As the most abundant biopolymer on Earth, cellulose is a key structural component of the plant cell wall. Cellulose is produced at the plasma membrane by cellulose synthase (CesA) complexes (CSCs), which are assembled in the endomembrane system and trafficked to the plasma membrane. While several proteins that affect CesA activity have been identified, components that regulate CSC assembly and trafficking remain unknown. Here we show that STELLO1 and 2 are Golgi-localized proteins that can interact with CesAs and control cellulose quantity. In the absence of STELLO function, the spatial distribution within the Golgi, secretion and activity of the CSCs are impaired indicating a central role of the STELLO proteins in CSC assembly. Point mutations in the predicted catalytic domains of the STELLO proteins indicate that they are glycosyltransferases facing the Golgi lumen. Hence, we have uncovered proteins that regulate CSC assembly in the plant Golgi apparatus.
Plant cell walls are essential for plant growth and development, and protect cells against external stress.2
During growth, plant cells are surrounded by a strong yet adaptable primary cell wall. Once growth has ceased, and depending on the function of the cell, an additional secondary wall may be deposited.

The bulk of plant cell wall polysaccharides are synthesized in the Golgi and secreted to the apoplast, with the exception of cellulose that is synthesized at the plasma membrane by cellulose synthase (CesA) complexes (CSCs).2-3 As the most abundant biopolymer on Earth, cellulose is a principal component of both primary and secondary cell walls. Genetic and biochemical studies have revealed that two hetero-trimers of CesA proteins (CesA1, 3, and the 6-like CesAs, as well as CesA4, 7 and 8) are involved in primary and secondary wall synthesis in Arabidopsis thaliana, respectively.4-6 The CesA subunits are synthesized in the endoplasmic reticulum (ER), and CSC rosettes with sixfold symmetry have been observed in the Golgi, but it remains unclear where the CesA oligomerization and assembly into CSCs occurs.7-9 The CSCs are trafficked to the plasma membrane via the trans-Golgi network (TGN), and/or via small post-Golgi vesicles that are known as small CesA compartments or microtubule-associated CesA compartments.10,11 Once the CSCs are delivered to the plasma membrane they begin to synthesize cellulose microfibrils. During synthesis, the nascent microfibrils become embedded in the cell wall and the activity of the CSCs thus provides a motile force that drives the complexes forward through the membrane.12 The direction of the CSC movement is guided by cortical microtubules, via associations with CesA interacting1 (refs 13–15).

Apart from the CesA proteins, several components that impact on cellulose synthesis have been identified, including the GPI-anchored protein COBRA, the endo-glucanase KORRIGAN, the chitinase-like proteins (CTL1 and 2) and the recently discovered companion of CesA1 and 2 (refs 16–21). Most of these proteins affect the activity of the CSCs once they have reached the plasma membrane.2 However, less is known about processes that influence cellulose synthesis in other cellular compartments. Proper maturation of N-linked glycans affects cellulose synthesis, as mutations in glucosidases (knopf and rsw3) and in a mannosyl-phosphate guanylyltransferase (cym1) resulted in cell elongation defects and reduced cellulose levels.22–24 More recent reports show that defects in the N-glycosylation machinery affect the glycosylation of the endo-glucanase KORRIGAN, and that mutations of the glycosylation sites led to reduced glucanase activity, and mis-localization, of the protein.25–27 Nevertheless, N-linked glycosylation is a process that affects a substantial fraction of the cell’s proteome, and components along the secretory pathway that distinctively affect the activity of CSCs therefore remain unknown.

Here we report on two Golgi-localized proteins, STELLO1 and 2 (STL1 and 2, classical Greek ‘to set in order, arrange, send’), that specifically affect cellulose production. STL1 and 2 can interact with the CesAs, and mutations in the proteins led to defects in CesA secretion and activity, and altered the distribution of the CesAs in the Golgi cisternae. We further demonstrate that the STLs affect CSC assembly, which impacts on cellulose synthesis efficiency.

Results

Mutations in STL1 and STL2 cause cellulose-related phenotypes. Genes that are co-expressed with the CesA genes tend to be associated with cellulose synthesis.28 Using the pfam-based co-expression tool FamNet (http://aranet.mpimp-golm.mpg.de/famnet.html, ref. 29), we found that the pfam domain of unknown function (DUF)288 was co-expressed with the pfam CesA (Supplementary Fig. 1a). The DUF288 pfam contains two Arabidopsis proteins, At2g41770 and At3g57420, which we named STL1 and STL2. STL homologues are present throughout the plant kingdom, but STL proteins are distinct from distantly related proteins in nematodes, fungi and molluscs (Supplementary Fig. 1b). Microarray data suggested that STL1 and STL2 have similar expression profiles, and are active in cells that are expanding or producing secondary cell walls (Supplementary Fig. 1c), which we confirmed with transgenic Arabidopsis plants expressing pSTL1:STL1-GFP (Supplementary Fig. 1d–i).

Homozygous stl T-DNA insertion lines (stl-1, SALK_029852; stl-2, GABI_733B10; stl-1, SALK_023535; and stl-2, SALK_095790) that expressed no full-length STL1 and STL2, respectively (Supplementary Fig. 2a–c), did not differ in growth compared with wild-type plants (Fig. 1a–e). To test whether the STLs are functionally redundant, we generated stl1stl2 double mutants (stl-1stl-2 and stl-1stl-2) in the wild type. The primary roots of light-grown seedlings and etiolated hypocotyls of the stl1stl2 mutants were significantly shorter compared with wild-type (Fig. 1a–c; Supplementary Fig. 2d–f). In addition, 8-week-old soil-grown stl1stl2 mutant plants exhibited stunted growth (Fig. 1e).

Primary wall cellulose-related mutants display increased sensitivity to cellulose synthesis inhibitors.3,21 The stl1stl2 mutants were similarly hypersensitive, displaying severe cell swelling, in response to either isoxaben or 2,6-dichlorobenzonitrile (DCB; Fig. 1a–d; Supplementary Fig. 2d–f). The STL proteins also affected secondary wall production, as the stl1stl2 mutants showed occasional collapsed xylem vessels, and the interfascicular fibre cell-wall thickness was substantially reduced (Fig. 2a–c), which can also be observed in secondary wall cellulose synthesis mutants.30 Cellulose synthesis is also important in seed columella development, and cellulose contributes to rays in the seed mucilage adherent layer.31–34 Indeed, seed columella shape was abnormal, and the cellulose rays and adherent mucilage were absent in the stl1stl2 mutants (Fig. 2d–m). Our data thus indicate that stl1stl2 mutant plants show widespread impairment in cellulose production.

