CRISPR/Cas9-mediated P-CR domain-specific engineering of CESA4 heterodimerization capacity alters cell wall architecture and improves saccharification efficiency in poplar

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Summary
Cellulose is the most abundant unique biopolymer in nature with widespread applications in bioenergy and high-value bioproducts. The large transmembrane-localized cellulose synthase (CESA) complexes (CSCs) play a pivotal role in the biosynthesis and orientation of the para-crystalline cellulose microfibrils during secondary cell wall (SCW) deposition. However, the hub CESA subunit with high potential homo/heterodimerization capacity and its functional effects on cell wall architecture, cellulose crystallinity, and saccharification efficiency remains unclear. Here, we reported the highly potent binding site containing four residues of Pro435, Thr436, Pro437, and Gly438 in the plant-conserved region (P-CR) of PalCESA4 subunit, which are involved in the CESA4-CESA8 heterodimerization. The CRISPR/Cas9 knock-out mutagenesis in the predicted binding site results in physiological abnormalities, stunt growth, and deficient roots. The homozygous double substitution of W436Q and P437S and heterozygous double deletions of W436 and P437 residues potentially reduced CESA4-binding affinity resulting in normal roots, 1.5–2-fold higher plant growth and cell wall regeneration rates, 1.7-fold thinner cell wall, high hemicellulose content, 37%–67% decrease in cellulose content, high cellulose DP, 25%–37% decrease in cellulose crystallinity, and 50% increase in saccharification efficiency. The heterozygous deletion of W436 increases about 2-fold CESA4 homo/heterodimerization capacity led to the 50% decrease in plant growth and increase in cell walls thickness, cellulose content (33%), cellulose DP (20%), and Crl (8%). Our findings provide a strategy for introducing commercial CRISPR/Cas9-mediated bioengineered poplars with promising cellulose applications. We anticipate our results could create an engineering revolution in bioenergy and cellulose-based nanomaterial technologies.

Introduction
The plant cell wall contains two different types of lignocellulosic matrices, including pectin-rich primary (PCW) and lignin-rich secondary (SCW) cell walls. Generally, PCW plays a pivotal role in determining cell shape, size, and cell division, while SCW provides structural support, and protection against biotic and abiotic stresses during plant growth and development (Burton et al., 2010; Cosgrove, 2005; Hu et al., 2018b; Majda et al., 2017). The unique lignocellulosic matrix of each CW is composed of cellulose, noncellulosic polysaccharides (neutral hemicellulose and acidic pectin), lignin, proteins, and other chemical compounds (Carpita et al., 2001; Li et al., 2016b, 2016a; Somerville et al., 2004). The SCW-related lignocellulosic biomass from woody plants is the most abundant biocompatible and biodegradable resource with broad application in bioenergy and high-value biomaterial industries (Tursi, 2019). However, lignocellulosic recalcitrance is a major challenge in cellulose-based industries that hinders the saccharification yield. To overcome this limitation, metabolic engineering and genetic manipulation of genes involved in SCW biosynthesis and lignocellulosic compounds in woody plants are the most magnificent fields that have been reported so far (Cao et al., 2020; De Meester et al., 2020; Himmel et al., 2007; Hori et al., 2020; Hu et al., 2018; Hu et al., 2018; Huang et al., 2019). Among woody plants, poplar (Populus sp.) is the best model plant for investigating cell wall characteristics and lignocellulose bioengineering due to its fast growth, worldwide distribution, small size, and sequenced whole genome, and high in vitro propagation and genetic transformation capacity (Confalonieri et al., 2003; Porth and El-Kassaby, 2015).

Cellulose microfibrils (CMFs) are the most abundant para-crystalline H-bond linear β-1, 4-linked glucans in the SCW lignocellulosic composition of Populus species that are synthesized by large transmembrane-localized multimeric CSCs known as hexagonal transmembrane rosettes (Lieth, 1975; Sjostrom, 1993; Somerville, 2006; Xi et al., 2017). Due to their unique para-crystalline nature, cellulose microfibrils play a crucial role in the structural and functional characteristics of the plant cell wall.
during plant morphogenesis (Arioli et al., 1998; Burn et al., 2002; Sethaphong et al., 2016; Williamson et al., 2001). Furthermore, the high biomass and unique physicochemical properties of CMFs from poplar make it a magnificent candidate for the production of bioethanol and cellulose-based materials (Fengel and Weigl, 2011; Sjostrom, 1993; Slabaugh et al., 2016). The wall cell architecture, lignocellulose composition, and biomass recycling are highly influenced by the physicochemical properties of CMFs, including crystallinity (CrI) and degree of polymerization (DP) in woody plants (Scanlon and Timmermans, 2013; Speicher et al., 2018). These properties are potentially controlled by the arrangements and interactions of cellulose synthase A (CESA) subunits in the formation of CSC assemblies, and the dynamic and performance of CSC rosettes during cell wall biosynthesis (Hagler et al., 2014; Nixon et al., 2016; Purushotham et al., 2020; Sethaphong et al., 2013; Turner and Kumar, 2018).

In higher plants, two types of PCW- and SCW-specific CSC rosettes have been identified that ensemble from dimerization and trimerization of 18, 24, or 36 CESA subunits in Golgi cisternae (Abbás et al., 2020; Hill et al., 2014; Nixon et al., 2016). In Arabidopsis, PCW-specific CSC rosette contains CESA1, CESA3, and CESA6-like (CESA2, CESA5, CESA6, and CESA9) subunits, whereas CESA4, CESA7, and CESA8 subunits are involved in the formation of SCW-specific CSC assemblies in the stoichiometry of 1 : 1 : 1 (Kumar et al., 2009; Kumar and Turner, 2015; Richmond and Somerville, 2000; Zhang et al., 2018). In P. trichocarpa genome, 18 CESA genes encoding CESA subunits with A/B isoforms were identified as involved in the formation of PCW and SCW-specific CSC assemblies with the stoichiometry of 3 : 2 : 1 (Abbás et al., 2020; Song et al., 2010; Xi et al., 2017; Zhang et al., 2018). CESAs subunits consist of three distinct regions, including N-terminal zinc-finger-binding domain (ZFBD) (~140–160aa long), eight transmembrane helices (TMH) (22aa long of each), and the central catalytic region (CCD), which contain a plant-conserved region (P-CR) (~118 residues) and class-specific region (CSR) (~124 residues) (Somerville, 2006). These domains potentially facilitate CESA homo/heterodimerizations and CSCs-CS1/POM2 complex formation during CSC rosette formations and indirect CSCs-microtubules interactions, respectively (Bringmann et al., 2012; Li et al., 2012; Park and Ding, 2020; Purushotham et al., 2020; Roberts and Roberts, 2009). However, it remains unclear whether altering the dimerization capacity of SCW-CESAs could potentially control the biomass recalcitrance, lignocellulose composition, cellulose crystallinity, and cell wall architecture during plant morphogenesis in poplar.

Recently, the generations of homozygous knockout poplars were reported using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) system as a powerful tool for gene editing approaches (Azeez and Busov, 2021; Bruegmann et al., 2019; Elorriaga et al., 2018; Fan et al., 2015; Triozzi et al., 2021; Wang et al., 2020; Xu et al., 2021). Here, through the protein–protein interaction analysis, we showed that the P-CR domain of the PalCESA4 subunit contains a specific protein–protein binding site that is potentially involved in CESAA4-CESA8 heterodimerization in poplar. Furthermore, we generated CRISPR/Cas9 T0 mutants to investigate the effect of dimerization capacity on cell wall architecture, plant morphophysiological properties, cell wall regeneration and growth rate, lignocellulose composition, biomass recalcitrance, cellulose DP and crystallinity and saccharification efficiency in poplar. The CRISPR/Cas9-knockout mutants in the predicted binding site of the CESAA-related P-CR domain showed morphophysiological abnormalities, stunt growth, and deficient roots. The homozygous substitution of W436Q and P437S and heterozygous deletion of both W436 and P437 residues in the P-CR domain of T0 mutant increase plant growth, cell wall regeneration in leaf mesophyll protoplasts, decrease cell wall thickness, vessel density, lignocellulose composition, cellulose DP, crystallinity, and improve lignocellulose saccharification efficiency. However, heterozygous deletion of single W436 residue causes a reverse effect on the plant growth, cell wall thickness, biomass recalcitrance, cellulose DP, and CrI properties.

