Cellulose synthase-like D1 controls organ size in maize

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Abstract

Background: Plant architecture is a critical factor that affects planting density and, consequently, grain yield in maize. The genes or loci that determine organ size are the key regulators of plant architecture. Thus, understanding the genetic and molecular mechanisms of organ size will inform the use of a molecular manipulation approach to improve maize plant architecture and grain yield.

Results: A total of 18 unique quantitative trait loci (QTLs) were identified for 11 agronomic traits in the F2 and F2:3 segregating populations derived from a cross between a double haploid line with a small plant architecture (MT03-1) and an inbred line with a large plant architecture (LEE-1). Subsequently, we showed that one QTL, qLW10, for multiple agronomic traits that relate to plant organ size reflects allelic variation in ZmCSLD1, which encodes a cellulose synthase-like D protein. ZmCSLD1 was localized to the trans-Golgi and was highly expressed in the rapidly growing regions. The loss of ZmCSLD1 function decreased cell division, which resulted in smaller organs with fewer cell numbers and, in turn, pleiotropic effects on multiple agronomic traits. In addition, intragenic complementation was investigated for two Zmcsld1 alleles with nonsynonymous SNPs in different functional domains, and the mechanism of this complementation was determined to be through homodimeric interactions.

Conclusions: Through positional cloning by using two populations and allelism tests, qLW10 for organ size was resolved to be a cellulose synthase-like D family gene, ZmCSLD1. ZmCSLD1 has pleiotropic effects on multiple agronomic traits that alter plant organ size by changing the process of cell division. These findings provide new insight into the regulatory mechanism that underlies plant organ development.

Keywords: QTL, ZmCSLD1, Organ size, Pleiotropic effects and intragenic complementation

Background

Maize (Zea mays ssp. mays) is the highest yielding and most widely grown crop in the world. Increasing grain yield has always been a primary goal of maize breeding. Plant architecture is a result of many trait interactions during plant development and growth, and is a critical factor that affects plant density and, consequently, grain yield. In general, maize plant architecture includes plant height (PH), leaf number (LN), leaf angle (LA), leaf area, and tassel traits. An ideal plant architecture includes a short stature to prevent lodging, an erect LA and a moderate leaf area to maximize light interception, and small tassels to partition more energy and nutrients for ear and seed development [1–4]. Most of the traits that contribute to plant architecture, such as PH, leaf length (LL), leaf width (LW), and tassel length (TL) can collectively be referred to as organ size, which is determined by cell number and cell size and results from the two successive processes of cell proliferation and cell expansion. Thus, understanding the genetic and molecular mechanism of organ size will inform the development of a molecular manipulation approach to improve maize plant architecture and grain yield.

Plant organ size is a product of the interaction of genotype and environmental influences [5]. The genetic architecture of organ size has been well studied in maize by using different genetic populations [6–11]. For example, a nested association mapping (NAM) population, which contained ~5,000 recombinant inbred lines and was developed by crossing 25 diverse lines to B73, was...
used to identify the quantitative trait loci (QTLs) of multiple traits that relate to organ size [7, 9, 10]. By using joint linkage analysis, a total of 89, 92, 37, 36, and 34 QTLs were detected for PH, ear height (EH), TL, LL and LW, respectively. Plant architecture traits were also studied in a natural population that contained 513 diverse maize lines by a genome-wide association study, and 185 SNPs were significantly associated with PH, EH, LL, LW and TL [11]. Recently, approximately 314 QTLs were identified to control the phenotypic variation in organ size-related traits (PH, EH, LL, LW) in 10 maize populations [8]. These QTL studies indicate that the formation of organ size is complex and characterized by small effects for most QTLs, large effects for several QTLs, and a certain number of pleiotropic QTLs.

Studies of the model plant Arabidopsis have revealed that the formation of plant organ size are modulated by several molecular mechanisms, including transcriptional regulation [12−15], hormone signalling [15−18], ubiquitin-mediated proteolysis [19, 20], and cell wall biosynthesis [21]. However, only several genes for organ size have been isolated and characterized in maize, such as BR2 [22, 23] and ZmGA3ox2 or D1 [24, 25] for PH; DIL1, GA20-OXIDASE1, GRF1 and GRF10 for LL and PH [26−29]; ZmGE2 [30] and BIGE1 [31] for embryo size; and ZmBRI1, CNR1, CNR2 and ZmPLA1 for overall organ size [32−34]. Similar to Arabidopsis, organ size in maize is controlled by multiple genes in various regulatory pathways. For the genes known to regulate organ size, the molecular mechanisms of most genes regulating organ size relate to hormone signalling [23, 25, 34]. As expected, all the genes control organ size by changing cell division and cell expansion to alter cell number or cell size, respectively. For example, overexpressing ZmPLA1 increased organ size by prolonging cell division, and knocking down ZmBRI1 reduced organ size by decreasing cell division and cell elongation [32, 34]. Besides, the regulation ways to organ growth were also different in the same tissue. Both BR2 and ZmGA3ox2 control PH, although, BR2 plays a role in cell division, while ZmGA3ox2 plays a role in cell expansion [23, 25]. Similarly, LL was regulated by GRFs, with overexpression of GRF10 and GRF10 increasing LL by cell division and expansion, respectively [28, 29]. In addition, not like the role of ZmGA3ox2 in stem, gibberellin greatly enlarged the cell division zone, and increased LL in GA20-OXIDASE1 overexpression plants [27, 35]. These results had provided summary knowledge about the genes that control organ size, although the regulatory pathways in which these genes operate are still poorly understood in maize.