The stl1stl2 mutant contains less cellulose. To confirm that cellulose levels were reduced in the stl1stl2 mutants, we measured cellulose content by glucose release using Saeman hydrolysis. Indeed, the cellulose content was reduced by ~50% in the young hypocotyls of stl1stl2 mutants, indicating that primary wall cellulose synthesis was significantly reduced (Fig. 3a). Additionally, cellulose was reduced by ~40% in secondary cell-wall-rich stems of stl1stl2 mutants (Fig. 3b). The solid-state 13C cross-polarization-magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectra of intact Arabidopsis stems showed substantial changes in the carbohydrate region of the mutant (Fig. 3c,d). Subtraction of the mutant spectrum from the wild-type spectrum showed loss of cellulose in the stl1stl2 mutant, consistent with the assessment by Saeman hydrolysis. Nevertheless, the peaks of cellulose carbons, including crystalline cellulose carbohydrate 4 at 89 p.p.m. were still visible in the mutant. The X-ray diffraction pattern of the stl1stl2 mutant showed less intensity than the wild type (Fig. 3e–h; Supplementary Fig. 3a–c), possibly due to the thinner mutant stems and reduced cellulose content. After background subtraction and intensity normalization, the mutant radial diffraction profile was only subtly different to that of the wild type, with a broader peak arising from the 5.5 Å d-spacing (Fig. 3h; Supplementary Fig. 3a,b), which comprises the (1–10) and (110) reflections indexed according to the β- crystal structure.27 Since the strategy for background subtraction was designed to reduce the differences between the
wild type and the mutant radial diffraction profiles, the actual differences may be greater than they appear in these profiles. Such fine differences in cellulose diffraction profiles are inherently difficult to interpret. We speculate that heterogeneity of cellulose environments and the altered proportions between cellulose and matrix polysaccharides might be contributing to the observed changes in diffraction profiles. Hence, these different approaches all indicate that the stl1stl2 mutant contained lower levels of cellulose as compared with wild type. In contrast, the composition and relative proportion of the matrix-phase polysaccharides did not appear to be substantially altered as determined by the amount of monosaccharides released by trifluoroacetic acid (TFA) hydrolysis of alcohol-insoluble residues.

To assess how the STLs are oriented in the Golgi membrane, we performed microsomal protease protection assays in the absence or presence of detergent to disrupt the membranes. In intact microsomes, the fusion protein was resistant to protease, but was digested if detergent was added, indicating that the C-terminus of both etiolated hypocotyls and stems (Supplementary Table 1).

**STL1 and STL2 are localized to the Golgi.** To evaluate where in the cell the STLs may function, we generated pSTL1:STL1-GFP, pUb10:STL1-mCherry and pUb10:STL2-GFP constructs. These constructs were functional as they partially restored growth of the stl1stl2 mutant (Fig. 1a–c; Supplementary Fig. 4a,b). We observed green fluorescent protein (GFP) and mCherry signals in ring-shaped cytosolic compartments that resembled Golgi in etiolated hypocotyls (Fig. 4a). Dual-labelled STL2-GFP and STL1-mCherry plants revealed that the two STLs co-localized in the ring-shaped compartments (Fig. 4a; Supplementary Fig. 4c).

To see if the ring-like compartments represented Golgi, we introgressed different red fluorescent protein (RFP)- or mCherry-tagged Golgi and TGN markers into the STL1- and STL2-GFP lines, respectively. We also crossed the STL-FP lines with plants expressing tdTomato-CesA6 (tdT-CesA6) or GFP-CesA3 to investigate possible co-localization of the proteins. The dual-labelled seedlings showed that STL-GFP proteins did not co-localize well with the TGN markers vacular ATPase subunit a1 (VHAa1)-RFP and SYP43-RFP (Fig. 4j–l; Supplementary Fig. 4c), supporting a localization of the STLs in the Golgi apparatus.

STL glycosyltransferase-like domain is in the Golgi lumen. The STL proteins contain a region of homology to proteins of the CAZy glycosyltransferase (GT) 75 family that includes the self-glycosylating protein UDP-arabinose mutase (RGP), which has been shown to have GT activity (Supplementary Fig. 5a, discussed in ref. 36). Hydrophobicity analysis suggested the STLs contain a putative N-terminal signal sequence that could target them to the endoplasmic reticulum (Supplementary Fig. 5a). To test if the A STL glycosyltransferase-like domain is in the Golgi lumen, we performed microsomal protease protection assays in the absence or presence of detergent to disrupt the membranes. In intact microsomes, the fusion protein was resistant to protease, but was digested if detergent was added, indicating that the C-terminus of

Figure 1 | Mutations in STL1 and STL2 impact on plant growth. (a) Six-day-old Col-0, stl1-1, stl2-2, stl1-1stl2-2, stl1-2stl2-1 and pSTL1:STL1-GFP(stl1-1stl2-2; STL1 fused C-terminally to GFP under its native promoter expressed in the stl1stl2 mutant background) seedlings grown in the dark on half MS media supplemented with 0.5 mM isoxaben (upper panel) or on half MS media supplemented with 0.5 mM isoxaben (lower panel). Scale bar, 0.5 cm. (b,c) Bar graphs of hypocotyl length on media supplemented with increasing concentration of isoxaben (b) or DCB (c). Values are mean (+ s.e.) from three biological replicates with more than 10 seedlings per replicate. ***P value < 0.001. Student’s t-test. (d) Close-up of hypocotyl cells of seedlings grown as in a using stereo microscopy. Scale bar, 200 μm. (e) Eight-week-old greenhouse grown plants. Scale bar, 5 cm.
the protein resides within the Golgi lumen (Fig. 5b). To confirm these results, we performed a bimolecular-fluorescence complementation (BiFC)-based Golgi protein membrane topology (GO-PROMTO) assay37. The N-terminal part of VENUS (Yn; the first 155 amino acids), or the C-terminal part of VENUS (Yc; the last 84 amino acids), was fused in frame either before (cytosolic reporter) or after (Golgi luminal reporter) the first 52 amino acids of the rat ST protein (TMD), which consists of a transmembrane domain targeted to the Golgi membrane (Supplementary Fig. 5b). Clear fluorescence complementation was observed on co-expression of both Yn/Yc-STL1 and Yn/Yc-STL2 with the cytosolic reporter, but not with the luminal reporter (Fig. 5c; Supplementary Fig. 5c). These results corroborate that the N-terminus of STL proteins faces the cytoplasm.