Results and discussion

Identification of SCW-related CESA genes in *Populus alba*

The NI phylogenetic tree of PalCESA genes showed a similarity of 97%–99% (query coverage: 95%–100%) with the CESA orthologs from *A. thaliana* and *P. trichocarpa* (bootstrap support value of 95% or higher; Figure S1). In agreement with Li et al. (2017), our alignment and phylogenetic analysis results indicate that the amino acid sequence data of CESAs are highly conserved among monocots and dicots. In agreement with Nawaz et al. (2019), our phylogenetic tree was categorized in two PCW- and SCW-related CESAs monophyletic clusters, which were subdivided into three clades each. Among aa sequence data of PalCESAs, XP_034916499 and XP_034912086 showed a close relationship with the mean of 85.5% in CESAl1 and CESAl3 clades, respectively. Kumar et al. (2009) reported the orthologous relationship between PtrCESA6A-F paralogues and AtCESA2/5/6/9 clade. However, we only found the sequence data of CESAl6 (XP_034912874) and PalCESA2 (XP_034915572) in the *P. alba* genome annotation database with a similarity of >95%. The five-sequence data of CESAl genes from *P. alba* showed the >99% similarity with the SCW-related PtrCESA subunits including PalCESA4 (XP_034916189), PalCESA7a (XP_034907672), PalCESA7b (XP_034895550), PalCESA8b (XP_034992271), and PalCESA8b (XP_034898820), which were considered for further analysis. In agreement with previous reports, PtrCESA1(A/B)/3(C/D) genes and PtrCESA4/7/(A/B)(A/B) genes correspond to PCW-specific SCs and SCW-specific CSCs, respectively (Abbás et al., 2020; Song et al., 2010).

Tertiary structure modeling of SCW-related PalCESA domains

The transmembrane localization’s nature of cellulose synthase subunit makes their structural analysis challenging due to their relative flexibility, instability, and hydrophobic characteristics (Carpenter et al., 2008). To overcome these limitations, after determining the TMH domains in the amino acid sequence of CESAl proteins (Figure S2), we accurately predicted the tertiary structure of ZFBD and CCD of each SCW-related PalCESA subunits using the homology modeling approach, which their crystallography structures are not available in the PDB database. As a result, the predictions are highly accurate in the range of 95%–100%, and the residues are predicted to be in their correct state. The modeled CESAl7/8-related CCD (Figure S3A–C) and ZFBD (Figure S3D–F) tertiary structures showed the highest homology and identity of 89% and 99.5% compared with the crystallographic structure of PtrCESA8 (PDB ID: 6WLB) reported by Purushotham et al. (2020). The distribution of Phi and Psi

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The potential capacity of SCW-related CESA hom/heterodimerizations was examined using the sequence-based and structure-based binding affinity analysis of ZFBD and CCD from PalCESA4/7/8 subunits (Table S1). As the sequence-based analysis results, the CESA4-ZFBD homodimerization showed the least $k_D$ of 4.4$e^{-11}$ and the most negative $\Delta G$ value of −14.12 compared with the CESA7/8-ZFBD homodimerization. In agreement with Trimmers et al. (2009) and Zangir (2012), we observed a significantly lower binding affinity in CESA7 and CESA8 homodimerization than that of CESA4 (Timmers et al., 2009; Zangir, 2012). However, the highest hydrophobic affinity was observed in the ZFBD CESA4-CESA8 heteroduplex. There is a significant high affinity between CESA4-related ZFBD and CESA8-related CCD with $k_D$ of 1.46$e^{-8}$ and $\Delta G$ of −10.68. Like UM or RING domain-containing transcription factors, the CESA4-related ZFBD is probably involved in the spontaneous formation of CESA4-CESA4/7/8 duplexes using the cysteine-rich conserved region (Feuerstein et al., 1994; Kurek et al., 2002; Park and Ding, 2020; Purushotham et al., 2020). Compared with CESA4-ZFBD, the highest binding affinity was observed in the CCD-heterodimer of CESA4-CESA8 complex with $k_D$ of 5.71$e^{-11}$ and $\Delta G$ of −13.92. The highest coefficient weight of electrostatic (−1892.7) and hydrophobic (−3039.7) were observed in the CESA4-CESA8 CCD heterodimer. These results indicate that the CESA4 subunit probably could establish both hom/heterodimers in the formation of SCW-related CSC assemblies using the ZFBD and noncatalytic domains in the CCD region, respectively. Supporting our findings, Trimmer et al. (2009) reported that CESA4 is the only subunit that was able to form a homodimer, and the rest of all formed heterodimers in Arabidopsis (Timmers et al., 2009).

Binding site prediction in cytosolic region of PalCESA4

The in vitro and in vivo biochemical PPI assays such as yeast two hybridization (Y2H), Co-immunoprecipitation (Co-IP), Förster resonance energy transfer (FRET), and bimolecular fluorescence complementation (BiFC) have their limitations that make PPI analysis of the transmembrane proteins much more challenging (Avila et al., 2015; Bao et al., 2009; Rao et al., 2014). Therefore, we have investigated the binding site of the active domain of the CESA4 catalytic domain involved in the heterodimerization process using a bioinformatics approach. We identified an efficient binding site containing 205 residues in the P-CR domain of the CESA4 cytosolic region with 99.5% accuracy and a C-score of 0.94 values (Figure 1a). To investigate the CCD heterodimerization model in the CESA4-CESA8 complex, 10 models along with their expected confidence values were predicted using ClusPro 2.0 and ZDOCK 3.0.2. To evaluate models, the residues within 5 Å between both chains were selected as possible binding sites. The results showed that there is a strong tendency between the P-CR domain of CESA4 and CESA8 cytosolic region in the formation of the CESA4-CESA8 complex (Figure 1c). In previous studies, the involvement of the P-CR domain in the CESA subunit dimerization and CSC assemblies have been reported (Purushotham et al., 2020; Sethapong et al., 2013). The docking results confirmed the predicted binding site position (Figure 1d) with about four residues, including Pro435, Trp436, Pro437, and Gly438 in the turn region between two alpha-helices in the P-CR domain of CESA4. Therefore, this highly potent binding site in the P-CR domain of PalCESA4 was considered as the gRNA target region for CRISPR/Cas9-mediated mutagenesis (Figure 1b, Figure 5S).

Targeted mutagenesis in the predicted binding site of PalCESA4 P-CR domain

We have generated CRISPR/Cas9-mediated T0 mutants using the pfGC-pcoCas9:CESA4-specific sgRNA cassette binary vector to investigate the functional effects of the predicted binding site of the CESA4 P-CR domain on plant growth, wood composition, cell wall architecture, physicochemical properties of cellulose, and biomass recalcitrance in poplar. The off-target analysis result showed there is no highly potent off-target site in the P. trichocarpa genome. The putative transgenic plants were validated by PCR amplification of BAR (552 bp), Cas9 (184 bp), and CESA4-specific sgRNA expression cassette (127 bp) genes with 93.62% positive amplification (Figure S6). In agreement with previous reports, our results showed the DSBs occurrence in the range of 2–4 bp upstream of the PAM motif with a CRISPR/Cas9 mutagenesis efficiency of 72.73% (Cong et al., 2013; Hilton and Gersbach, 2015; Ran et al., 2013). As the genotyping analysis result, we obtained 52.27% homozygous T0 mutants (Figure S7 and Table S2). As the result, the small indels (1–3 bp) and 5–9 bp deletions occurred in 80.56% of T0 mutants, while the large deletions of −15 bp were observed in the DSB region (Figures S8 and S9). Among them, the homozygous T0 mutant L1 showed a 4 bp mutation in the target site causes the double amino acid substitutions of W436Q and P437S in the predicted binding site position (Figure S8). We also observed the amino acid deletions of W436 in heterozygous mutant L2 and W436-P437 deletion in the heterozygous mutant L3 (Figure S9). In the rest of the T0 mutants (90.63%), CRISPR/Cas9 mutagenesis resulted in translational frameshifts that led to premature stop codons close to the gRNA target region (Figures S8 and S9). The Sanger sequencing chromatogram of the CESA4-specific target site from 32 T0 mutants and WT plant were illustrated in Figure S10.

The targeted mutagenesis of the P-CR domain alters the PalCESA4 binding affinity in the formation of SCW-related CSC complex

Among 32 T0 mutants, we only examined the binding affinity of PalCESA4 subunits with amino acid substitution and deletions, including the homozygous PalCESA4$^{W436Q,P437S}$ (L1), the heterozygous PalCESA4$^{W436Q,P437S,W436del}$ (L2), and the PalCESA4$^{W436Q,P437S,W436del,P437del}$ (L3) T0 Mutants compared with the WT plant. The rest of the T0 mutants were not considered in the binding affinity prediction due to the presence of premature stop codons in the CESA4 target region resulting in nonfunctional and unfolded protein structures. The sequence-based and structure-based binding affinity analysis results associated with the protein–protein interactions between the CESA4-related CCD domain and PalCESA4/7/8 domains from three T0 mutants were presented in Table S3. As the result, the substitution of W436Q and P437S (L1 T0 mutant) and deletion of W436 (L2 T0 mutant) cause a significant decrease in the binding affinity of CESA4-related P-CR domain and formation of hom/heterodimers in SCW-related CSC assembly. However, the heterozygous deletion of W436 and P437 residues (L2 T0 mutant) showed a significantly higher binding affinity than that of WT. In agreement with our findings, P-CR domains are potentially involved in CESA hom/heterodimerizations during CSC rosette formations (Bringmann et al., 2012; Li et al., 2012; Park and Ding, 2015).
2020; Purushotham et al., 2020; Roberts and Roberts, 2009). With regards to the consequences, it could be concluded that any nucleotide change in the P-CR domain from CESA4 can alter the dimerization capacity of the CESA4 subunit.