In this study, we developed a double haploid line with a small plant architecture, MT03-1, which was crossed with an inbred line with a large plant architecture, LEE-12, to determine the genetic architecture of the maize agronomic traits that relate to organ size. A major QTL for organ size, qLW10, was identified in the F2 and F2:3 segregating populations derived from a cross between MT03-1 and LEE-12. This locus was further cloned to be ZmCSLD1, which encodes a cellulose synthase-like D1 protein that affects plant growth through cell division or expansion. The molecular mechanism of ZmCSLD1 that affects plant organ size was extensively addressed through genetic analysis, subcellular localization, an expression profile and the analysis of cell number and cell size.

Results
A large-effect QTL cluster controls multiple agronomic traits
MT03-1 is a double haploid line with a relatively small plant architecture (Additional file 1: Table S1). To investigate the genetic basis of these traits, QTL mapping was performed for the 11 measured traits by using a genetic map of 1833.66 cM (Additional file 1: Tables S1; Additional file 2: Tables S2), and a total of 19 and 25 QTLs were detected in the F2 and F2:3 populations, respectively (Fig. 1a; Additional file 3: Table S3). These QTLs were distributed in 18 genomic regions across 8 of the 10 chromosomes, except for chromosomes 6 and 9, and each QTL explained 3.27 to 75.05% of the phenotypic variation. Out of 18 unique QTLs, 6 and 2 were associated with at least two traits in the F2 and F2:3 populations, respectively, which indicates that they had pleiotropic effects or were closely linked loci.

Among these QTLs, one QTL cluster with a large effect on chromosome 10, which was flanked by SYN17100 and PZEI10041758, was identified for nine traits in both the F2 and F2:3 populations. On average, this locus explained 27.35% of the phenotypic variation in all nine traits and differed from 75.05% for LW to 9.18% for EH. In addition, all the alleles at this locus associated with decreased size came from MT03-1. This result was highly consistent with the phenotypic correlation among traits (Fig. 1b). All nine traits with the QTL identified at this locus had a moderate to high correlation with one another ($r = 0.44−0.94$, F2:3 population), whereas the two remaining traits without the identified QTL at this locus, namely, the LA and 100-kernel weight (KW100), were weakly correlated with the nine traits ($r = 0.2−0.34$, F2:3 population). This result suggests that the nine traits may share a common pleiotropic genetic variant at this locus.

ZmCSLD1 is the causal gene for qLW10
To clone the causal gene for the QTL cluster, we selected LW as the trait for positional cloning because the
greatest difference was observed for LW between the parents, LEE-12 and MT03-1 (Fig. 2a and b; Additional file 1: Table S1), and this locus had the largest effect on LW (Fig. 2c; Additional file 3: Table S3). This locus was then designated qLW10. The position of qLW10 was refined to a 3.3-Mb segment flanked by markers IDP9050 and MTL02 by using 3,292 BC2F1 and 669 BC3F1 plants (Fig. 2d). To confirm the narrowed interval, a heterozygous inbred family BYK-HIF, which segregated in LW (Additional file 4: Figure S1A-C) was also used to fine map qLW10. Nine recombinant types were identified in 720 F2 plants selfed from BYK-HIF, and the progeny test of homozygous segregants narrowed the interval to a 3.8-Mb region flanked by the markers IDP9050 and IDP7754 (Additional file 4: Figure S1D).

The nucleotide sequence analysis based on B73 reference sequences predicted 33 and 36 genes in the 3.3- and 3.8-Mb intervals, respectively. Simultaneously, two pairs of near-isogenic lines (NILs) were developed. qLW10MTL and qlw10MTL carried 3.3-Mb fragments from LEE-12 and MT03-1, respectively. qLW10BYK and qlw10BYK harboured different alleles at the target locus, with only a 0.4% difference in the genome background. Compared with qLW10MTL and qLW10BYK, qlw10MTL and qlw10BYK had smaller plant architectures (Additional file 5: Figure S2; Additional file 6: Figure S3).

Among these genes in the qLW10 interval, GRMZM2G015886 (ZmCSLD1) has been previously reported to narrow LW and reduce growth after a loss of function [36], which is very similar to the effect of qLW10. Therefore, ZmCSLD1 was proposed as the best candidate gene for qLW10. To confirm this hypothesis, we first sequenced the full-length ZmCSLD1 in two pairs of NILs. Compared with the qLW10MTL allele, the qlw10MTL allele contained a 467-bp insertion and a 63-bp deletion in the third exon, resulting in a frameshift that prevented the production of the mature protein because of the introduction of a premature stop codon (Fig. 2e). For the qLW10BYK and qlw10BYK lines, there was a G to A transition in the third exon, causing a conserved amino acid with a major change from glycine to aspartic acid (Fig. 2e; Additional file 4: Figure S1E; Additional file 7: Figure S4).

To confirm that ZmCSLD1 is the causal gene for qLW10, two additional Zmcsld1 mutants, Zmcsld1MO17 and Zmcsld1W22, were developed (Fig. 2e). A genomic sequence analysis revealed that Zmcsld1MO17 had a T-to-C substitution (tryptophan-to-arginine) in the last transmembrane domain of ZmCSLD1 (Additional file 7: Figure S4), and Zmcsld1W22 had a Mu-transposon insertion in the second exon. As expected, the LW in each mutant was significantly decreased compared with their corresponding wild-type lines, similar to qlw10MTL and qlw10BYK (Fig. 2f). The allelic effect was the largest for Zmcsld1W22, followed by qlw10MTL, qlw10BYK and Zmcsld1MO17. To further evaluate the allelic effect, Zmcsld1W22 was crossed with qlw10MTL, qlw10BYK, Zmcsld1MO17 and their wild types, as designated in Additional file 8: Table S4. Compared with the F1 hybrids with a normal allele, all three F1 hybrids with both mutant alleles had
narrower leaves (Fig. 2g), which was further validated in the six F₂ populations produced by the four mutant combinations (Additional file 9: Figure S5A and C). These results indicate that ZmCSLD1 is the causal gene for qLW10.