To investigate what parts of the protein are relevant for the functions, we generated truncated protein versions where either the C-terminal or N-terminal parts of the STL1 were removed (Supplementary Fig. 5d). Both the C-terminal and N-terminal constructs were still localized to compartments reminiscent of Golgi, but only the C-terminal end of the protein was able to restore growth of the stl1stl2 mutant seedlings on isoxaben-containing media (Supplementary Fig. 5e–i). The C-terminal construct was also localized to the peripheral part of Golgi, while the N-terminal construct did not (Supplementary Fig. 5e,f). To investigate if the putative GT domain was important for STL function, we generated point mutations of amino acids that might contribute to the GT activity (Fig. 5a; Supplementary Fig. 5a). Mutations of conserved aspartates in the DXD motif putative sugar nucleotide binding region (DD205-206AA and DVD297-299AVA) impaired the function of the STLs, as the constructs were unable to complement stl1stl2 mutant growth (Fig. 5d). However, the conserved arginine corresponding to a self-glycosylation site in RGPs (R271A), and aspartates elsewhere in the protein (DD591-592AA) were not required as the mutated proteins restored stl1stl2 plant growth. The inability of some of the STL-GFP constructs to complement was not due to changes in protein localization, as all of the proteins were visible as ring-like Golgi compartments (Fig. 5e–i). Therefore, STL membrane topology and site-directed mutagenesis experiments are consistent with STL proteins having a GT activity in the Golgi lumen.

**STL1 and STL2 can interact with the CesA proteins.** The stl1stl2 cellulose defects and the co-localization of the tdT-CesA6 with STL2-GFP and GFP-CesA3 with STL1-mCherry prompted us to
We confirmed the yeast-based interactions by BiFC assays in tobacco epidermal leaf cells. As for the yeast assay, we used the Got1p *Arabidopsis* homologue (also identified as Wave line 18; At3g03180) as a negative control. Got1p localizes to the Golgi apparatus and has a similar membrane topology as that of the STLs (Fig. 5c; ref. 38). The N- (Yn) or C- (Yc) terminal part of VENUS was fused to the N-terminus of STL1, STL2, CesA1, CesA3, CesA6 and Got1p. We observed interactions between the CesAs and STL1 or STL2 (Fig. 6b; Supplementary Fig. 6a,b), but not between the Got1p and the CesA or the STL proteins. In addition, the STL and Got1p proteins showed homo-dimerization capability, which served as a positive control for the constructs (Fig. 6b; Supplementary Fig. 6b). Our data thus support that the CSC can interact with the STL proteins.

**STLs influence the Golgi localization of CesA3 protein.** The observed interaction of the STL proteins and the CesAs suggested that the STLs could influence the CSCs in the Golgi. Interestingly, the Golgi distribution of the GFP-CesA3 fluorescence was changed in the *stl1stl2* mutant as compared with wild type (Fig. 7a–e). The typical ring-shaped GFP-CesA3 signal, prominent in wild-type cells, was less pronounced in the *stl1stl2* mutant cells and instead resembled solid fluorescent spheres (Fig. 7a–c). We observed much more of the Golgi-localized GFP-CesA3 signal as solid spheres in the *stl1stl2* (54% of 820 Golgi) as compared with wild type (8% of 870 Golgi scoring scheme as outlined in Fig. 7c). In addition, quantification of the fluorescence distribution revealed a significant reduction of object diameter in *stl1stl2* mutants compared with the control (Fig. 7d). Surprisingly, similar changes in fluorescence distribution were not observed for the Golgi-localized protein Got1p-YFP nor for IRX9L-RFP which are localized to the Golgi periphery (Fig. 7a,d; Supplementary Fig. 6c,d). Moreover, co-localization analyses of the ST-RFP and GFP-CesA3 in the *stl1stl2* mutant revealed improved coincidence of the two fluorophores (Fig. 7e), corroborating a specific change of the GFP-CesA3 Golgi distribution in the *stl1stl2* mutant.

We confirmed the altered CesA Golgi distribution by performing immuno-gold labelling with anti-GFP and a gold-conjugated secondary antibody in GFP-CesA3-expressing plants. Gold labelling, representing GFP-CesA3, was detected at cell walls of primary and secondary cell walls (Figs 7f–h). Individual values and the mean from four (a) and two (b) biological replicates are shown.

**Figure 3 | Cellulose levels are reduced in the *stl1stl2* mutant.** (a,b) Cellulose content of primary cell walls (a; six-day-old etiolated seedlings, ~300–400) and secondary cell walls (b; six-week-old greenhouse-grown stem material, >10 stems). Individual values and the mean from four (a) and two (b) biological replicates are shown.

*P value < 0.05. ***P value < 0.001. Student’s t-test. (c) 1H (850 MHz)-13C solid-state cross-polarization-MAS NMR spectra of wild-type and *stl1stl2* stems. The signal intensity was normalized to the aliphatic/protein region (35–25 p.p.m.) with the assumption that proteins and waxes were not substantially changed in the mutant. (d) Subtracted 13C solid-state NMR spectrum (wild-type-*stl1stl2*) to reveal signals cellulose carbons C1–C6 specifically reduced in *stl1stl2*. The microfibril surface (s) and internal (i) C4 and C6 are labelled. (e–g) X-ray fibre diffraction patterns from wild-type (e) and *stl1stl2* mutants with lower (f) or higher (g) spurious diffraction intensities attributed mainly to wax. Arrows in e point to line corresponding to d-spacing of native cellulose (5.5, 4.0 and 2.6 Å); Arrows in f point to rings corresponding to d-spacing of wax (4.13 and 3.77 Å). The 4.13-Å ring is also visible in e, h Normalized diffraction intensity along the equatorial direction after subtraction of CRAFS-resolved background. The diffractograms are averages from three (wild-type) and two (*stl1stl2*) biological replicates. Only diffractions patterns with lower spurious signal (such as the one in f) were included in the average *stl1stl2* diffractograms presented in h.
the plasma membrane demonstrating specific labelling (Supplementary Fig. 6e). Gold label was also found in the Golgi apparatus (Fig. 7f). Similar to the live-cell imaging results, we found that GFP-CesA3 was confined to a more narrow Golgi localization in \textit{stl1stl2} as compared with that of wild type (Fig. 7f,g; Supplementary Fig. 6f,g). Importantly, no significant differences in Golgi shape, nor in the number of Golgi cisternae, were observed in the \textit{stl1stl2} mutant as compared with wild type (Fig. 7h,i; Supplementary Fig. 6h,i).