CESA4-specific P-CR domain modulates poplar morpho-physiological properties

In total, we obtained seven different phenotypes among 32 T0 mutants in four-month-old plants compared with WT plants (Figure 2). The results associated with the binding affinity analysis and morpho-physiological measurements indicate that any nucleotide mutation in the two conserved residues of tryptophan (W436) and proline (P437) in the CESA P-CR domains causes different phenotypes with improved and valuable plant morpho-physiological traits by alteration in the CESA4 dimerization capacity (Table S3 and Figure 2).

The substitutions of W436Q and P437S promotes rapid growth and development

The homozygous PalcesA4W436Q_P437S T0 mutant (L1 line) (Figure 2b) showed a fast-growing phenotype with a significant increase in shoot morphological properties, including shoot height (69%), trunk diameter (48%), internodal stem length (42.28%), node number, and leaf number (18.99%) compared with the WT plant (Figure 2a, Figures S11-S15). The root phenotypes have been illustrated at the top-right corner of Figure 2a (WT) and Figure 2b (L1 mutant). The L1 T0 mutant showed a significant decrease of about 29.26% in root number, but a three-fold root length compared with the WT plant (Figure S17 and S18). Although the shoot and root fresh-dry biomass ratios are 2–3-fold lower than that of WT (Figures S16 and S19), the root–shoot biomass ratio did not show any changes compared with the WT plant (Figure S20). Furthermore, the L1 T0 mutant produced 45.29%, 44%, and 45.45% more total chlorophyll, chlorophyll a, and chlorophyll b than the WT plant, respectively (Figures S21-S23). The Plastochron Index (P.I.) measurements showed an almost 2-fold higher leaf initiation rate in L1 than that of the WT (Figure S24). Li et al. (2017) reported the ethyl methanesulfonate (EMS)-mediated OsCESA9 mutant with amino acid substitutions of W481C and P482S residues in the OsCESA9-related P-CR domain of O. sativa with normal growth and grain yields compared with WT as well as a remarkable improvement in the agronomical traits such as logging resistance. In comparison, our finding indicates that the substitutions of the W436Q and P437S residues increase plant growth rate and improve biomass production in poplar due to the reduction of CESA4 dimerization capability.

Figure 1 Illustration of the binding site pocket in the secondary structure of the PalCESA4 catalytic domain and potent residues involved in the formation of CESA4-CESA8 heterodimer. (a) A probable binding site and its related residues within 5 Å of the binding site for CESA4 cytosolic region predicted by PSIVER, NSP-HomPPI, and FTsite web server tools. The residues were highlighted in red and their name and position were illustrated in rectangular. (b) The potent residues in the P-CR domain of PalCESA4 involved in the protein-protein interaction, which was considered as the gRNA target region for CRISPR/Cas9-mediated mutagenesis (green-colored region). (c) The interaction between PalCESA4 catalytic domain and PalCESA8 catalytic domain was predicted by ZDOCK 3.0.2 and ClusPro 2.0 docking software. Residues that are within 5 Å of the binding site are represented by stick representation of side-chain atoms. (d) Close-up view of binding site region in CES4 P-CR domain. The atoms are colored by element with carbon colored the same as the corresponding binding site (light blue color).

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The W436 deletion in the CESA4 P-CR domain negatively regulates the morphophysiological properties of poplar

Compared with the WT (Figure 2a), an obvious decrease in growth rate was observed during in vitro regeneration (Figure 2c) and also two months after transplantation of the heterozygous PalcesA4W436del T0 mutant (L2) (Figure 2i). Although the shoot height and trunk diameter, node numbers, and leaf numbers showed a significant decrease of about 8.85–17.06%, the internodal length of the L3 T0 mutant was almost similar to the WT plant (Figures S11–S15). Compared with WT, the L2 T0 mutant showed a 50% and 19.66% decrease in P.I. (Figure 5f) and chlorophyll content (Figures S21–S23). Furthermore, the leaf initiation rate of the L3 T0 mutant is almost 1.5-fold higher than that of the WT (Figure S24). In the L3 T0 mutant, a considerable decrease of about 29.26% and 57.5% were observed in the root number and the root length (Figures S17 and S18), respectively. The morpho-physiological properties of the L3 T0 mutant were almost the same as that of the L1 T0 mutant.

The positive regulation of growth rate in the heterozygous PalCESA4W436del_P437del T0 mutant

The heterozygous deletions of 436W and 437P residues of the CESA4-specific P-CR domain, which were identified in the PalcesA4W436del P437del T0 mutant (L3) (Figure 2d and i) resulted in a significant increase in trunk diameter (17.06%) and chlorophyll content (24.78%) (Figures S12, S21–S23). Furthermore, the leaf initiation rate of the L3 T0 mutant is almost 1.5-fold higher than that of the WT (Figure S24). In the L3 T0 mutant, a considerable decrease of about 29.26% and 57.5% were observed in the root number and the root length (Figures S17 and S18), respectively. The morpho-physiological properties of the L3 T0 mutant were almost the same as that of the L1 T0 mutant.

CRISPR/Cas9-Knockout Palcesa4 T0 mutants critically influence the root architectures in poplar

Among 29 homo/heterozygous Palcesa4 T0 mutants, we observed a total of four different root phenotypes with stunt growth, deficient roots, and shoot elongation about 2–5 cm in...
length without leaf initiation (Figures 4e-h). Figures S12-S24 show the morphological measurements of the knockout mutants. The homozygous knockout T0 mutants, including L6, L10, L14, L19, and L22 showed about 2-fold higher root induction with approximately 0.5–1 cm in length compared with the WT (Figures 2e, S17I, S18). However, in some of the homozygous/ biallelic T0 mutants, including L9, L24, L26, L27, L30, L32, L40, and L47, the root growth was extremely limited to only three to four short branches (2–3 cm in length) that contained several thick lateral roots (Figure 2f). The heterozygous mutants of L16, L29, and L45 showed two roots with 1–2.5 cm length and 0.3 mm thickness (Figure 2g). The rest of the mutants showed a few short roots with stunted growth (Figure 2h).

In agreement with our result, several studies have been reported either EMS-induced mutagenesis or overexpression of CESA genes in Arabidopsis, rice, barley, and aspen with defective phenotypes, including short roots, dwarf plants, and morphological abnormalities (Chen et al., 2005; Joshi et al., 2011; Tan et al., 2015; Tanaka et al., 2003; Taylor et al., 2003; Zhong et al., 2003). Furthermore, the evaluation of the RNAi knockdown and CRISPR/Cas9-knockout SCW-related PtcCESA4 subunits indicates stunted growth, necrosis, reduced shoot length, decreased cell wall thickness, and collapsed vessels in P. trichocarpa (Abbas et al., 2020; Xu et al., 2021).

The wood anatomical changes in T0 mutants

The anatomical changes in the xylem tissues of L1-L3 T0 mutants compared with WT were examined using light microscopy (LM) and FE-SEM (Figure 3). The wood anatomical measurements were also presented in Table 1. Compared with WT (Figure 3a), the vessel density of L1 (Figure 3b) and L3 (Figure 3d) T0 mutants were significantly decreased by 18.85% and 86.75%, respectively (Table 1). However, we observed a 3.14% increase in the wood morphology of the L2 T0 mutant (Figure 3c). The area fractions of the cell wall (50%), ray cells, fiber, and vessel lumens showed a significant decrease in L1 and L3 T0 mutants. In the L2 T0 mutant, although there is a substantial increase in the area fractions of the cell wall (13.78%) and fiber lumen (16.64%), the area fraction of vessel lumen showed a 10% decrease compared with the WT (Table 1).

As the FE-SEM microscopy result, the cell wall thickness of L1 (0.96 ± 0.004 µm; Figure 3f) and L3 (1.36 ± 0.003 µm; Figure 3h) T0 mutants were significantly less than that of the WT (1.64 ± 0.007 µm; Figure 3e). By decreasing cell wall thickness, the vessel diameters of cell lumen in L1 and L3 T0 mutants showed a remarkable increase of 26.56% and 9.05% compared with WT, respectively. However, in comparison with WT, the L2 T0 mutant showed a 29.26% increase in the cell wall thickness resulting in a remarkable decrease in the lumen diameters of fiber cells (22.68%) and xylem vessels (36.98%) (Figure 3g, Table 1). In agreement with our results obtained for the L1 and L3 T0 mutants, Li et al. (2017) reported a significant decrease in the cell wall thickness of OsCESA9 mutant with W481C and P482S substitutions in the P-CR domain compared with that of WT (Li et al., 2017). According to the previous report, RNAi-mediated knockdown of PtcCESA4 gene expression caused a significant decrease of about 40% in the cell wall thickness of fiber cells (Abbas et al., 2020).

