell dimensions and cell numbers along leaf axes. Non-mutant plants included both wildtype and heterozygous individuals from segregating progeny after 3 back-crosses into the W22 inbred. Cell numbers were quantified (each designated by a tic mark on impressions such as those shown to the right) along longitudinal and lateral axes of defined length. Mean cellular dimensions were determined by dividing number of cells along a given axis by the length of that axis. (mutant N=14; non-mutant N=10; longitudinal axis 1.88mm; lateral axis...
Axes used for analyses of epidermal cell size from mutant leaves were done with transects that did not cross cells in the ballooning protrusions. Approximate cell numbers across and along leaf blades were estimated by comparing cell size estimates with average leaf length and width measurements (see Figure 3). These analyses did not include the midrib (0.5 cm was subtracted from total leaf width). Mean width of epidermal cells was 17% greater in csld1 mutants, whereas mean cell numbers were 48% less across the lateral axis and 16% less along the longitudinal axis. Values are marked with * where they differ significantly from those of non-mutant plants (p < 0.05).

**Figure 5.** Levels of Zm-CslD1 mRNA in diverse tissues from wildtype plants of the W22 inbred. A, Expression of Zm-CslD1 in diverse organs. B, Zm-CslD1 mRNA levels in leaf blades and blade-regions at different stages of development. Levels of mRNA were quantified by CyberGreen quantitative Real Time RT-PCR. Three biological replications were analyzed for each tissue. Yellow areas of leaf drawing represent immature, non-green tissue. Standard errors are indicated by vertical bars.

**Figure 6.** High-resolution X-ray micro CT analysis of csld1 mutant and non-mutant stalks. Stem sections from the third internode of greenhouse-grown csld1 mutant and non-mutant plants were scanned at 3.5µm resolution using high-resolution X-ray micro-CT. Sections for scanning (approximately 3 x 2 x 10 mm) were hand-cut from the edges of stems from mutant and non-mutant plants (four each). Non-mutant plants included both wildtype and heterozygous individuals from segregating progeny after 3 back-crosses into the W22 inbred. Three-dimensional analyses revealed significant differences in density of wall material (lower left) and distribution of wall thickness (right). Mutant stems had more dense, but generally thinner walls, even when analyses were limited to the rind only. Volumetric analyses were calculated with an algorithm developed for trabeacular bone (Hildebrand and Regsegger, 1997). Standard errors are indicated with vertical bars. Values from csld1 mutant plants that are significantly different from non-mutants are indicated as, (p < 0.05)** and (p<0.1)*.

**Figure 7.** Confocal images showing defects early in development of csld1 mutant leaf epidermis. Propidium iodide-stained cell walls from fresh, immature csld1 mutant and non-
mutant leaves. A, Pre-differentiation zones from the basal 3 mm of non-mutant leaf blades contrasting with the abnormal clsd1 cell size, shape, and organization. Leaf blades were approximately 10-cm long. B, Post-differentiation zones (from 3 to 10 mm above the base of the blade) showing persistent effects of altered cell division, with presence of large, misshapen, less ordered cells of the clsl1-1 leaf epidermis. Leaf blades were approximately 10-cm long. C, A typical file of large, irregular cells bounded by individuals with incomplete cell walls protruding into them from their outer edges, consistent with clonal inheritance of large cells size in the clsl1 mutants. D, Serial optical sections revealing irregular gaps in cell walls (arrow) that occur frequently in epidermal cells of the clsl1 mutant.

**Figure 8.** Nuclei of immature clsl1 mutant and non-mutant epidermis. A, Confocal imaging of fixed cells stained with propidium iodide showing the abundance of large nuclei in clsl1 mutant epidermis, and the correspondence between these and larger cell size. B, Percent of total nuclei with 4N, 8N, and 16N as determined by flow cytometry of isolated nuclei stained with propidium iodide. Nuclei were from immature tissue of basal portions of clsl1 mutant and non-mutant leaves. The majority of nuclei were 2N (not shown). Standard errors are indicated by vertical bars. Significantly different from non-mutant (p < 0.05) indicated by *.
### TABLES

**Table 1.** Cell Wall Sugar Composition of Leaf Blade and Epidermal Peels from *csld1* Mutant and Non-mutant Plants.

| Sample     | Ara   | Rha   | Fuc   | Xyl   | Man   | GalA  | Gal   | Glc   | GlcA  | Cel.   |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| Whole leaf |       |       |       |       |       |       |       |       |       |        |
| WT         | 11.43 | 0.53  | nd    | 81.40 | nd    | 1.36  | 0.85  | 4.43  | nd    | 26.6   |
|            | (0.33) | (0.02) | (0.01) | (0.07) | (0.69) | (0.41) | (1.8) |
| *csld1*    | 11.84 | 0.57  | nd    | 81.18 | nd    | 1.59  | 0.80  | 4.03  | nd    | 22.9   |
|            | (0.18) | (0.05) | (1.31) | (.016) | (0.04) | (1.41) | (3.1) |
| Epidermal  |       |       |       |       |       |       |       |       |       |        |
| WT         | 10.66 | 0.31  | nd    | 85.04 | nd    | 0.95  | 0.65  | 2.39  | nd    | nt     |
|            | (0.54) | (0.02) | (0.69) | (0.29) | (0.08) | (0.34) |       |
| *csld1*    | 11.93 | 0.34  | nd    | 84.37 | nd    | 0.71  | 0.69  | 1.97  | nd    | nt     |
|            | (0.63) | (0.03) | (0.96) | (0.05) | (0.08) | (0.22) |       |

Legend: Alcohol-insoluble residues were prepared from both whole-leaf-blade sections and isolated epidermal strips of greenhouse-grown plants identified as mutant or wildtype by PCR of a segregating family. Analysis of non-cellulosic cell wall sugars was done at the CCRC at the University of Georgia. Values for non-cellulosic sugars are given as mole percent, with standard error in parentheses. Cellulose content was estimated based on remaining weight after hydrolysis of hemicelluloses and lignin. Cellulose content is given as weight percentage of alcohol-insoluble cell wall fraction, with standard error in parentheses. None of the differences between *csld1* mutant and wildtype samples were statistically significant at p < 0.05 (N = 4, for each sample). Key: Ara = arabinose, Rha = rhamnose, Fuc = fucose, Xyl = xylose, Man = mannose, GalA = galacturonic acid, Gal = galactose, Glc = glucose, GlcA = glucuronic acid, Cel = cellulose, nd = not detected, nt = not tested.
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