**STLs impact CSC assembly and plasma membrane motility.** To investigate whether the CesA behaviour was further affected in the \textit{stl1stl2} mutant, we first assessed the secretion of the CesAs by fluorescence recovery after photo-bleaching (FRAP) experiments of GFP-CesA3 at the plasma membrane focal plane. Only punctate structures in the central subregion were counted to avoid false positives due to active CesA complexes migrating into the region of quantification. Our FRAP experiments revealed that the CesAs are typically delivered with a rate of $4.8 \pm 1.2$ fluorescent CesA foci per $\mu m^2$ per hour ($\pm$ indicates s.d., $n \geq 11$ cells from more than 6 seedlings per genotype) in wild-type hypocotyls, which was significantly reduced in the \textit{stl1stl2} mutants to $2.2 \pm 0.9$ fluorescent CesA foci per $\mu m^2$ per hour (Fig. 8a,b).

Plasma membrane-based motility of fluorescently labelled CesAs is typically used as a proxy for complex activity. To assess complex activity we therefore also measured the GFP-CesA3 movement at the plasma membrane using Imaris analyses of spinning-disc confocal image series. We found that while the GFP-CesA3 proteins typically migrate with a speed of $255 \pm 128$ nm min$^{-1}$ ($\pm$ indicates s.d., $n \geq 10$ cells from 4 seedlings per genotype) in wild-type hypocotyls, the speed was significantly reduced in the \textit{stl1stl2} mutants to $191 \pm 92$ nm min$^{-1}$ (Fig. 8c,d).

The changes of CSC behaviour in the \textit{stl1stl2} mutant suggested that the STL proteins might affect the CSC assembly in the Golgi. To investigate this we performed blue-native polyacrylamide gel electrophoresis (BN-PAGE) analyses of microsomes from seedlings, or plant stems, of wild type and the \textit{stl1stl2} mutants. The CSCs can be seen as multimeric protein complexes when analysed by BN-PAGE (refs 9,39). Indeed, we detected bands at high molecular weight (Fig. 8e,g); however, the intensity of these bands was substantially reduced in the \textit{stl1stl2} mutants as compared with wild-type plants (Fig. 8e,g; Supplementary Fig. 7a). Similar reductions in band intensities were observed for both primary (anti-GFP; GFP-CesA3), and secondary (CesA8 antibodies), wall CSCs in the \textit{stl1stl2} mutants (Fig. 8e,g; Supplementary Fig. 7a). In contrast, the GFP-CesA3 levels were not, and the CesA8 levels only moderately, reduced in the \textit{stl1stl2} mutants.
mutant in the SDS-PAGE analysis (Fig. 8f,h,i; Supplementary Fig. 7a). This reduction was significantly less prominent as compared with the multimeric CSC band intensities (Fig. 8e–i).

Hence, relatively less CesAs are incorporated into multimeric CSCs, and the delivery rate of CSCs to the plasma membrane was reduced in the mutant as compared with wild type, corroborating that the cellulose crystals synthesized might have altered structure that the cellulose stacks remain scarce, a recent study proposes that cargo proteins are excluded from the central region of the trans-Golgi cisternae. The confinement of the CSCs to the central region of the Golgi stacks remain scarce, a recent study proposes that cargo proteins are excluded from the central region of the trans-Golgi cisternae. The confinement of the CSCs to the central region of the Golgi stacks would thus imply that they are not properly set for secretion, supporting that the GT-related activity of the STLS is necessary for accurate CSC assembly. Indeed, the CesAs were less well incorporated into multimeric CSCs, and the delivery rate of CSCs to the plasma membrane was reduced in the mutant. In addition, the CSC activity at the plasma membrane was impaired and X-ray fibre diffraction observations revealed that the cellulose crystals synthesized might have altered structure in the mutant as compared with wild type, corroborating that the CSCs are not properly arranged in the stl1st2 cells.

The STLS could also have a role in maintaining the CSCs in an inactive state until they have reached the plasma membrane. If so, the STLS and the CesAs would be delivered to the cell surface and how the CSCs are assembled and trafficked to the plasma membrane. While reports on altered spatial distributions of proteins within Golgi stacks remain scarce, a recent study proposes that cargo proteins are excluded from the central region of the trans-Golgi cisternae. The confinement of the CSCs to the central region of the Golgi stacks would thus imply that they are not properly set for secretion, supporting that the GT-related activity of the STLS is necessary for accurate CSC assembly. Indeed, the CesAs were less well incorporated into multimeric CSCs, and the delivery rate of CSCs to the plasma membrane was reduced in the mutant. In addition, the CSC activity at the plasma membrane was impaired and X-ray fibre diffraction observations revealed that the cellulose crystals synthesized might have altered structure in the mutant as compared with wild type, corroborating that the CSCs are not properly arranged in the stl1st2 cells.

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together. Here, the CSCs ought to become activated and the STLs would therefore need to be effectively removed from the plasma membrane. Indeed, we did not observe any detectable fluorescence associated with STL-GFP at the plasma membrane, supporting such a function. However, if the CSCs were active at the Golgi, as then would be the case in the stl1stl2 mutants, we would expect severe defects in Golgi structure and function due to aberrant cellulose deposition, which we did not observe in our transmission electron microscopy (TEM) studies. We therefore favour a scenario in which the STL proteins affect the assembly of the CSCs (Fig. 9).

It is plausible that the STLs could glycosylate the CSC or associated components that impact on CSC function and localization. Glycosylation of CesA proteins has not been reported, and they are not thought to be N-glycosylated22, but non-conventional types of protein glycosylation of CesA cannot be ruled out. In contrast, KORRIGAN, which is closely associated with the CSC, has N-glycosylations important for function26,27.