The rate of cell wall regeneration surrounding the mesophyll protoplasts of T0 mutants

The cell wall regeneration and cell division rates of the mesophyll protoplasts from WT and three T0 mutants were investigated by inducing cell wall regeneration in the CIM medium for 72 h. The FE-SEM images during 72-h cell wall regeneration of the mesophyll protoplast from WT and three T0 mutants were illustrated in Figure 4. The mesophyll protoplasts of L3 and WT have a spherical shape with 2880.46 ± 0.003 nm and 2119.67 ± 0.02 nm in diameter, respectively. However, the L1 and L2 T0 mutants contain cylindrical shape mesophyll protoplast with 1772.24 ± 0.05 nm and 2016.45 ± 0.002 nm in diameter, respectively.

In agreement with the morphophysiological analysis results, the significant difference was not observed in the initiation rate of cell wall deposition surrounding the plasma membrane of the mesophyll protoplasts from L3 T0 mutant compared with WT. In comparison with WT, the cell wall regeneration of the mesophyll protoplasts of L1 T0 mutant was initiated after 24 h of callus induction, which refers to high cell wall regeneration rates in the plant cell and plant growth. However, the cell wall regeneration rate of L2 protoplasts was significantly lower than that of the WT (Figure 4). According to the binding affinity analysis results of CESA4 mutants (Table S3), we found a strong association between CESA4 homo/heterodimerization capacity and cell wall regeneration rate of mesophyll protoplasts. Our results reveal that low binding affinity in the CESA4 subunit due to the double amino acid substitutions of W436Q and P437S probably causes a 2-fold increase in cell wall regeneration rate of mesophyll protoplast from L1 T0 mutant. In contrast, strong protein-protein interaction between the mutated CESA4 subunit with W436 deletion and other SCW-related subunits could significantly reduce cell wall regeneration rate in L2 T0 mutant poplar.

Moreover, previous studies have been reported a strong correlation between cell wall deposition surrounding the plasma membrane and cell division in plants (Grout, 1975; Schilde-Rentschler, 1977; Suzuki et al., 1998; Tagawa et al., 2019). Suzuki et al. (1998) suggested that the thin cell wall regeneration is a prerequisite cytokinesis process and cell division in P. alba L. (Suzuki et al., 1998). In agreement with previous reports, our findings reveal that either increase or decrease in the rate of the cell wall deposition in mesophyll protoplasts could cause significant alterations in cell division followed by plant growth in the GE poplars.

Lignocellulose composition changes in T0 mutants

The effects of alteration in PaCESA4 dimerization capacity on wood compositions were evaluated by measuring the lignocellulose composition in the three T0 mutants (Table 2). The α-cellulose contents of L1 and L3 were 67% and 34.77% less than that of the WT. The L1 and L3 T0 mutants showed a 6% increase in klonas and acid-soluble lignin contents. The reduction of the α-cellulose contents also significantly altered hemicellulose contents with a remarkable increase of about 2.45-fold (L1) and 75% (L3) compared with the WT. Furthermore, the low cellulose content led high water and extractive oil contents in the T0 mutants. However, the L2 T0 mutant showed ~33% increase in the α-cellulose content and a decrease in hemicellulose and extractive contents. There are no obvious differences in the lignin and water content between the WT and L2 T0 mutant. In agreement with our results, the measurement of cell wall composition in OsCESA9W481C_P482S mutant rice showed an 18% reduction in cellulose level with a 16% increase in hemicellulose content. However, the lignin content was almost similar to the WT rice (Li et al., 2017). In contrast, the RNAi-mediated knock-downs of PtcCESA4/7/8 genes caused a significant decrease in cellulose
FE-SEM microscopy of cellulose fibrils in T0 mutants

The FE-SEM microscopy analysis showed a significant variation in cellulose size and thickness between T0 mutant and WT plants (Table S4). The length (mean ± SE of 290.12 ± 2.04 μm) and thickness (mean ± SE of 7.55 ± 0.09 μm) of the cellulose fibrils in the L1 T0 mutant (Figure 5b) were ~40% less than that of WT (Figure 5a). In contrast, the cellulose fibrils of the L2 T0 mutant (Figure 5c) showed a 17.43% and 53.94% increase in length and thickness compared with WT, respectively (Figure 5a). In the L3 T0 mutant (Figure 5d), a significant decrease of about 27.3% and 17.47% were observed in the length (mean ± SE of 338.08 ± 2.46 μm) and thickness (mean ± SE of 10.25 ± 0.06 μm) of the cellulose fibrils compared with WT (Figure 5a), respectively.

CRI of CMFs in the T0 mutants

The semi-crystalline nature of cellulose determines the mechanical and thermal properties of cellulose microfibrils (Chen et al., 2011). Here, to investigate crystalline morphology and behavior of cellulose fibers, X-ray diffractometry (XRD) was performed on the α-cellulose fibrils extracted from the WT and the three T0 mutant poplars (Figure 5e). The diffractograms showed sharp peaks around 2θ = 16° and 22.6°, which represent amorphous and crystalline regions of cellulose microfibrils, respectively. Compared with WT, the L1 and L3 T0 mutants showed a decrease of 25.7% and 12.71% in cellulose CrI, respectively. However, an 8.23% increase was observed in the cellulose CRI of the L2 T0 mutant. In agreement with our results for the L1 T0 mutant, Li et al. (2017) showed a 36% decrease in CRI of cellulose.
microfibrils from the OsCESA9\textsuperscript{W481C_P482S} mutant rice (Li et al., 2017). However, the crystallinity of cellulose was not changed in RNAi-mediated knockdown or the CRISPR/Cas9 T0 mutants. The digital images were taken by FE-SEM at 200× magnifications. Arrows show the regenerated CMFs deposited from PMs. Bars indicate 0.5–1 μm.

Figure 4 The FE-SEM images of the cell wall regeneration in the mesophyll protoplasts from the fourth leaf of regenerated WT, the PalCes\textsuperscript{A4W436Q_P437S} (L1), the PalCes\textsuperscript{A4W436del_P437del} (L2), and the PalCes\textsuperscript{A4W436del_P437del} (L3) T0 mutants during 72-h regeneration treatments. The analysis was performed using randomly selected five digital LM and FE-SEM images of the wild-type and the CRISPR/Cas9 T0 mutants. The digital images were taken by FE-SEM at 30 000–50 000x magnifications. Arrows show the regenerated CMFs deposited from PMs. SE of three biological replicates. The bars represent SE.

| Plants | Dry-Fresh weight ratio (w/w) | Water content (w/w) | Extractive content (w/w) | Hemicellulose (w/w) | α-Cellulose (w/w) | Total lignin (w/w) | Klason lignin (w/w) | Acid-soluble lignin (w/w) |
|--------|-----------------------------|---------------------|--------------------------|-------------------|----------------|----------------|----------------|---------------------|
| WT     | 0.16 ± 0.01a                | 84.13 ± 0.59c       | 4.44 ± 0.05c             | 21.19 ± 0.52c     | 51.73 ± 0.20b | 22.31 ± 0.18b | 19.30 ± 0.16b | 3.01 ± 0.02a       |
| L1     | 0.082 ± 0.003c              | 91.83 ± 0.34a       | 7.37 ± 0.078a            | 51.92 ± 0.19a     | 17.05 ± 0.08d | 23.65 ± 0.19a | 20.71 ± 0.17a | 2.94 ± 0.02b       |
| L2     | 0.16 ± 0.002a               | 83.9 ± 0.21c        | 3.83 ± 0.04d             | 5.39 ± 0.39d      | 68.8 ± 0.27a  | 21.97 ± 0.18b | 18.41 ± 0.15b | 3.56 ± 0.03a       |
| L3     | 0.095 ± 0.003b              | 90.53 ± 0.28b       | 5.97 ± 0.06b             | 36.7 ± 0.25b      | 33.74 ± 0.13c | 23.58 ± 0.19a | 20.65 ± 0.17a | 2.93 ± 0.02b       |

Table 2 Lignocellulose composition in T0 mutants compared with WT poplar

DP of CMFs in The T0 mutants

The crystallinity level of cellulose microfibrils has a strong correlation with its DP (Li et al., 2017; Zhang et al., 2013). Therefore, we focused on examining the cellulose DP of the T0 mutants compared with WT using gel permeation chromatography (GPC) analysis (Figure 5f-h). The number-average DP (DP\textsubscript{n}) and weight-average DP (DP\textsubscript{w}) of cellulose from the WT were respectively in the range of 1011 and 4547 glucose as described by Yoo et al. (2017). Compared with WT, the L1 T0 mutant showed a higher DP\textsubscript{n} and DP\textsubscript{w} of cellulose. The L3 T0 mutant showed only a significant difference in DP\textsubscript{w} with a mean of 4644 units compared with WT. The L2 T0 mutant showed a remarkable increase in the DP\textsubscript{n} (20%) and DP\textsubscript{w} (12%) of cellulose (Figures 5f and g). However, Li et al. (2017) reported a significant decrease in DP of cellulose microfibrils from OsCESA9\textsuperscript{W481C_P482S} mutant rice (Li et al., 2017). The high polydispersity index (PDI) indicates a high size variation in cellulose microfibrils in L1 and the L3 T0 mutants compared with WT as described by (Gilbert et al., 2009). In the L2 T0 mutant, we observed a significant reduction in the PDI due to low size variation in cellulose microfibrils (Figure 5h).