Characterization of ZmCSLD1
ZmCSLD1 is a member of the conserved family of CSLD proteins in maize that are important for cell growth and development [36]. ZmCSLD1 contains a “D,D,D,QXXRW” motif with a RING-type zinc finger-like domain and two transmembrane domains at the N-terminus and six transmembrane domains in the C-terminal region (Additional file 10: Figure S6). The phylogenetic tree of ZmCSLD1 orthologues across 32 angiosperms indicate that ZmCSLD1 is highly conserved in different classes such as asterids, core eudicots, rosids and poales (Additional file 10: Figure S6).

To examine the subcellular localization of ZmCSLD1, we transiently expressed pSuper:ZmCSLD1-GFP in maize...
protoplast cells under the blank control of pSuper:GFP and co-transformed it with mCherry-HDEL-RFP (endo-plasmic reticulum (ER) marker), Man1-mCherry-RFP (cis-Golgi marker) and RFP-SYP61 (trans-Golgi marker) as the controls for subcellular localization (Fig. 3a–p). A punctate pattern of GFP signals was observed in the protoplasts when ZmCSLD1-GFP was expressed (Fig. 3c, f and n), and this pattern was different from the ER marker location pattern (Fig. 3e). The partial overlap of ZmCSLD1-GFP with the cis-Golgi marker and complete merging with the trans-Golgi marker indicate that ZmCSLD1 localizes to the trans-Golgi (Fig. 3k and e); this finding is consistent with the well-known subcellular localization of its orthologues in rice (OsCSLD4) and Arabidopsis (AtCSLD5) [21, 37].

In previous studies, xylan and homogalacturonan synthase activity were reduced in Atcsld5 [21], and the content of xylan, cellulose and homogalacturonan were

![Image of subcellular localization](image_url)

**Fig. 3** Subcellular localization of ZmCSLD1. a–b Empty Super:GFP vector expression observed by fluorescence (a) and bright-field (b; BF) microscopy. c–d CSLD1-GFP fusion protein expression. e–f The co-expression of the ER marker mCherry-HDEL and the CSLD1-GFP fusion protein (f), Merge (g) and BF (h) are shown. i–l The co-expression of the cis-Golgi marker Man1-mCherry (i) and the CSLD1-GFP fusion protein (j), Merge (k) and BF (l) are shown. m–p The co-expression of the trans-Golgi marker RFP-SYP61 (m) and the CSLD1-GFP fusion protein (n), Merge (q) and BF (p) are shown. Scale bar = 5 μm.
confirmed that ZmCSLD1 might participate in the cell wall polysaccharides biosynthesis due to their protein identity. To test the association between ZmCSLD1 and cell wall compositions, the cell wall monosaccharide composition and cellulose content of the ear leaves in ZmCSLD1<sup>W22</sup> and Zmcsld1<sup>W22</sup> were measured at 7 days after flowering. Compared with ZmCSLD1<sup>W22</sup>, Zmcsld1<sup>W22</sup> showed a significant increase of mannose and glucose content, but a decrease tendency in xylose, galactose and cellulose content (Additional file 11: Table S5). These results confirmed that ZmCSLD1 played an important role in the cell wall polysaccharide biosynthesis.

**ZmCSLD1 has pleiotropic and heterogeneous effects**

To validate that ZmCSLD1 is also the causal gene that affects the other eight traits, 16 traits were examined in qLW10<sup>MTL</sup> and qlw10<sup>YTK</sup>, qLW10<sup>BYK</sup> and qlw10<sup>YTK</sup>, ZmCSLD1<sup>W22</sup> and Zmcsld1<sup>W22</sup> and ZmCSLD1<sup>Mo17</sup> and Zmcsld1<sup>Mo17</sup> (Additional file 12: Figure S7). Twelve traits showed large differences between qLW10<sup>MTL</sup> and qlw10<sup>YTK</sup>, and the qlw10<sup>YTK</sup> allele conferred overall decreases in these traits of 8.99% (veinlet number, VN) to 33.47% (LW). The same QTL at the ZmCSLD1 locus was identified for each of the nine common traits in the LEE-12 × MT03-1 F2 population, which further indicates the pleiotropic effects of ZmCSLD1. Furthermore, 10 of the traits, including narrow leaves, dwarf plants, and thin stems, showed consistent patterns in all the lines with the loss-of-function alleles, whereas some traits showed significant differences between qLW10<sup>BYK</sup> and qlw10<sup>YTK</sup> and between ZmCSLD1<sup>W22</sup> and Zmcsld1<sup>W22</sup> but not between qLW10<sup>MTL</sup> and qlw10<sup>YTK</sup> and ZmCSLD1<sup>Mo17</sup> and Zmcsld1<sup>Mo17</sup>. The tassel branch number (TBN), for example, which is determined by the branching ability of the shoot apical meristem, varied in the different genomic backgrounds. Even for the traits with striking effects of the loss-of-function alleles, the effects varied among the different alleles, e.g., the effects of LW ranging from 17 to 42% (Additional file 12: Figure S7). This phenomenon may be due to the heterogeneous effects of different alleles because the causal sites occurred in different protein domains (Fig. 4a). Both qlw10<sup>YTK</sup> and Zmcsld1<sup>W22</sup> contained large insertions before the “D,D,D,QXXRW” motif and transmembrane domains that were predicted to lead to a complete loss of the ZmCSLD1 function, whereas the missense mutations in the “D,D,D,QXXRW” motif of qlw10<sup>YTK</sup> (G839D) and in the last transmembrane domain of Zmcsld1<sup>Mo17</sup> (W1184R) had weak effects on the traits.