While it is perhaps unlikely that a GT75-related GT would be involved in modifying these types of glycans, it is possible that the STLs could affect glycans attached to KORRIGAN. This could be mediated via a direct interaction with KORRIGAN, or via interactions with other GTs in the Golgi, which in turn could lead to defects in cellulose synthesis as observed in the stl1stl2 mutants. However, we did not see any differences in protein size or the cellular location of GFP-labelled KORRIGAN in the stl1stl2 mutant as compared with wild type (Supplementary Fig. 7b,c). While these data do not rule out the possibility that the STLs function on KORRIGAN, it appears more likely that they act on other components of the CSC machinery.

Our results support a model in which the STL proteins regulate the assembly of the CSCs, and perhaps maintain the CSCs in a certain state during their transit from the Golgi to the plasma membrane (Fig. 9). Perturbations in the actin cytoskeleton, V-ATPase activity at the TGN and lesions in the kinesis FRA1 do affect the trafficking of the CSC to the plasma membrane41–43. However, these defects affect a range of other cellular processes and specific components that regulate trafficking and integrity of the CSCs have therefore been wanting. Hence, the STLs open new avenues into understanding the function and regulation of cellulose synthesis and provide a cornerstone in how we comprehend a plant’s ability to regulate this process.

Methods

Plant materials and growth condition. T-DNA insertional lines for stl1-1 (SALK_029852), stl1-2 (GABI_733B10), stl2-1 (SALK_023355) and stl2-2 (SALK_095790) were obtained from NASC (http://Arabidopsis.info; ref. 44). Primers used for genotyping are listed in Supplementary Table 2. The pCesA4:xylosyltransferase-RFP, SYP43-RFP, GFP-KOR and Got1p-YFP marker lines were described previously5,27,40,45–49. The pCesA3:GFP-CesA3 line in Arabidopsis plants has been grown on half mass spectrometry (MS) media with 1% sucrose in long day condition (16 h light and 8 h dark) or dark. For drug treatments, the seeds were directly germinated and grown for indicated days on plates supplemented with various amounts of Isoxaben (Dr Ehrenstorfer GmbH) or DCB (Sigma).

Sequence analysis. The amino acid sequence of Arabidopsis STL1 was used to search the publicly available databases at NCBI (http://www.ncbi.nlm.nih.gov/) and Phytotome (http://phytotome.jgi.doe.gov/pz/portal.html). The identified protein sequences from A. thaliana, Populus trichocarpa, Brachypodium distachyon, Selaginella moellendorffii, Physcomitrella patens, Lottia gigantea, Masas cuneatae, Minthostachys verticillata, Ostreococcus tauri, Caenorhabditis elegans and Chlamydomonas reinhardi were trimmed and aligned with the MUSCLE algorithm. An unrooted phylogenetic tree was built with MEGA 6.0 (http://www.megasoftware.net/) using the maximum-likelihood Le and Gascuel (LG) model with Gamma distributed Invariant sites. One-thousand bootstrap replicates were completed to evaluate branch support length. The multiple sequence alignment based on predicted secondary structures of STL and RGP1 was performed using the HHpred program (http://toolkit.tuebingen.mpg.de/hhpred).

Constructs. The ORFs of STL1 and STL2 were amplified from a seedling complementary DNA (cDNA) library with primer pairs STL1_for/STL1_rev and STL2_for/STL2_rev, respectively, and cloned into pENTR using the pENTR/D-TOPO Cloning Kit (Invitrogen, USA). For pUB10-STL2-GFP construct, London resin (LR) reactions of the Gateway cloning system (Invitrogen) were performed to sub-clone error-free STL2 into pUGC-GFP-DEST (ref. 50). The GFP fragment in pUGC-GFP-DEST was replaced by mCherry with restriction enzymes SpeI and PslI, which was then used for LR reactions with STL1-pENTR to generate pUB10-STL1-mCherry construct.

The 1,000 bp STL1 upstream sequence including the 5’-UTR and the coding region including introns were amplified via primers pSTL1_for/pSTL1_rev and

Figure 6 | STL1 and STL2 can interact with the CesA proteins. (a) Split-ubiquitin assays using STLs as bait and CesAs as prey in yeast. Values are the percentage of yeast colonies that displayed growth after 4 days on selective medium at 28 °C. ALG5 (yeast ALG5 dolicholphosphoglucose synthetase) was fused with wild-type N-terminal part of ubiquitin (NubI) as positive control. Mutated N-terminal part of ubiquitin (NubG) was used in the rest of the vectors including the auto-activation control (empty vector), negative control (ALG5 and Got1p) and CesAs. (b) BiFC assays detecting the interaction of CesAs and STLs in N. benthamiana epidermal leaf cells. The N-terminal (Yn) or C-terminal (Yc) part of VENUS was fused in frame with CesA1, CesA3, CesA6, STL1, STL2 and Got1p (negative control), respectively. The combination of constructs is indicated in each figure panel. The nuclear marker CFP-N7 (cyan) was included as a positive transformation control in all experiments. Scale bar, 50 μm.
**Figure 7 | Mutation of STLs changes the CesA distribution in Golgi.** (a) Images of 3-day-old etiolated hypocotyl cells expressing ST-RFP and GFP-CesA3, or Got1p-YFP in Col-0 or stl1stl2 mutant. Scale bar, 5 µm. (b) 3D heat-map renderings of fluorescence intensity of the GFP-CesA3 signal (white rectangles in a). (c) Relative fluorescent signal intensity across transects in a. Black solid lines indicate fitting of a double-Gaussian distribution to the intensity sections (red lines). Object diameter was calculated by summing the peak-to-peak distance and the width of the individual Gaussians (dotted lines). (d) Relative object diameter of GFP-CesA3 or Got1p-YFP in Col-0 and stl1stl2 mutant. Centre lines equal medians; box limits equal 25th and 75th percentiles; whiskers display minimum and maximum values. For GFP-CesA3, n = 870 Golgi (25 cells from 12 control plants); n = 820 Golgi (26 cells from 13 stl1-1stl2-2 plants). For Got1p-YFP, n = 567 Golgi (16 cells from 8 control plants); n = 583 Golgi (14 cells from 7 stl1-1stl2-2 plants). **P value < 0.01, Student’s t-test; NS, not significant. (e) Quantitative estimates of co-localization (Mander’s coefficient) of GFP-CesA3 and ST-RFP in Col-0 or stl1stl2 seedlings. n ≥ 7 cells per genotype. *P value < 0.05, Student’s t-test. (f) Representative TEM images of immuno-gold staining of GFP-CesA3 in wild-type and stl1stl2 plants. Scale bar, 500 nm. (g) Violin plots of Golgi gold periphery distributions. Values show the distance of each gold particle from the centre, relative to the total length, of the Golgi. n ≥ 190 gold particles per genotype from two independent immunoTEM experiments. **P value < 0.01, Student’s t-test. (h) Representative TEM images for Golgi morphology in Col-0 and stl1stl2 plants. Scale bar, 500 nm. (i) Violin plots of Golgi lengths. n = 200 Golgi per genotype from three independent cryofixation experiments. No significant difference between the wild-type and stl1stl2 plants was observed (P = 0.76, Student’s t-test). White circles show medians in g, i; box limits equal 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values.
cloned into a pGreenII0000 vector containing the Oleosin-GFP from pFAST-G01 as selection marker. The sequence was inserted between the ApaI at the 5’ and Sall at the 3’. The stop codon was removed to C-terminally fuse in-frame the mGFP and a nopaline synthase (NOS) terminator. The mGFP was amplified with primers mGFP_for/mGFP_rev and cloned between mGFP and a nopaline synthase (NOS) terminator. The mGFP was amplified with primers NOS_for/NOS_rev and inserted between NOS at the 5’ and Sacl at the 3’. The point mutations were generated by Genescript.