Improved biomass recalcitrance and high Saccharification efficiency in PalCes\textsuperscript{A4W436Q_P437S} and PalCes\textsuperscript{A4W436del_P437del} T0 mutants

The lignocellulose composition, high cellulose DP and CrI could increase the biomass recalcitrance and hinder saccharification efficiency (De Meester et al., 2020; Himmel et al., 2007; Hori et al., 2020; Hu et al., 2018a; Hu et al., 2018b; Jang et al., 2021; Martarello et al., 2021; Speicher et al., 2018). Here, the saccharification potential of WT and three T0 mutant plants were further investigated based on cellulose-to-glucose conversion efficiency under the limited saccharification conditions, including acidic (1 M of HCl, 80 °C, 2 h), alkaline (62.5 mM of NaOH, 90 °C, 3 h), and no pretreatment. As the result, the saccharification efficiencies of L1 and L3 T0 mutants were ~1.5-fold higher than that of
WT in all three pretreatments. The low CrI of cellulose and high hemicellulose content are the most important factors that influence the saccharification efficiency of these T0 mutants as described by (Li et al., 2013a, 2017; Xu et al., 2012). In the L2 T0 mutant, the significant decrease in cellulose to glucose conversion yield was observed probably due to high cellulose crystallinity and DP (Figure 5). In agreement with Li et al. (2017), our findings reveal that alteration of CESA4 dimerization capacity by mutagenesis in the binding site of the P-CR domain could improve cellulose recalcitrance and saccharification yield for bioethanol production.

In summary, the P-CR domain of the PalCESA4 subunit contains a specific protein–protein binding site that is potentially involved in CESA4-CESA8 heterodimerization. The targeted mutagenesis in the binding site could potentially alter CESA4 dimerization capacity in the formation of the SCW-related CSC complex. The amino acid substitutions of W436Q and P437S residues in the CESA4 P-CR domain cause a reduction in the binding affinity of CESA4 in the formation of homo/heterodimers, resulting in decreased cellulose crystallinity, 67% lower cellulose content, high hemicellulose content, high PDI, and improved saccharification efficiency. In comparison, the heterozygous deletion of W436 and P437 residues in the P-CR domain of the CESA4 subunit indicates similar effects on lignocellulose composition, physicochemical properties of cellulose, and saccharification efficiency. However, the influence of this type of mutation was less than that of the amino acid substitution of W436Q and P437S residues in the P-CR domain. In both T0 mutants, we observed positive morphophysiological and wood anatomical changes such as high growth rate and biomass production with thinner cell walls in the vessel and the fiber cells. Due to the high binding affinity of the CESA4 subunit, the heterozygous PalCESA4(436/437del) T0 mutant showed a low plant growth rate, thick cell wall, high cellulose content with high DP (12–20%), and CrI that negatively affected the saccharification yield. It could be concluded that the P-CR domain of the CESA4 subunit could be considered as the potential factor to control the secondary cell wall architecture, lignocellulose composition, cellulose DP, CrI of cellulose, and plant morphological and physiological properties in white poplar. For future perspective, we strongly recommended further biochemical and functional validations in the highly potent binding site of the P-CR domain from CESA subunits in Populus species. Identification of CRISPR/Cas9-mediated CESA4 mutants with amino acid substitution and/or indel mutation in the P-CR domain-binding site could provide an opportunity to obtain GE P. alba L. with biophysically interested cellulose microfibrils for use as suitable biomaterials in cellulose-based industries.

Methods

Phylogenetic analysis

The total number of 80 amino acid sequences of CESA genes from Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa), maize (Zea mays), canola/rapeseed (Brassica rapa and Brassica napus), eastern balsam-poplar (Populus trichocarpa), and cotton (Gossypium hirsutum) were obtained from TAIR database (http://www.arabidopsis.org), Rice Annotation Project database (RAP-db) (https://rapdb.dna.afrc.go.jp), PopGenIE v.3.0 (http://www.popgenie.org), and Phytozone v.12.1.6 (https://phytozone.jgi.doe.gov/pz/portal.html) as the orthologous organs. The predicted amino acid sequence data of CESA genes from P. alba L. were retrieved from NCBI Populus alba genome annotation database Release 100 using BLASTp tool at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). CLUSTALW program was used to carry out multiple sequence alignments (MSA) among all CESA query sequences at the amino acid level (Thompson et al., 1994). The phylogenetic tree of the CESA protein family was constructed by the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) under the Jones-Taylor-Thornton + Gamma distribution of 0.63 (JTT+G) (Jones et al., 1992) using MEGA (Kumar et al., 2018).

Protein–protein interaction and binding site prediction in CESA dimers

The tertiary structures of ZFBD and CCD domains of PalCESA4/7 (a/b)/8(a/b) were predicted using the homology-based modeling method (Method S1). The binding affinity change in Gibbs free energy (ΔΔG) of the CESA homodimer and heterodimers in ZFBD and catalytic domain regions were determined using a sequence-based method of PPA-Pred2 tool (https://www.itran.ac.in/bioinfo/PPA_Pred/prediction.html); Yugandhar and Gromiha, 2014, 2015) and structure-based method of ClusPro 2.0 server available at https://cluspro.bu.edu/ for proper interactions (Comeau et al., 2004a, 2004b; Kozakov et al., 2006, 2013) and PRODIGY (https://wennmr.science.uu.nl/prodigy; Vangone and Bonvin, 2015; Xue et al., 2016). The binding site prediction of CESAs was performed using Protein–protein interaction Sites prediction server (PSIVER) (https://mizuguchilab.org/PSIVER; Murakami and Mizuguchi, 2010), Non-Partner-Specific HomPPI (NPS-HomPPI) (http://aialab-projects2.ist.psu.edu/NPSHOMPPI); Xue et al., 2011), and FTSite server from Boston University (available online at http://ftsite.bu.edu/). The protein–protein interactions (PPI) of the selected CESAs were confirmed using ClusPro 2.0 (https://cluspro.bu.edu/home.php) and ZDOCK ver.3.0.2 (http://zdock.umassmed.edu; Pierce et al., 2014).

Plant material and growth condition

A white poplar (Populus alba L.) plant from the Botanical Research Garden of the University of Tabriz in Tabriz, Iran (38°03’30.4″N latitude and 46°20’07.8″E longitude with an average rainfall of 330 mm) was used as a donor plant for experimental analysis. The sterile transverse thin cell layer (tTCL) explants (0.3-mm thickness and 0.5 mm in diameter) from pulvini of 20th–7th petioles were used for Agrobacterium-mediated transformation (Method S2).

sgRNA design

The CRISPRdirect (https://crispr.dbcls.jp/; Naito et al., 2015), CHOPCHOP (https://chopchop.cbu.ibn.net; Labun et al., 2019), and CRISPOR (http://crispor.tefor.net) web tools (Concordet and Haeussler, 2018) were used to identify PalCESA4-binding site-specific synthetic guide RNA (sgRNA) in query sequence of PalCESA4 gene (XM_035060298.1). The sequence data of sgRNA seed region +NGG PAM motif (underlined letters) follows as 5'-TGGATGTTTCCAGGCACTTG-GG-3'. The specific off-target mutation analysis was described in Method S3 in detail.

Construction of CRISPR/Cas9 expression vector

The Escherichia coli strain DH5α (Invitrogen, USA) and Agrobacterium tumefaciens strain LBA4404 harboring the disarmed low virulent helper plasmid pTiACh5 in the ACh5 chromosomal background (Hoekema et al., 1983; Ooms et al., 1982) were used as host strains for bacterial and plant genetic transformation approaches, respectively. The pUC19 cloning vector (CAT. No. SD0061, Thermo Scientific™) and pFGC-pcoCas9 binary vector

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(Addgene plasmid #52256) were employed (Li et al., 2013b, 2015) as the plasmid backbones. The crRNA-free sgRNA expression cassette comprising of AU6-1 promoter (305 bp long), tracrRNA (Scaffold region with 76 bp long), and poly-dT (7 bp in length) terminator was synthesized by General Biosystems (Anhui, Co. (General BIOL®, Chuzhou, Anhui, P.R China). The CESA4-specific crRNA was assembled into the crRNA-free sgRNA expression cassette using the Splicing by Overlap-Extension PCR (SOEing PCR) method (Horton et al., 1989). After sequencing analysis, the CESA4-specific sgRNA expression cassette (449 bp) was subcloned into the pFGC-pcoCas9 binary vector (Figure S25). The recombinant plasmid was transformed into Agrobacterium tumefaciens strain LBA4404 by electroporation method at 2500 volts (Bio-Rad, Micropluser, Hercules, CA; Lin, 1995; Lurquin, 1997; Method S4).