To further confirm the pleiotropic and heterogeneous effects of ZmCSLD1, the 16 traits were also investigated in four F1 hybrids, including the three pairs described above that were crossed with Zmcsld1<sup>W22</sup> and two other combinations, namely, Zmcsld1<sup>Mo17</sup> × qlw10<sup>YTK</sup> and ZmCSLD1<sup>Mo17</sup> × qLW10<sup>YTK</sup> (Additional file 8: Table S4). As expected, the performance of all the measured traits in all three F1 hybrid crosses between the strongest allele and three other alleles supported the pleiotropic and heterogeneous effects of ZmCSLD1. Unexpectedly, the defective phenotype for most traits in the Zmcsld1<sup>Mo17</sup> × qlw10<sup>YTK</sup> F1 hybrid was completely complemented (Fig. 4b), similar to the phenomenon of intragenic complementation in the cellulose synthase gene in Arabidopsis AtCESA3 [38]. A further comparison of the LW among genotypes in six F2 populations indicated that the effect of the qlw10<sup>YTK</sup> allele was comparable to the effect of the Zmcsld1<sup>W22</sup> allele because both alleles produced an abnormal ZmCSLD1 protein, whereas the qlw10<sup>YTK</sup> and Zmcsld1<sup>Mo17</sup> alleles had weaker effects (Additional file 9: Figure S5). In addition, the leaves of the individuals with heterozygous (C/A) genotypes were significantly wider than the leaves of the individuals with both homozygous genotypes (C/C and A/A) in the ZmCSLD1<sup>Mo17</sup> × qlw10<sup>YTK</sup> F2 population (Additional file 9: Figure S5A); this finding strongly supports the ZmCSLD1<sup>Mo17</sup> × qlw10<sup>YTK</sup> F1 intragenic complementation.

To test whether ZmCSLD1 could form a homodimer at molecular level, we cloned two fragments, S1,<sup>331</sup> and S400–984, from 1 to 331 amino acids and S400–984 from 400 to 984 amino acids of ZmCSLD1, into both pGBK7T7-BD and pGADT7-AD vectors. The yeast two hybrid analysis indicated that segment S1,<sup>331</sup> could interact with itself, but segment S400–984 couldn’t (Fig. 4c). In addition, the mutation of qlw10<sup>YTK</sup> occurs in the cytoplasmic domain of ZmCSLD1, which possesses glycosyltransferase activity, and the Zmcsld1<sup>Mo17</sup> mutation is located in the last transmembrane domain of ZmCSLD1. Taken together, we raised a model to explain the intra-genic complementation between qlw10<sup>YTK</sup> and Zmcsld1<sup>Mo17</sup> (Fig. 4d). Both catalytic and transmembrane domains are necessary to the function of ZmCSLD1. Either mutation in these two domains in homologous lines will weaken the function of ZmCSLD1, while the function of ZmCSLD1 will be complemented in F1 hybrids of two mutation alleles in different domains.

**Decreased cell division rate contributes to a reduced organ size**

Cell division and expansion are the primary ways of plant growth. The relationship between ZmCSLD1 and cell division or expansion raises the question of whether ZmCSLD1 has pleiotropic effects on the size of multiple organs in maize. To investigate the relationship between organ size and ZmCSLD1, the LL and LW of all unfold leaf blades were measured to assess the leaf morphology of 40-day-old seedlings (Fig. 5a). An increasing difference between qLW10<sup>MTL</sup> and qlw10<sup>YTK</sup> was observed
for LW after the second leaf stage and for LL after the third leaf stage, and the fourth LW and length of qLW10MTL reached 1.36 and 1.21 times the LW and LL of qLw10MTL, respectively. This result was highly consistent with the morphology of mature leaves (Fig. 2a and b). To address the relationship between LW and cell division or cell expansion, the cell size was quantified and the cell number was calculated for the fourth leaf, which had the greatest variation in the leaf stages (Additional file 13: Figure S8). Compared with qLW10MTL, cell width increased by 19.52% in qLw10MTL, whereas cell length decreased by 4.44% (Fig. 5b); the cell number in both the lateral and longitudinal axes was notably reduced by 40.55% and 13.79%, respectively (Fig. 5c). Similarly,
all of three other alleles, qlw10BYK, Zmcsld1W22, and Zmesd1Mo17, showed significant decrease in LW and cell number in the lateral axes of the third leaf from 20-day-old seedlings (Additional file 14: Figure S9). These results imply that the narrowing of the qlw10MTL leaf was mainly caused by a decrease in cell number due to a reduction in cell division.