For the split-ubiquitin assays, the CDS of STL1 and STL2 were amplified with primer pairs (STL1_pTUB1_f/STL1_pTUB1_r and STL2_pTUB1_f/STL2_pTUB1_r, respectively) and cloned into the bait vector pTFB1 (Dualsystems Biotech AG, Switzerland) via the restriction enzyme sites Stul and SpeI. The constructs with CesA1, 3, 6, 4, 7 and 8 in the prey vector pADSL-Nx were described previously52,53. BiFC constructs were generated by amplifying PCR products using primers specified in Supplementary Table 2 and cloning them into SfoI and BamHI linearized vectors pAMON (a modified pGREEN II binary vector containing the first 155 amino acids of VENUS driven by CaMV35S promoter) and pSUR (C-terminal version containing the last 84 amino acids of VENUS) using the Gibson assembly method54. To improve the signal and the signal-to-noise ratio of the above systems, amino acid 152 in VENUS was changed from isoleucine to leucine, as this lowers self-assembly when co-expressed with VC155. All generated constructs were sequence-verified and transformed into Agrobacterium tumefaciens strain AGL1 by electroporation with the helper plasmid pSoup.

Cross-section and staining of stems. Sections of basal mature stem were fixed and embedded in LR White as described below for electron microscopy. Sections were stained with 0.1% methylene blue and viewed on an Olympus BX61 microscope.

Cell wall composition analysis. The basal part of the inflorescence stems from 6-week-old plants (5 cm from the base of the stem) were incubated in 96% (v/v) ethanol, twice with 2:3 (v/v) chloroform:methanol, and once with 65% (v/v), 80% (v/v) and absolute ethanol each and air-dried at room temperature. The monosaccharide composition analysis of the matrix cell wall polysaccharides was performed by high performance anion exchange (HPAEC) of acid hydrolysed cell walls. The alcohol-insoluble residue from stems (100 µg) was incubated in TFA (stl1stl2 mutants). Fourteen-day-old wild-type and stl1stl2 mutants expressing GFP-CesA3 (stl1stl2). The samples were analysed by BN-PAGE (stl1stl2) or SDS-PAGE (stl1stl2) followed by western blot with anti-GFP or anti-CesA8 antibody. Ponceau S staining of the membranes, coomassie blue (CBB) staining of protein gels, western blot with anti-actin or unspecific bands are shown for protein loading control. (I) Relative band intensity of western blot in stl1stl2-2 samples (normalized with the band intensity in control). Values are mean (± s.e., n = 3). **P value < 0.01 and *P value < 0.05, Student’s t-test.
Figure 9 | Schematic model of how the STL proteins function in cellulose production. In growing wild-type cells, the STL proteins are located to the Golgi. Here the proteins control the assembly of the CSCs. Once the CSCs are delivered to the plasma membrane they begin to synthesize cellulose. In the absence of the STL proteins the assembly of the CSCs is impaired, which cause aberrant localization of the complexes in the Golgi and that lead to defects in CSC trafficking to, and activity at, the plasma membrane.

(2 M, 1 ml) for 1 h at 121 °C. AIR of hypocotyls (1 mg) was hydrolysed by heating in 1.45 ml 4% sulfuric acid (121 °C, 60 min). Glucose in cellulose was measured after hydrolysis in 72% sulfuric acid at room temperature for 30 min and 1 M sulfuric acid for 3 h followed by neutralization with excess barium carbonate. The monosaccharide samples were separated using a CarboPac PA20 column on a HPAEC-PAD system (Dionex BioLC)\(^\text{55}\). The experiment was repeated three times, on three sets of independently grown plants.

**Electron microscopy.** Sections of basal mature stem were fixed in 4% (w/v) formaldehyde, 1% (v/v) glutaraldehyde, 80 mM Pipes-HCl (pH 6.9) at 4 °C overnight. Following dehydration in ethanol, the sections were embedded in London Resin (LR) white and allowed to polymerize at 60 °C. Thin sections were prepared on EM grids using a microtome, and samples were viewed using a FEI Philips CM 100 TEM (Multi-Imaging Centre, University of Cambridge, UK).

Three-day-old etiolated hypocotyls were high-pressure frozen, freeze-substituted, embedded and sectioned according to McFarlane et al.\(^\text{56}\). Briefly, samples were cryofixed using a Leica HPM 100 in B-type sample holders (Ted Pella) with 1-hexadecene (Sigma) as a cryoprotectant. For morphology, samples were freeze substituted in 2% osmium tetroxide (Electron Microscopy Sciences) and 8% 2, 2-dimethoxypropane (Sigma) in acetone. Freeze substitution in a Leica AFS2 held samples for 5 days at -85 °C, after which samples slowly warmed to room temperature over 2 days, and were infiltrated with Spurr’s resin over 4 days. Samples were sectioned to ~70 nm using a Leica UCT microtome, suspended on copper grids (Gilder) coated with 0.3% formvar (Electron Microscopy Sciences), stained with 2% uranyl acetate in 70% methanol and Reynolds’ lead citrate, then viewed on a Zeiss EM910 at 80 kV and images were collected using an Olympus Quemesa CCD camera. Golgi properties were quantified using ImageJ and the blind image analysis plugin BAR. Violin pots were generated using the BoxPlotR tool\(^\text{57}\).