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation of pulvini-tTCL explants from white poplar was performed as described in Method S5.

Screening of transgenic plants

The total genomic DNA (gDNA) was extracted from leaves of the WT and 47 independent T0 plants using the Cetyl Trimethyl Ammonium Bromide (CTAB) reagent method (Doyle and Doyle, 1987). The integration of the transgene in the genome of poplar plants was confirmed by PCR analysis using sgRNA expression cassette, Cas9, and BAR gene-specific primer sets (Table S5, Method S6).

Genotyping of T0 plants

The gDNA from WT and the total 44 number of the PCR-positive T0 poplars were used to visualize targeted-mutagenesis using heteroduplex genotyping (Guo et al., 2018), mutation sites-based specific primers PCR (MSBSP-PCR) amplification (Bhattacharya and Van Meir, 2019), and Sanger sequencing methods (Method S7).

Prediction of binding affinity in The CESA4-targeted T0 mutants

The binding affinity change in Gibbs free energy (∆∆G) of the CESA homodimer and heterodimers in ZFBD and catalytic domain regions were determined using a sequence-based method of PPA-Pred2 tool (https://www.iitm.ac.in/bioinfo/PPA_Pred/prediction.html) (Yugandhar and Gromiha, 2014, 2015) and structure-based method of ClusPro 2.0 server available at https://cluspro.bu.edu/home.php for proper interaction (Comeau et al., 2004a, 2004b; Kozakov et al., 2006, 2013) and PRODIGY (https://wenmr.science.uu.nl/prodigy) (Vangone and Bonvin, 2015; Xue et al., 2016).

Plant morphological measurements

After screening of CRISPR/Cas9 T0 mutants, we generated at least 20 clones from viable and healthy T0 mutants, including WT, L1, L2, and L3 and 4–5 clones from dwarf and viable mutants using the node culture method (Method S8). The propagations of healthy and viable T0 mutants were repeated three times to obtain a sufficient sample for further downstream phenotyping analysis. The morphological properties, including the shoot length (SH), internode length (IL), the stem base (trunk) diameter (TD), root length (RL), number of nodes (NN), leaves (NL), and roots (NR), the total root-shoot ratio biomass (BR), and Ploastochron index (P.I.) were measured in 4-months-old in vitro regenerated WT and T0 mutant plants. The P.I. was calculated during 20 days as described by Erickson and Michelini (1957). Each experiment was performed in three biological replicates (clones).

Chlorophyll content

Total chlorophyll was extracted from the 4th leaf close to the apical node in 4-months-old WT and T0 mutants using the dimethylsulfoxide (DMSO) method (Hiscox and Israelstam, 1979). The absorbances of the extracts were spectrophotometrically measured at 647 and 663 nm wavelengths, which were measured in three biological replicates (clones). Each biological replicates was run in three technical replicates. The total chlorophyll, chlorophyll a, and b contents were calculated as previously described by Lichtenhaler (1987).

Microscopy analysis of cell wall regeneration in leaf mesophyll protoplasts

The mesophyll protoplasts of the fully expanded leaves from the 4th node close to the apical buds of control and three T0 mutants were isolated using Cellulase Onozuka RS (CAS. No. 9012-54-8, Duchefa Biochemie, Netherlands) and Macerozyme (CAS. No. 9032-75-1, Duchefa Biochemie, Netherlands) as described by Guo et al. (2012). Immediately, the protoplasts were cultured on callus induction medium (CIM) (NH4NO3-free modified liquid MS medium supplemented with TDZ (0.01 mg/L), IBA (0.2 mg/L), B5A 1 g/L, glucose monophosphate 3.4%, CaCl2 0.67 g/L, Casein Hydrolysate 0.5 g/L and pH 5.6) at a cell density of 1–5 x 105 cells/mL for 0, 24, 48, and 72 h. The cell wall regeneration rate in leaf mesophyll protoplasts from WT and three T0 mutants were analyzed using Field Emission Scanning Electron microscopy (FE-SEM) (Method S9).

Wood anatomy measurements

For light microscopy (LM), the 20-µm-thick cross-sections from the 30th internode (1 cm in length) from WT and three T0 mutants were stained with Safranin 0.5% (w/v) and analyzed using a light microscope (BX41, Olympus, Tokyo, Japan) equipped with a digital camera (DPT2, Olympus) at ×100 and ×400 magnification. The fixation, embedding, and sectioning of tissues were described in Method S10. From each slide, five digital images were selected randomly along the radius at ×400 magnification for assessment of vessel density, number of fiber cells, number of rays, vessel lumen area fraction (%), fiber lumen area fraction (%), cell wall area fraction (%), and ray area fraction (%). The 8-µm-thick internodal stem sections were prepared for FE-SEM microscopy (Method S10). From each specimen, four areas were randomly selected and at least 100 cells were considered for measurements using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring).

Lignocellulose composition analysis

The 0.5 g fresh stem segment of the 15th internode to the trunk from WT and T0 mutant plants was air-dried and fine powdered according to TAPPI T257 cm-02 standard protocol. The extractive contents of the wood sample were measured using Soxhlet apparatus with a 2 : 1 (w/v) mixture of toluidine: ethanol (TAPPI T204 cm-07). To remove lignin, the extractive-free samples were treated with acidified sodium chloride (NaClO2) (0.6 g/g, pH = 4.0) at 75 ± 2 °C for three times about 2 h each and neutralized using sterile deionized water (Abe and Yano, 2009). The Klasson and acid-soluble lignin contents were measured as described by Li et al. (2011). The dry weight difference was considered as total dry matter.
lignin content (TAPPi T222 om-02). The hemicellulose was removed from holocellulose content using KOH 6%(w/v) described by Abe and Yano (2009). The hemicellulose and cellulose contents were determined according to standards of ASTM D 1104-56 and TAPPi T203 cm-99, respectively. All experiments and measurements were conducted in three technical replicates.

**FE-SEM microscopy of cellulose fibrils**

The size and thickness of cellulose fibrils from WT and T0 mutant plants were measured using FE-SEM microscopy imaged by FE-SEM microscope (TESCAN MIRA3 FEG-SEM, Kohoutovice, Czech Republic) at x200–x300 magnifications at 15 kV accelerating voltage and ~2 nA beam current. From each specimen, 50 fibers were randomly selected for measurements using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring).

**Cellulose crystallinity index (CrI)**

The XRD patterns of chemical-purified α-cellulose fibers from WT and T0 mutant plants were measured using an XRD diffractometer (D8-Advance, Brucker, Germany). The system operated at 40 kV and 30 mA, equipped with CuKα radiation at a wavelength of 1.54056 Å and a nickel monochromator filtering wave. The scattering angle was in the range of 2θ = 5–40° with a scanning rate of 5°/min at 25 °C. The crystallinity index (CrI) was calculated as described by Oun and Rhim (2016), Park et al. (2010).

**DP analysis of CMFs by GPC**

For GPC analysis of CMFs from WT and T0 plants, the 10 mg cellulose samples were tricarbanilated as described by Hubbell and Ragauskas (2010). The samples were dissolved in Tetra Hydro Furane (THF) (1 mg/mL) and filtered by a 0.45 µm nylon filter. The molecular weight distributions of the cellulose tricarbanilated samples were then analyzed on a PSS-Polymer Standards Service (Warwick, RI) Waters Breeze 2 HPLC system featuring Agilent HPLC 1200 components equipped with one with an online 5USORi GPC column, Agilent refractive index (RI) detector, and Agilent UV detector (270 nm) using THF as the mobile phase (1.0 mL/min) with injection volumes of 50 µL. A calibration curve was constructed based on eight narrow polystyrene standards ranging in molecular weight from 1.5 × 10^3 to 3.6 × 10^6 g/mol. DPw and DPn were calculated by dividing Mn and Mw by 519 g/mol (the molecular weight of the tricarbanilated cellulose repeat unit), respectively. The PDI was calculated based on Mn/Mw ratio. All values for molecular weight and degree of polymerization were the average of duplicate samples for each type of cellulose.

**Saccharification efficiency analysis**

The 10 mg dried and fine-powdered wood samples from WT and three T0 mutant plants were pretreated with hot water (no pretreatment), NaOH 0.25% (w/v) (Alkaline pretreatment) or HCl 3.7% (w/v) (Acidic pretreatment) and finally washed with ethanol 70% and acetone solutions. After pretreatment, the samples were saccharified with the enzymatic mixture of cellulase (CAS No. 9012-54-8, Sigma-Aldrich) and β-glucosidase (CAS No. 9001-22-3, Sigma-Aldrich) in the ratio of 5 : 3 at 50 °C for 72 h. To determine the concentration of glucose units in the samples, the glucose oxidase-peroxidase (GOD-POD) reaction was used and the absorbance was spectrophotometrically measured at a wavelength of 405 nm as described by De Meester et al. (2020).