In addition, based on the expression levels of ZmCSLD1 in 13 tissues at various developmental stages [39–43], ZmCSLD1 has been determined to be expressed in most maize tissues (Additional file 15: Figure S10). ZmCSLD1 expression was high in the ear primordia, tassel primordia, shoot apical meristem, third leaf base and ovule but was low in the root and embryo at 10 days after pollination (DAP), in the endosperm at 6 DAP, and in the seeds at 3 DAP, and no expression was detected in the third leaf tip, pre-emergence tassel, silk or anthers (Additional file 15: Figure S10A). In addition, ZmCSLD1 was highly expressed in the early stage of seed development, and dropped successively during the seed maturation process (Additional file 15: Figure S10B), which corresponds to a phase of mitotic cell proliferation during maize embryogenesis and endosperm development [40, 44, 45]. To further validate the association of ZmCSLD1 with cell proliferation, we sampled three root tissues and shoot tissue from 5-day-old seedlings, and four leaf tissues of the third leaf tissues from 10-day-old seedling in qlW10MTL and qlw10MTL (Fig. 6a and b). As expected, ZmCSLD1 were highly expressed in primary root tip, shoot base and the third leaf base tissues, and expressed at low levels in primary root middle, third leaf middle (proximal and distal) and tip (Fig. 6c). The expression pattern of ZmCSLD1 was highly consistent with that of three cell circle related genes CycB1;4, CycD3;1b and histone H2B which expressed during G2/M transition, G1/S transition and S phase in cell cycle, respectively [46, 47] (Fig. 6d; Additional file 16: Figure S11A and B). Specially, the expression level and pattern of ZmCSLD1 was most similar to CycB1;4 (r = 0.91), suggesting that ZmCSLD1 may also be expressed in the G2/M transition-phase during cell division. Additionally, ZmCSLD1 was also highly expressed in primary root base tissues which might be caused by the development of lateral roots. Collectively, ZmCSLD1 was highly expressed...
in the early stages during rapid growth in all tissues, whereas this expression was low in the mature stages (Fig. 6c; Additional file 15: Figure S10B), which is consistent with the function of ZmCSLD1 that relates to cell division and development.

**Discussion**

**ZmCSLD1 participates in the cell wall formation during cell division**

Organ size growth is a complex process that consists of cell division and expansion. The cell wall is an integral structure in plant cells and mainly comprises cellulose, hemicellulose and pectin. These components play essential roles in plant cell division and expansion, plant growth and, consequently, morphogenesis [48]. There are ten subfamilies in the cellulose synthase (CESA) superfamily according to protein sequence similarity, including nine cellulose synthase-like (CSL) families and one CESA family. These subfamilies contain a “D,D,D,QXXRW” motif thought to catalyse the synthesis of the β-glycosyl unit structure of cellulose and hemicellulose [49–51]. All CESA proteins localize to the plasma membrane to synthesize cellulose on the cell surface [52, 53]. Most CSL proteins, such as AtCSLA9, AtCSLC4, BdCSLF6, HvCSLH1 and AtCSLD5, accumulate in the Golgi, where they are thought to produce hemicellulose and pectin polysaccharides for subsequent vesicular secretion to the extracellular space [21, 54–56]. Heterologous expression has demonstrated that the CSLA family is related to β-mannan or glucosamann polysaccharide synthesis. CSLC proteins function in assembling the β-1,4 glucan backbone of xyloglucan, and the grass-specific families of CSLF and CSLH encode the mixed linkage glucan synthases [55, 57–59]. The other CSL families, namely, CSLB, CSLD, CSLE, CSLG and CSLJ, also likely participate in synthesizing hemicellulose polysaccharides, although it is still unclear which products they synthesize.

Among the CSL families, the CSLD proteins display the greatest amino acid sequence similarity to the CESA
family [50]; therefore, the CSLD proteins were predicted to have similar functions in synthesizing cell wall components. This prediction was confirmed by the finding that the CSLD proteins participate in the synthesis of cell wall polysaccharides, such as non-crystalline cellulose and xyloglucan [21, 37, 60, 61]. Our finding that ZmCSLD1 is a trans-Golgi protein is consistent with the subcellular localization of its orthologues AtCSLD5 and OsCSLD4; this finding suggests that ZmCSLD1 is likely to participate in the synthesis of hemicelluloses and non-crystalline cellulose in the Golgi apparatus, or cellulose with the assembly of CSLD proteins in the Golgi followed by transport to the plasma membrane [21, 37, 60, 62–64]. In addition, the cell wall composition changed in Zmsclsd1, especially a decrease tendency in the main components of cell wall backbone, xylose and cellulose content (Additional file 15: Table S5). These results, together with a previous study by Hunter et al. [36], suggest that ZmCSLD1 participates in the cell wall polysaccharide biosynthesis of cell plate during cell division. Whereas, the nature of the function of ZmCSLD1 as an enzyme, including its substrates and products, remains to be identified.

ZmCSLD1 affects cell division and organ size and has pleiotropic effects

Pleiotropic effects, which refer to one gene that can affect many traits, have been identified in many species [65]. These effects play an important role in development and evolution because of the complexity of the biochemical and developmental networks [66]. Pleiotropic effects often cause genetic correlations in bi- or multi-parent-derived populations [6, 8]. In our study, nine traits with QTL identified at the qLW10 locus had a moderate to high correlation with one another ($r$ = 0.44–0.94, F2,3 population) (Fig. 1b), which differs from other studies [6–11]. For example, the correlation between LW and LL was 0.62 in our F2,3 population but only 0.08 in an NAM population [10]. In addition, the NILs and mutants of ZmCSLD1 showed pleiotropic effects (Additional file 12: Figure S7), which also confirms that ZmCSLD1 underlies the pleiotropic effects of qLW10.