For immunoTEM cryofixation, freeze substitution and embedding were performed as above, except that freeze substitution was performed with 0.25% glutaraldehyde, 0.1% uranyl acetate and 8% 2, 2-dimethoxypropane (Sigma) in acetone and samples were embedded in LR White resin (London Resin Company). Sections (70 nm) were suspended on nickel grids (Gilder) with 0.3% formvar and immunolabelling was performed according to McFarlane et al.\(^\text{56}\). Samples were blocked with 5% bovine serum albumin in tris-buffered saline tween buffer (TBST; 10 mM Tris, 250 mM NaCl, 0.1% Tween20, pH 7.4) for 30 min, washed three times in TBST, then incubated in the primary antibody, 1/100 rabbit polyclonal anti-GFP (Life Technologies, A6455), diluted in TBST with 1% BSA for 1 h at room temperature. After 5 washes with TBST, samples were incubated in secondary antibody, 1/100 goat-anti-rabbit conjugated to 18 nm gold (ImmunoResearch Inc), diluted in TBST with 1% BSA for 1 h, then washed 3 times with TBST and 3 times with D₂O. Samples were post-stained with uranyl acetate and Reynolds’ lead citrate as above. Samples were viewed using a Philips CM120 BioTWIN at 120 kV and images were collected using a Gatan MultiScan 791 CCD camera. Gold particle distribution was measured using ImageJ and the blind image analysis plugin BAR. For scanning electron microscopy of the seed columella layer, the dry mature seeds were mounted on surface of aluminium stubs and subsequently sputter coated with 10 nm of gold, and viewed using a Philips FEI XL30-FEG SEM operated at 5 kV (Multi-Imaging Centre, University of Cambridge, UK).

**MAS solid-state NMR.** MAS solid-state NMR experiments were performed on a widebore Bruker (Karlsruhe, Germany) AVANCE 850 MHz solid-state NMR spectrometer operating at 20T, corresponding to \(^{1}H\) and \(^{13}C\) Larmor frequencies of 850.2 and 213.8 MHz, respectively, using a 4-mm double-resonance MAS probe. Experiments were conducted at room temperature at a MAS frequency of 12.0 kHz ± 5 Hz. The \(^{13}C\) cross-polarization-MAS spectra were acquired using a 1-ms ramped contact time, a spectral window of 100 kHz (468 p.p.m.), a recycle delay of 2 s and 28,800 transients were co-added for each sample. The \(^{1}H\) 90° pulse duration was 3.4 μs. Two-pulse phase-modulated decoupling was applied during acquisition at a \(^{1}H\) nutation frequency of 83 kHz (ref. 58). The \(^{13}C\) chemical shift was determined using the carbonyl peak at 177.8 ppm of l-alanine as an external reference with respect to TMS. Two independent stem samples were analysed.

**X-ray diffraction.** Arabidopsis stems were aligned perpendicular to the CuKα X-ray beam (\(\lambda = 1.5418\) Å) generated by a Rigaku ultra-X-18HF rotating anode with VarianMax HR monochromating optics. Diffraction patterns were collected by a mar345 image plate positioned 200 mm behind the specimen. Instrumental line broadening and scattering angle 20 were calibrated with an α-alumina reference sample. The CRAFS model was employed to resolve the isotropic polynomial background contributing to each diffraction pattern, thus separating the intensity arising from cellulose crystals\(^\text{59}\). In CRAFS modelling, we first obtained crystal parameters (unit cell, crystal size and peak profile shape) from analysis of Col-0 and then these crystal parameters were imposed for stl1stl2 mutant. With this strategy, background subtraction attenuates differences between Col-0 and stl1stl2, thus assuring that remaining differences are not background subtraction artifacts.

Confirmatory experiments (Supplementary Fig. 3) with additional biological replicates were performed with the same modelling and X-ray detection set-up installed in the MX1 beamline (\(\lambda = 1.5435\) Å) of the Brazilian Synchrotron Light Laboratory\(^\text{60}\).

**Fluorescence imaging and analysis.** The sub-cellular localization and dynamic behaviour of GFP, RFP or mCherry-tagged proteins were imaged with a confocal microscope equipped with a CSU-X1 Yokogawa spinning disc head fitted to a Nikon Ti-E inverted microscope, a CFI APO TIRF 100 numerical aperture 1.47 oil immersion objective and an evince charge-coupled device camera (Photometrics Technology). Image acquisitions were performed using Metamorph online premier (version 7.5). Seeding samples were mounted in half MS media between a cover glass and a 1-mm-thick agar (1%) pad affixed on a circular cover slip (Roth), thus stabilizing the sample and preventing it from compression and mechanical damage.

The M1 & M2 coefficients were analysed for co-localization quantification using the JACOP plugin\(^\text{41}\). Parameters were adjusted according to the specifications of the microscope and the optimal detection thresholds were manually determined by choosing the threshold at which the most objects and least noise pixels were found. The velocity of GFP-CesA3 at plasma membrane was calculated in Imaris software (Bitplane)\(^\text{15}\).

The 2D heat-map and line plot of fluorescence intensity was analysed in ImageJ (http://imagej.nih.gov/). To measure the object diameter of Golgi bodies, their cross-sectional intensity profiles were measured in ImageJ and analysed using a custom-made Matlab programme (Supplementary Data 1). The script used a peak-finding algorithm to determine the number of peaks in a cross-section.

When the script found one or two peaks, we termed these Golgi bodies as 'solid' and 'ring', respectively. For solid Golgi body, we assumed that the two Gaussians were too closely positioned to be resolved individually. Thus, we fitted the
cross-sections with a double-Gaussian distribution with the distance between the peaks being (i) a free parameter for ring-shaped Golgi and (ii) fixed to the resolution limit of our microscope (estimated to 266 nm) for solid Golgi. This approach yielded accurate estimations of the positions and widths of the two Gaussians. We defined the object diameter as the sum of the distance between the two peaks and the widths of each Gaussian.