**Statistical analysis**

The statistical analysis was performed using Microsoft Excel software 2019 software (Microsoft, Redmond, WA) and SPSS ver.16.0 (SPSS Inc., Chicago, IL). The results were analyzed using one-way ANOVA, followed by the post hoc Tukey’s test for multiple comparisons in all experiments. The level of significance was set at 5%.

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**Conflict of interest statement**

The authors declare that they have no conflict of interest.

**Author contributions**

S. N., Methodology, Validation, Investigation, Formal analysis, Writing-Original Draft, Writing-Review & Editing, Visualization. B. B. K., Conceptualization, Methodology, Validation, Resources, Writing-Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. A. A., Validation, Resources, Writing-Review & Editing and Supervision, Funding acquisition. N. M., Writing-Review & Editing, Resources.

**Data availability statement**

The nucleotide and protein sequence data of recombinant expression vector of pFGC-pcoCas9:sgRNA cassette is available in GenBank at NCBI under the accession number of MZ466588.

**References**

Abbas, M., Peszlen, I., Shi, R., Kim, H., Katahira, R., Kafle, K., Xiang, Z. et al. (2020) Involvement of CesA4, CesA7-A/B and CesAB-A/B in secondary wall formation in Populus trichocarpa wood. Tree Physiol. 40(1), 73–89.

Aber, K. and Yano, H. (2009) Comparison of the characteristics of cellulose microfibril aggregates of wood, rice straw and potato tuber. Cellulose, 16(6), 1017–1023.

Arioli, T., Eng, L., Betzner, A.S., Burn, J., Wittke, W., Herth, W., Camilleri, C. et al. (1998) Molecular analysis of cellulose biosynthesis in Arabidopsis. Science, 279(5351), 717–720.

Avila, J.R., Lee, J.S. and Torii, K.U. (2015) Co-Immunoprecipitation of membrane-bound receptors. Arabidopsis Book, 13, e0180. https://doi.org/10.1199/tab.0180.

Azez, A. and Busov, V. (2021) CRISPR/Cas9-mediated single and biallelic knockout of poplar STERILE APETALA (PopSAP) leads to complete reproductive sterility. Plant Biotechnol. J. 19(1), 23–25.

Bao, L., Redondo, C., Findlay, J.B.C., Walker, J.H. and Ponnambalam, S. (2009) Deciphering soluble and membrane protein function using yeast systems

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Feuerstein, R., Wang, X., Song, D., Cooke, N.E. and Liebhaber, S.A. (1994) The LiMVdouble zinc-finger motif functions as a protein dimerization domain. Proc. Natl Acad. Sci. USA, 91(22), 10655–10659. https://doi.org/10.1073/pnas.91.22.10655

Gilbert, R., Hess, M., Jenkins, A., Jones, R., Kratochvil, P. and Stepto, R. (2009) Dispersity in polymer science. Pure Appl. Chem. 81(2), 351–353.

Grout, B.W. (1975) Cellulose microfibril deposition at the plasmamembrane surface of regenerating tobacco mesophyll protoplasts: a deep-etch study. Planta, 123(3), 275–282.

Guo, J., Li, K., Jin, L., Xu, R., Miao, K., Yang, F., Qi, C. et al. (2018) A simple and cost-effective method for screening of CRISPR/Cas9-induced homozygous/ biallelic mutants. Plant Methods, 14(1), 1–10.

Guo, J., Morrell-Falvey, J.L., Labbé, J.L., Muchero, W., Kalluri, U.C., Tuskan, G.A. and Chen, J.-G. (2012) Highly efficient isolation of Populus mesophyll protoplasts and its application in transient expression assays. PLoS One, 7(9), e49908.

Haigler, C.H., Grimison, M.J., Gervais, J., Le Moigne, H., Höfte, H., Monasse, B. and Navard, P. (2014) Molecular modeling and imaging of initial stages of cellulose fibril assembly: evidence for a disordered intermediate state. PLoS One, 9(6), e93981.

Hill, J.L., Hammoudi, M.B. and Tien, M. (2014) The Arabidopsis cellulose synthase complex: a proposed hexamer of CESAs trimers in an equivolular stoichiometry. Plant Cell, 26(12), 4834–4842.

Hilton, I.B. and Gersbach, C.A. (2015) Enabling functional genomics with genome engineering. Genome Res. 25(10), 1442–1455.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W. and Foust, T.D. (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science, 315(5813), 804–807.

Hiscox, J. and Israelstam, R. (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57(12), 1332–1334.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J. and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir-and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature, 303(5913), 179–180.

Hori, C., Takata, N., Lam, P.Y., Tobimatsu, Y., Naganos, S., Mortimer, J.C. and Cullen, D. (2020) Identifying transcription factors that reduce wood recalcitrance and improve enzymic degradation of xylem cell wall in Populus. Sci. Rep. 10(1), 1–13.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene, 77(1), 61–68.

Hu, H., Zhang, R., Feng, S., Wang, W., Wang, Y., Fan, C., Li, Y. and Li, Y. (2018a) Three AtCESA6-like members enhance biomass production by distinctively promoting cell growth in Arabidopsis. Plant Biotechnol. J. 16(5), 976–988.

Hu, H., Zhang, R., Tao, Z., Li, X., Li, Y., Huang, J., Li, X. et al. (2018b) Cellulose synthase mutants distinctively affect cell growth and cell wall integrity for plant biomass production in Arabidopsis. Plant Cell Physiol. 59(6), 1144–1157.

Huang, J., Xia, T., Li, G., Li, X., Li, Y., Wang, Y., Wang, Y. and Yang, F. (2019) Overproduction of native endo-β-1,4-glucanases leads to largely enhanced biomass saccharification and bioethanol production by specific modification of cellulose features in transgenic rice. Biotechnol. Biofuels, 12(1), 1–15.

Hubbell, C.A. and Ragauskas, A.J. (2010) Effect of acid-chlorite delignification on cellulose degree of polymerization. Biores. Technol. 101(19), 7410–7415.

Jang, H.-A., Bae, E.-K., Kim, M.-H., Park, S.-J., Park, H.-J. and Kim, H.-Y. (2011) Perturbation of wood cellulose synthesis causes wood disorganization and dynamics. In Carpita, N., Tierney, M. and Campbell, M. (Eds.) (2001) Molecular biology of plant cell walls: chemistry, structure and function of plant cell walls. New York: Walter de Gruyter.

Joshi, C.P., Thammannagowda, S., Fujino, T., Gou, J.-Q., Avci, U., Haigler, C.H., McDonnell, L.M. et al. (2011) Perturbation of cellulose synthesis causes pleiotropic effects in transgenic aspen. Molecular Plant, 4(2), 331–345.

Kazokas, D., Beglov, D., Bohnhud, T., Motta, S.E., Xia, B., Hall, D.R. and Vajda, S. (2013) How good is automated protein docking? Proteins: Struct., Funct., Bioinf. 81(12), 2159–2166.

Kazokas, D., Brenke, R., Comeau, S.R. and Vajda, S. (2006) PIPER: an FT-based protein docking program with pairwise potentials. Proteins: Struct., Funct., Bioinf. 65(2), 392–406.
Kumar, M., Thammannagowda, S., Bulone, V., Chiang, V., Han, K.-H., Joshi, C.P., Mansfield, S.D. et al. (2009) An update on the nomenclature for the cellulose synthase genes in Populus. Trends Plant Sci. 14(5), 248–254.

Kumar, M. and Turner, S. (2015) Plant cellulose synthesis: CESA proteins crossing kingdoms. Phytochemistry, 112, 91–99.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35(6), 1547–1549.

Kurek, I., Kawagoe, Y., Jacob-Wilk, D., Doblin, M. and Delmer, D. (2022) CRISPRDirect: softwares for designing CRISPR/Cas guide RNA with reduced off-target sites. Bioinformatics, 31(7), 1120–1123.

Nawaz, M.A., Lin, X., Chan, T.-F., Imtiaz, M., Rehman, H.M., Ali, M.A., Baloch, F.S. et al. (2019) Characterization of cellulose synthase A (CESA) gene family in eudicots. Biochem. Genet. 57(2), 248–272.

Nixon, B.T., Mansouri, K., Singh, A., Du, J., Davis, J.K., Lee, J.-G., Slabbaert, E. et al. (2016) Comparative structural and computational analysis supports eighteen cellulose synthases in the plant cellulose synthesis complex. Sci. Rep. 6(1), 1–14.

Ooms, G., Hooykaas, P.J., Van Veen, R.J., Van Beelen, P., Regensburg-Tuink, T.J. and Slippers, R.A. (1982) Octopine T-plasmid deletion mutants of Agrobacterium tumefaciens with emphasis on the right side of the T-region. Plasmid, 7(1), 15–29.