Pleiotropic genes often participate in transcription regulation, hormone signalling, ubiquitin-mediated proteolysis and cell wall biosynthesis pathways, which control organ size by regulating cell proliferation [5, 67]. The size and growth of organs are determined by the characteristics of their cells, such as their width, length and number. Because ZmCSLD1 encodes an enzyme in cell wall biosynthesis, it is interesting to investigate whether the pleiotropic effects of ZmCSLD1 due to organ size variation are caused by cell division or expansion. In the current study, the reduction in cell number in both the lateral and longitudinal leaf axes was far more pronounced than the reduction in cell size (Fig. 5b and c; Additional file 14: Figure S9). Thus, the reduction in cell number appears to be the main cause of the narrow organ phenotype, which is highly consistent with the results of Hunter et al. [36]. Furthermore, ZmCSLD1 was highly expressed in immature tissues, such as in the root tip, immature leaf base, ear primordia, shoot apex and tassel primordia and young-embryo endosperm and seed (Fig. 6c; Additional file 15: Figure S10A), but the transcript was nearly undetectable in mature tissues (Fig. 6c; Additional file 15: Figure S10B). Similar expression patterns of its orthologues in Arabidopsis and rice were validated by a β-glucuronidase (GUS) expression from the AtCSLD5 and OsCSLD4 promoters, respectively [21, 37]. The common characteristics of these tissues in which ZmCSLD1 is highly expressed is that these tissues are regions of rapidly dividing cells. In addition, OsCSLD4 is specifically expressed during the M phase of the cell cycle and the AtCSLD5 functions in the cell plate formation period, both of which are critical phases in mitosis [68, 69]. The sequence conservation of ZmCSLD1 with OsCSLD4 and AtCSLD5 strongly supports the role of ZmCSLD1 in cell division.

Mechanism of intragenic complementation between different ZmCSLD1 alleles

The recovery of the narrow-leaf phenotype in the heterozygotes (Fig. 4b) indicates that the Zmsclsd1Ko1 and qlw10Byk alleles of the ZmCSLD1 gene exhibit intragenic complementation. Intragenic complementation has been reported in plants, e.g., among the pattern formation gene GNOM [70], the abscisic acid biosynthesis gene ABA2 [71], the cytokinin receptor gene WOL/CRE1 [72], the brassinosteroid receptor gene BRI1 [73], and the cellulose biosynthesis genes AtCESA1 [74] and AtCESA3 [38]. The common characteristics of these genes is that they encode proteins with multiple functional domains or proteins that form homomeric or higher order homomultimeric complexes. For example, AtCESA1 and AtCESA3 are a part of the large cellulose synthase complex that contains approximately 36 CESA polypeptides in Arabidopsis and that is divided into six rosette subunits, each of which consists of three CESA isoforms [53, 54]. The three CESA isoforms form cellulose synthase complexes through various heterodimeric and/or homodimeric combinations to synthesize the cellulose microfibrils of the cell wall [75–77]. Therefore, these CESA genes can complement one another when the product of one allele functionally compensates for the product of a second allele in the same complex.
Similarly, the proteins of the CLSD family also likely form a heterodimeric or homodimeric enzyme complex that functions in generating non-crystalline cellulose or the β-1,4 glucan backbone of hemicellulose [78]. Notably, the mutations in qLW10BYK and Zmcsld1MO17 result in different amino acid residues in the “D,D,D,QXXRW” motif (G839D) and the last transmembrane domain (W1184R), respectively (Fig. 4a). Thus, the mechanism of intragenic complementation in the F1 hybrids between qLW10BYK and Zmcsld1MO17 by the formation of the homodimers of the two mutant alleles that compensate for the defective cytosolic catalytic domain and the disabled transmembrane domain in the same complex, which consequently reduces or recovers the narrow-leaf phenotype in the heterozygotes (Fig. 4b–d). However, further studies are necessary to confirm how many ZmCLSD1 subunits are present in the complex and to determine whether other protein subunits are involved.

**Conclusion**

Our results show that qLW10 was resolved to be ZmCSLD1 through map-based cloning. ZmCSLD1 encodes an enzyme in cell wall biosynthesis and controls organ size by altering cell division, which results in pleotropic effects on multiple traits in maize. The subcellular localization of ZmCSLD1 in the trans-Golgi shows that ZmCSLD1 participates in cell wall polysaccharides formation during cell division. In addition, ZmCSLD1 may form a homomeric or higher order homomultimeric complex to catalyse polysaccharide biosynthesis because of intragenic complementation.

**Methods**

**Plant materials and growth conditions**

Two maize double haploid lines, MT03-1 with a small plant architecture and LEE-12 with a large plant architecture, were used to develop an F2 population including 197 individuals. A heterozygous inbred family, BYK-HIF, was also used, which was derived from a By815 × K22 recombinant inbred line population. A pair of NILs, ZmCSLD1MO17 and Zmcsld1MO17, were developed from a line in a Mo17 × X26–4 (Zea mays ssp. mexicana) BC2F5 population by backcrossing once with Mo17 and selfing four times. A UniformMu line (UFMu-04904) was ordered from the Maize Genetics Cooperation Stock Center and backcrossed twice with W22 to reduce the background, which generated ZmCSLD1W22 and Zmcsld1W22. The plants were grown in the fields for QTL mapping, fine mapping and allelism tests.

**Construction of a genetic linkage map and QTL mapping**

All 197 F2 individuals and F2:3 families in the LEE-12 × MT03-1 F2 population, along with both parents, were grown in Beijing (40°N, 116°E), China, in 2012 and Shenyang (42°N, 123°E), China, in 2013, respectively. All F2 individuals and their parents were genotyped by using the MaizeSNP3K subset (3072 SNPs) of the Illumina MaizeSNP50 BeadChip [79]. SNP genotyping was performed on the Illumina GoldenGate SNP genotyping platform [80] at the National Maize Improvement Center of China, China Agricultural University. The quality of each SNP was checked manually, and poor-quality SNPs were excluded. Using 767 high-quality polymorphic SNPs, a genetic map of 1833.66 cM with an average interval of 2.42 cM between adjacent markers was constructed by R/qtl. QTL mapping for 11 measured traits (Additional file 1: Table S1; Additional file 2: Table S2) in both the F2 and F2:3 populations was performed by using composite interval mapping [81] that was implemented in Windows QTL Cartographer 2.5 [82]. The threshold logarithm of odds (LOD) value to declare the putative QTL was estimated by permutation tests with a minimum of 1,000 replicates at a significance level of p < 0.05 [83]. The confidence interval of the QTL position was determined by using the 1.5-LOD support interval method [84].