**FRAP assays.** FRAP experiments of GFP-CesA3 in control and stl1stl2 mutants were performed using a FRAP system ILAS (Roper Scientific) integrated into the spinning-disk set-up. Fluorescence at the PM was photobleached using the 488-nm laser and the delivery of new CesA particles was detected every 5 s for a period of 10 min. We scored the insertion events based on two criteria: first, before the delivery, the immediate region surrounding the delivery should be devoid of any CesA fluorescent particles, and second, after insertion, the particles should show the characteristic tracking behaviour in subsequent frames as examined using kymographs. New CesA insertion events were identified in the central regions to avoid the CesAs moving from outside into the photobleached area.

**Split-ubiquitin-based Y2H.** Interactions of STL1 and STL2 with different CesAs were analysed using the split-ubiquitin mbYTh system. Plasmids (200 ng each) for prey and bait were mixed and transformed into the yeast strain YTM51 (HypB::His3) and YTM52 (HypB::His3). Yeast cells were grown in YPD medium for 3 days at 30°C. 

**BifC and GO-PROMTO assay.** Agrobacterium tumefaciens strain AGL1 carrying the BIFC constructs and another strain generating the P19 protein to suppress gene silencing were resuspended in infiltration medium (10 mM MgCl2, 10 mM MES and pH 5.7) to a final concentration of OD600 0.03. After 2 h of incubation at room temperature, the cells were infiltrated into the abaxial surface of N. benthamiana leaves using a 1 ml needleless syringe. After 62 h, leaf sectors were excised, mounted in UHQ water and imaged on an inverted Leica SP2 confocal microscope using a 63 × HCX PL Apo objective (numerical aperture of 1.4). Images were collected using the average of 16 linear scans and all images were taken with the same settings. Each inoculation was performed on triple leaves and all transformations were performed on at least two separate occasions. The nuclear marker CFP-N7 (cyan) was a generous gift from Dr Tezq Quon (Monash University, Melbourne, Australia) and was infiltrated with all combinations as a positive control to assess transformation efficiency.

To confirm the membrane topology for STL1 and STL2, we used the GO-PROMTO BIFC assay and transiently expressed combinations of constructs with either cytosolic or lumenal fluorescent reporters in N. benthamiana leaves. The combination that was transiently co-expressed included the STL1/STL2 BIFC constructs (YN-STL1, YN-STL2, Yc-STL1 or Yc-STL2), and the GO-PROMTO luminal (Yn-TMD) and cytosolic (TMD-Yn and TMD-Yc) reporter constructs.

**Protease protection assay.** For topology analysis, STL1 was expressed under its own promoter with 4 × myc tag at the C-terminus in Arabidopsis callus. Microsome preparation was performed from callus tissue homogenized in an equal volume of homogenization buffer (250 mM Suc, 100 mM MES, pH 6.5, 1 mM dithiothreitol and Complete Protease Inhibitor Cocktail Tablets; Roche). The homogenate was centrifuged twice for 30 min at 2,200 g. The supernatant was centrifuged for 2 h at 4°C. Cross-sections with a double-Gaussian distribution with the distance between the peaks being (i) a free parameter for ring-shaped Golgi and (ii) fixed to the resolution limit of our microscope (estimated to 266 nm) for solid Golgi. This approach yielded accurate estimations of the positions and widths of the two Gaussians. We defined the object diameter as the sum of the distance between the two peaks and the widths of each Gaussian.

**RT-PCR and qRT-PCR.** Total RNA was isolated from 10-day-old seedlings by using the RNAse plant mini kit (Qiagen) and treated with DNase I (Ambion) to avoid genomic DNA contamination. First-strand cDNA was synthesized from 1.5 µg total RNA samples using the Superscript III Reverse Transcriptase (Invitrogen). The resulting cDNA was used as template for RT-PCR with a programme of 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 2.5 min, followed by 72°C for 10 min.

**Mucilage phenotype analysis.** Dried mature seeds were first imbibed in excess of millipore filtered deionized water for 1 h, then incubated in a 0.01% (w/v) ruthenium red solution for 30 min and rinsed with water. The adherent mucilage was observed with a Zeiss Axiosmager M2 microscope (>20 magnification using High quality differential interference contrast imaging). The imbedded seeds were separately stained with 0.01% (w/v) Pontamine fast scarlet 4B, a cellulose-specific dye (S479896; Sigma) for 1 h followed by rinsing in water. The stained cellulose in the seed-adherent mucilage was observed with a Leica TCS SP8 confocal laser scanning microscope, the Pontamine Fast Scarlet 4B dye was excited at 561 nm and detected in the 570–640 nm range.

**Statistical analysis.** A t-test (two tailed) was performed for statistical analysis if not otherwise specified. An F-test was used to determine equal or unequal variance in the t-test. *,” ** and *** indicate P value <0.05, <0.01 and <0.001, respectively.

**Data availability.** Solid-state NMR data is available at: https://www.repository. camus.org/handle/1810/254646. The authors declare that all other relevant data supporting the findings of this study are available within the article and its Supplementary Information Files or on request from the corresponding authors.

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Author contributions

Y.Z., N.N., M.S., T.V., S.P. and P.D. conceived the research and designed the experiments. Y.Z. did isoxaben treatment, split-ubiquitin, FRAP, western blots for CesA5 and truncated version complementation experiments. N.N. identified and generated mutants, did phylogenetic and protein sequence analysis, analysed stem and callus cell wall and mucilage phenotypes, and carried out protease protection assays. M.S. performed the expression and the point mutagenesis studies. T.V. performed DCB treatment experiments, measured cell wall content in etiolated hypocotyls and calculated GFP-CesA3 speed at P.M. Y.Z. and M.S. did the sub-cellular localization analysis. H.E.M. conducted the electron microscopy. C.K. conducted the BiFC and GO-PROMTO with E.L. and M.S.D., R.S. provided MatLab script and did the object diameter measurements of the Golgi, J.C.M. conducted HPLC, C.D. conducted and interpreted the XRD, C.K. conducted the RFAP data, and R.D. conducted the NMR with S.P.B. Y.Z., N.N., M.S., T.V., X.Y. and R.L. generated materials. S.P. and P.D. supervised the project, interpreted experimental data and co-wrote the paper with Y.Z. All authors commented on the manuscript.

Additional information

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