Oon, A.A. and Rheim, J.-W. (2016) Isolation of cellulose nanocrystals from grain straws and their use for the preparation of carboxymethyl cellulose-based nanocomposite films. Carbohydr. Polym. 150, 187–200.

Park, S., Baker, J.O., Himmel, M.E., Pariia, P.A. and Johnson, D.K. (2010) Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. Biotechnol. Biofuels, 3(1), 1–10.

Park, S. and Ding, S.Y. (2020) The N-terminal zinc finger of CELLULOSE SYNTHASE6 is critical in defining its functional properties by determining the level of homodimerization in Arabidopsis. Plant J. 103(5), 1826–1838.

Pierce, B.G., Wiehe, K., Hwang, H., Kim, B.-H., Vreven, T. and Weng, Z. (2014) ZDOCK server: interactive docking prediction of protein–protein complexes and symmetric multimers. Bioinformatics, 30(12), 1771–1773.

Porth, I. and El-Kasaby, Y.A. (2015) Using Populus as a lignocellulosic feedstock for bioethanol. Biotechnol. J. 10(4), 510–524.

Purushotham, P., Ho, R. and Zimmer, J. (2020) Architecture of a catalytically active homotrimeric plant cellulose synthase complex. Science, 369(6507), 1089–1094.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Designing CRISPR/Cas guide RNA with reduced off-target sites. Nat. Biotechnol. 31(12), 688–691.

Li, J.-F., Fernie, A.R. and Persson, S. (2016a) Transition of primary to secondary cell wall synthesis in Arabidopsis. Plant Physiol. 171(2), 2281–2308.

Rao, V.S., Srinivas, K., Sujini, G. and Kumar, G. (2014) Protein-protein interaction detection: methods and analysis. Int. J. Proteom. 2014, 1–12.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. Plant Physiol. 124(2), 495–498.

Rushforth, E., Ho, R. and Zimmer, J. (2020) Architecture of a catalytically active homotrimeric plant cellulose synthase complex. Science, 369(6507), 1089–1094.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8(11), 2281–2308.

Rao, V.S., Srinivas, K., Sujini, G. and Kumar, G. (2014) Protein-protein interaction detection: methods and analysis. Int. J. Proteom. 2014, 1–12.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. Plant Physiol. 124(2), 495–498.

Rushforth, E., Ho, R. and Zimmer, J. (2020) Architecture of a catalytically active homotrimeric plant cellulose synthase complex. Science, 369(6507), 1089–1094.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8(11), 2281–2308.

Rao, V.S., Srinivas, K., Sujini, G. and Kumar, G. (2014) Protein-protein interaction detection: methods and analysis. Int. J. Proteom. 2014, 1–12.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. Plant Physiol. 124(2), 495–498.

Rushforth, E., Ho, R. and Zimmer, J. (2020) Architecture of a catalytically active homotrimeric plant cellulose synthase complex. Science, 369(6507), 1089–1094.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8(11), 2281–2308.

Rao, V.S., Srinivas, K., Sujini, G. and Kumar, G. (2014) Protein-protein interaction detection: methods and analysis. Int. J. Proteom. 2014, 1–12.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. Plant Physiol. 124(2), 495–498.

Rushforth, E., Ho, R. and Zimmer, J. (2020) Architecture of a catalytically active homotrimeric plant cellulose synthase complex. Science, 369(6507), 1089–1094.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8(11), 2281–2308.

Rao, V.S., Srinivas, K., Sujini, G. and Kumar, G. (2014) Protein-protein interaction detection: methods and analysis. Int. J. Proteom. 2014, 1–12.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. Plant Physiol. 124(2), 495–498.

Rushforth, E., Ho, R. and Zimmer, J. (2020) Architecture of a catalytically active homotrimeric plant cellulose synthase complex. Science, 369(6507), 1089–1094.
Saccharification Improvement via CES4 Engineering

Zangr, N.M. (2012). Characterising the cellulose synthase complexes of cell walls. Thesis, Wageningen University, the Netherlands.

Zhang, W., Yi, Z., Huang, J., Li, F., Hao, B., Li, M., Hong, S. et al. (2013) Three lignocellulose features that distinctively affect biomass enzymatic digestibility under NaOH and H2SO4 pretreatments in Miscanthus. Biores. Technol. 130, 30–37.

Zhang, X., Dominguex, P.G., Kumar, M., Bygdell, J., Miroshnichenko, S., Sundberg, B., Winges, G. et al. (2018) Cellulose synthase stoichiometry in aspen differs from Arabidopsis and Norway spruce. Plant Physiol. 177(3), 1096–1107.

Zhong, R., Morrison, W.H., Freshour, G.D., Hahn, M.G. and Ye, Z.-H. (2003) Expression of a mutant form of cellulose synthase ATCESA7 causes dominant negative effect on cellulose biosynthesis. Plant Physiol. 132(2), 786–795.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic relationship of CES4 proteins from Arabidopsis thaliana (At), Brassica napus (Bn), Brassica rapa (Br), Gossypium hirsutum (Gh), Oryza sativa (Os), Populus trichocarpa (Pt), Populus alba (Pal), and Zea mays (Zm).

Figure S2 Schematic diagrams of TMH domains probability in the amino acid sequence data of PaCESA4/7/8 subunits (A–E).

Figure S3 Schematic cartoon 3D models of zinc-finger and catalytic domains of PaCES4/7/8 proteins.

Figure S4 Ramachandran plots of zinc-finger and catalytic domains of PaCESA4/7/8 proteins.

Figure S5 The 2D topological diagram of a cytosolic region of PaCESA4 enzyme with 552 aa in length generated by Phyre2 software.

Figure S6 PCR analysis of the independently regenerated Basta®-resistant plantlets using BAR gene (550 bp), Cas9 gene (184 bp), and PaCE4-specific sgRNA cassette specific primers to screen the T0 white poplars.

Figure S7 Identification of CRISPR/Cas9-mediated PaCESA4 T0 mutants of P. alba.

Figure S8 Illustration of multiple sequence alignments between wild-type (WT) and the homoygous CRISPR/Cas9-mediated T0 mutants (L1-L47) and their sequence variation in the PaCE4-specific gRNA target region at the amino acid and nucleotide levels.

Figure S9 Illustration of multiple sequence alignments between wild-type (WT) and the heterozygous/biallelic CRISPR/Cas9-mediated T0 mutants (L1-L47) and their sequence variation in the PaCE4-specific gRNA target region at the amino acid and nucleotide levels.

Figure S10 Representative chromatograms showing the sequence analyses of edited sites in 32 T0 mutants (L1-L47) compared with wild-type (WT) P. alba.

Figure S11 The means of shoot height measurements in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants.

Figure S12 The means of trunk diameter measurements in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants.

Figure S13 The means of internodal-stem length in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants.

Figure S14 The means of node number in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants.

Figure S15 The means of leaf number in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants.
Figure S16 The means of fresh: dry shoot biomass ratio in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S17 The means of roots number measurements in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S18 The means of root length measurements in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S19 The means of fresh-dry root biomass ratio in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S20 The means of root: shoot biomass ratio in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S21 The means of total chlorophyll content (mg/g) in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S22 The means of chlorophyll a content (mg/g) in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S23 The means of chlorophyll b content (mg/g) in wild-type and 32 CRISPR/Cas9-mediated T0 white poplar mutants. Figure S24 The P.I. plot for the untransformed wild-type P. alba (WT), T0 mutant of PalCESA4W436Q_P437S (L1), T0 mutant of PalCESA4W436del (L2), and T0 mutant of PalCESA4W436del_P437del (L3). Figure S25 Schematic representation of the CRISPR/Cas9 binary vector of pFGC-pcoCas9 with PalCESA4-specific sgRNA cassette (449 bp long).

Table S1 Binding affinity and molecular docking of the zinc finger and catalytic domains of PalCESA4 homodimer and heterodimers of PalCESA7 and PalCESA8 domains.

Table S2 Mutational status of stable CRISPR/Cas9-mutagenic lines targeting PalCESA4 P-CR domain in P. alba. Table S3 Binding affinity and molecular docking of the homo/heteroduplexes between the mutated PalCESA4 catalytic domain and PalCESA4/7/8 domains from WT and three T0 mutants. Table S4 The mean of length and thickness of CMFs from WT and three T0 mutants including L1-L3. Table S5 The list of primer sets used in the experiments. Method S1 The homology modeling for tertiary structure of PalCESA subunits. Method S2 Surface sterilization of pulvini-tTCL explants. Method S3 Analysis of off-target mutations. Method S4 SOEing-PCR and molecular cloning of CESA4-specific sgRNA expression cassette. Method S5 Agrobacterium-mediated transformation of pulvini-tTCL explants. Method S6 Screening of transgenic poplars using PCR method. Method S7 Genotyping analysis using MSBSP-PCR and heteroduplex genotyping methods. Method S8 Node culturing of the regenerated T0 mutants. Method S9 FE-SEM microscopy of mesophyll protoplasts. Method S10 Fixation, embedding, and sectioning of internodal-stem cross-sections.