**Positional cloning of qLW10**

Fine mapping of qLW10 was based on 3,292 BC2F1 and 669 BC3F1 plants bred from the backcross between MT03-1 and LEE-12 with MT03-1 as the recurrent parent. Additionally, an F6 family, BYK-HIF, in the By815 × K22 RIL population that had heterozygous alleles at the qLW10 locus and that varied in LW was also used to fine map qLW10 based on 720 F2 plants descended from BYK-HIF. The markers used for positional cloning by using the two populations are listed in Additional file 17: Table S6.

**Genotyping of various Zmcsld1 alleles**

To determine the causal variants of the four Zmcsld1 alleles, full-length ZmCSLD1 was sequenced from two pairs of NILs, qLW10MO17/qLW10MTL and qLW10BYK/qLW10BYK, and two pairs of mutants and their wild type, Zmcsld1MO17/ZmCSLD1MO17 and ZmCSLD1W22/Zmcsld1W22. The genotype of Zmcsld1W22 was confirmed with the Mu-transposon primer TIR6 and gene-specific primer CSLD1-P3. A list of primers for this genotyping is given in Additional file 17: Table S6. Furthermore, four Zmcsld1 alleles and their wild-type counterparts were genotyped by using the MaizeSNP6K subset (5,259 SNPs) of the Illumina MaizeSNP50 BeadChip to check their genetic backgrounds.

**Allelism tests**

Ten F1 hybrids were generated from eight lines with different ZmCSLD1 alleles to evaluate the allelic effects as
described in Additional file 8: Table S4. All the hybrids were planted in a randomized complete block design with two replications in Sanya (18°N, 110°E), China, in 2015. Each hybrid was grown in a two-row plot with 5-m rows and 0.67 m between rows. The 16 traits were measured in at least 30 plants per hybrid as described in Additional file 2: Table S2. In addition, ~120 individuals of six F2 populations, including qLw10MTL × qLw10BYK, qLw10MTL × Zmcsld1W22, qLw10BYK × Zmcsld1W22, Zmcsld1Mo17 × qLw10MTL, Zmcsld1Mo17 × qLw10BYK and Zmcsld1W22 × Zmcsld1W22, were planted in Sanya (18°N, 110°E) in 2015. These individuals were genotyped by SNP and Mu-transposon insertion markers (Additional file 17: Table S6) and were phenotyped for LW, LL, PH, EH (Additional file 2: Table S2).

Construction of the phylogenetic tree
The predicted protein sequences of ZmCSLD1 orthologs were searched by using BLAST against the UniProt database (http://www.uniprot.org/blast/) for sequences with alignment scores >3,870, and one sequence in each database (http://www.uniprot.org/blast/) for sequences. The bootstrap replications in MEGA version 6.0 [86]. Yeast two-hybrid assays

Yeast two-hybrid assays
Two segments of ZmCLSD1 (S1–331: from 1 to 331 amino acids, and S300–984: from 400 to 984 amino acids of ZmCSLD1 protein sequence) were amplified by using primers AD-GS1, BD-GS1 and AD-GS2, BD-GS2 (Additional file 17: Table S6), respectively. The PCR products were cloned into the pGBK7-T-BD and the pGADT7-AD vector via EcoRI and BamHI restriction sites using a Hieff Clone one-step PCR cloning kit (Yisheng, China), respectively. Four different combinations were cotransformed into the yeast strain AH109. Yeast cells harboring both pGADT7-AD and pGBK7-BD vectors were selected on SD/–Leu/–Trp (–LW) medium. Interactions of all different combinations were tested on SD/–Leu/–Trp/–His/–Ade (–LWHA) medium (Clontech), pGADT7-T/pGBK7–53 was used as a positive control. Plates were incubated at 30 °C for 5 days.

Subcellular localization
The coding sequence of ZmCSLD1 was amplified by using a cDNA template from 5-day-old B73 seedlings. The PCR product was amplified by using primer rCSLD1–03 (Additional file 17: Table S6) and cloned into the SUP1300 vector at the XbaI and Spel sites to encode a ZmCSLD1-GFP fusion protein with the Super promoter. Maize protoplasts were obtained as described by Burdo et al. [87], except that B73 was used instead of B73 × Mo17 F1. Plasmids (15 μg) were transformed into protoplasts and incubated for 16 to 20 h in the dark at 25 °C before monitoring their GFP expression by using confocal microscopy (Zeiss 710, Germany).

Measurement of leaf and cell size
To measure leaf size and cell number, the NILs, qLw10MTL and qLw10MTL, were grown in the field, and the completely extended fourth leaf from 40-day-old seedlings was sampled. Simultaneously, the LL and LW of the six unfolded leaves (L2–7) were measured. For the remaining qLw10BYK/qLw10BYK, and two additional mutants and their wide type, Zmcsld1MO17/ZmCSLD1MO17, and ZmCSLD1W22/Zmcsld1W22, were planted in greenhouse, and the leaf and cell size of the third leaf from 20-day-old seedlings was measured. The replication of the abaxial epidermal surface in the middle leaf was carried out as described by Moon et al. [88]. Briefly, colorless nail polish was applied to the leaf abaxial surface and allowed to dry completely, and then the dried nail polish replicas were peeled off by transparent adhesive tape and pasted to glass slides. Leaf epidermal cells were observed and photographed by using light microscopy (Leica DM2000 LED, Germany) with a Leica DFC450 camera. For each sample, cell sizes were measured using ImageJ 1.45 s (ImageJ, National Institutes of Health, USA) in a 0.585 mm by 0.785 mm field, and at least 50 normal cells except stomata guard cell, smaller and irregular cells were measured per sample.

RNA isolation and qRT-PCR
qLw10MTL and qLw10MTL were planted in greenhouse with 16-h-light/8-h-dark photoperiod for expression analysis. The primary root tip (5 mm, cell division region), middle (5 mm, cell elongation region), base (5 mm, root hair region), and shoot base (2 mm, including shoot apical meristem) from 5-day-old seedlings (Fig. 6a), and the third leaf base (1 cm), proximal middle (1 cm), distal middle (1 cm), and tip (1 cm) from 10-day-old seedlings (Fig. 6b) were sampled for analyzing ZmCSLD1, CycB1;4, CycD3;1b and histone H2B expression. Each tissue had three independent biological replicates with ten plants per biological replicate. Total RNA was extracted using a TianGen plant RNA extraction kit (China). First-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Japan). qRT-PCR was carried out in triplicate for each sample using the SYBRGreen I kit (TaKaRa) on a 7500 Real-Time PCR System (Applied Biosystems, USA). Maize TUBG was used for normalization between samples [89]. Quantification of relative expression was based on the comparative threshold cycle method.
Cell wall components analysis
The ear leaves of ZmCSLD1w22 and Zmcsld1w22 were sampled at 7 days after flowering. Three independent biological replicates with five plants per biological replicate and five technical replicates for each sample were analyzed. Leaf veins were excluded before grinding to powder. Analysis of cell wall monosaccharide composition and cellulose content was carried out at the Institute of Genetics and Developmental Biology at Chinese Academy of Sciences (Beijing, China). Alcohol-insoluble residues (AIR) were prepared as previously described [37]. The Cell wall monosaccharide composition was determined with a gas chromatograph-coupled mass spectrometer (7890A-5975C, Agilent), and the crystal-line cellulose content was quantified by an anthrone method as described previously [91].

Additional files

Additional file 1: Table S1. Summary statistics of 11 agronomic traits in parental, F2 and F2:3 populations. (DOCX 15 kb)

Additional file 2: Table S2. Traits analyzed in this study. (DOCX 14 kb)

Additional file 3: Table S3. Summary of QTLs for 11 agronomic traits in the LEE-12 x MT03-1 F2 and F2:3 populations. (DOCX 27 kb)

Additional file 4: Figure S1. Positional cloning of qlw10 using the HIF family BYK-HIF in the By815 x K22 recombinant inbred line population. (DOCX 323 kb)

Additional file 5: Figure S2. Characteristics of plant architecture in qlw10mt and qlw10mt+. (DOCX 683 kb)

Additional file 6: Figure S3. Characteristics of plant architecture in qlw10mt and qlw10mt+. (DOCX 783 kb)

Additional file 7: Figure S4. Schematic representation of the ZmCSLD1 protein. (DOCX 498 kb)

Additional file 8: Table S4. Genetic design of the allelism tests. (DOCX 14 kb)

Additional file 9: Figure S5. Box plots showing the four Zmcsld1 allelic effects in six F2 populations. (DOCX 358 kb)

Additional file 10: Figure S6. Phylogenetic tree of CSLD orthologs across 32 angiosperms. (DOCX 256 kb)

Additional file 11: Table S5. Comparisons of cell wall monosaccharide composition and cellulose of leaf blades without leaf veins. (DOCX 16 kb)

Additional file 12: Figure S7. The pleiotropic effects of Zmcsld1 estimated by comparing Zmcsld1 and ZmCSLD1 homologous lines among different alleles. (DOCX 114 kb)

Additional file 13: Figure S8. Epidermal impressions of the fourth leaf abaxial surfaces from 40-day-old seedlings. (DOCX 1468 kb)

Additional file 14: Figure S9. Comparisons of leaf and cell size between ZmCSLD1 and Zmcsld1 in other three alleles. (DOCX 311 kb)

Additional file 15: Figure S10. Expression pattern of ZmCSLD1 in various tissues at different developmental stages. (DOCX 238 kb)

Additional file 16: Figure S11. Relative expression of histone H2B and CjcD2;1b in root, shoot and leaf tissues in qlw10mt and qlw10mt+. (DOCX 166 kb)

Additional file 17: Table S6. List of primers used in this study. (DOCX 18 kb)

Abbreviations
AIR: Alcohol-insoluble residues; CESA: Cellulose synthase; CSL: Cellulose synthase-like; DAP: Days after pollination; EH: Ear height; ER: Endoplasmic reticulum; HIF: Heterozygous inbred family; KW100: 100-kernel weight; LA: Leaf angle; LL: Leaf length; LN: Leaf number; LW: Leaf width; NAM: Nested association mapping; PH: Plant height; QTL: Quantitative trait locus; TBN: Tassel branch number; TL: Tassel length; VN: Veinlet number

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Availability of data and materials
The GenBank accession numbers of CSLD1 coding DNA sequences in 4 maize lines are: KX710098 (MT03), KX710099 (K22), KX710100 (W22) and KX710101 (Mo17). All materials used in the current study are available for request.

Authors’ contributions
XY and WS designed the study; WL, ZY and JY performed the experiments and data analysis; JL, XY and WL developed the materials; WL and XY wrote the paper. All the authors read and approved the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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