

Independent Functional Expression of *Populus trichocarpa* Cellulose Synthase Isoforms CesA1 and CesA8 in *Escherichia coli*

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Abstract

Cellulose synthase (CesA) drives cellulose biosynthesis in plants, yet the isoform-specific roles of CesA subunits in cellulose assembly remain elusive. To dissect their distinct functions, two critical isoforms of PtCesA1 and PtCesA8 from *Populus trichocarpa* were heterologously expressed in *Escherichia coli*.

Both PtCesA1 and PtCesA8 proteins were expressed correctly in *E. coli*. Transmission electron microscopy (TEM) revealed both PtCesA1 and PtCesA8 formed disordered needle-like networks more than vector control. These architectures were validated by ATR-FTIR spectroscopy and cellulase hydrolysis, confirming β -1,4-glucan synthesis distinct from bacterial polysaccharides. Immunofluorescence localized PtCesA1 to linear terminal complexes, whereas PtCesA8 showed disorganized distributions, suggesting divergent assembly mechanisms.

To our knowledge, this study pioneers the independent prokaryotic expression of plant CesA isoforms in *E. coli*, circumventing the cooperative subunit interactions required in native systems. By bridging plant and bacterial cellulose synthesis paradigms, we establish a microbial platform for producing structurally tailored cellulose, advancing both fundamental understanding and bio-inspired materials engineering.

Highlights

We report the first prokaryotic expression of CesA isoforms PtCesA1 and PtCesA8 in .

Both PtCesA1 and PtCesA8 formed disordered needle-like networks.

Both isoforms independently synthesize β -1,4-glucan, confirming their intrinsic cellulose-producing activity.

This work establishes a microbial platform for dissecting plant CesA functions and engineering structurally tailored cellulose, bypassing energy-intensive biomass extraction.

Our findings bridge plant and bacterial cellulose synthesis paradigms, offering insights for sustainable production of bio-inspired materials.

1 Introduction

Cellulose, the most abundant biopolymer on Earth, constitutes 30–50% of the global biomass and is synthesized naturally at an annual rate of 10^{11} – 10^{12} tons[1]. As a linear polymer of β -1, 4-linked D-glucopyranose units[2], it serves the primary structural component of plant cell walls and critical resource for materials such as paper, textiles and bioenergy[3]. Beyond plants, cellulose is also synthesized by bacteria and even some animals[4, 5]. Despite its ubiquity, conventional extraction from lignocellulosic biomass relies on energy-intensive processes (e.g., delignification, bleaching), which generate sulfurous wastewater and greenhouse emissions[6–8]. Researchers have dedicated extensive efforts to developing energy-efficient strategies, including the optimization of solvent systems[9] and

advanced equipment [10], as well as the application of genetic engineering techniques[11] to enhance microbial metabolic pathways. While nanocellulose production via mechanical or chemical "top-down" approaches have advanced, these approaches remain limited by high energy demands and low yields[12] Bacterial cellulose, though lignin free, suffers from scalability challenges and high costs. In contrast, bottom-up biosynthesis strategies-heterologous expression of cellulose synthase A (CesA) subunits in microbial hosts-offer a sustainable route to structurally tailored cellulose[13]. However, existing systems predominantly yield amorphous cellulose or metastable cellulose II[14–17], failing to replicate the native crystalline architecture (cellulose I) produced by plant CesA complexes or bacteria cellulose synthase with other assistant genes[4, 18–21].

In plants, cellulose is synthesized by the Cellulose Synthase Complex (CSC) on the plasma membrane also called terminal complexes (TCs). The CSC is comprised CesA subunits, though the exact stoichiometry remains debated. Recent studies propose a model of 18 CesAs per CSC, based on the trimeric subunit assembly and microfibril dimensions[22]. Quantitative proteomic further reveal that CesA1 dominates primary cell walls, governing polarized cellulose deposition and glucan chain cross-linking, while CesA8 prevails in secondary walls, exhibits higher catalytic activity and direct control over microfibril diameter[23, 24]. Despite these insights, the cooperative mechanisms of CesA isoforms within CSCs and their roles in crystalline cellulose assembly remain unresolved[25]. Prior attempts to express single CesA subunits in heterologous systems (e.g., *Acetobacter*, yeast) generated β -1,4-linked glucan but lacked structural fidelity, with irregular crystallinity and disordered microfibril organization [15].

Here, we report the independent heterologous expression of *Populus trichocarpa* CesA1 (PtCesA1) and CesA8 (PtCesA8) in *Escherichia coli* (*E. coli*). By optimizing protein expression conditions and product purification protocols, β -1,4-glucan were synthesized successfully. Structural characterization using transmission electron microscopy (TEM), ATR-FTIR spectroscopy, and cellulase hydrolysis confirmed cellulose production. Notably, both PtCesA1 and PtCesA8 formed disordered crystalline networks. This work establishes *E. coli* as a tractable platform for dissecting isoform-specific CesA functions and underscores the necessity of subunit cooperativity for native cellulose assembly. Our findings bridge plant and bacterial cellulose synthesis paradigms, offering a foundation for engineering sustainable, bio-inspired materials.

2 Materials and Methods

2.1 Construction of heterologous expression vectors

The cDNA sequences encoding *Populus trichocarpa* cellulose synthase subunits PtCesA1 (GenBank: PtStettler14.18G024900) and PtCesA8 (GenBank: PtStettler14.18G056900) were retrieved from Phytozome v12(<https://phytozome.jgi.doe.gov/pz/portal.html>). These sequences were amplified via restriction enzyme digestion [26] using primers flanked by *Sma* I and *Hind* III restriction sites:

PtCesA1 forward primer 5'-TACCCCGGGATGGAAGCGAATGCTGG-3' and reverse primer 5'-CCAAGCTTCTAGCAATTGACGCCACAT-3';

PtCesA8 forward primer 5'-CCCGGGATGATGGAATCTGGGGCTC-3' and forward primer 5'-TGCATTTCTATAGATTGCTGAAAGCTT-3'.

Amplified fragments were ligated into the pQE-80L vector (Qiagen Inc.), which encodes an N-terminal 6×His-tag for protein purification. Ligation reactions were performed using T4 DNA ligase (New England Biolabs) according to the manufacturer's protocol. The resulting constructs were verified by DNA sequencing (GenScript Nanjing, Jiangsu, China). The agents used without marking are purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2 Bacterial transformation and culture

Recombinant plasmids were transformed into *E. coli* XL1-Blue (SinoMol, Nanjing, China). Bacterial cells were pre-cultured on LB agar plates [10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl (Nanjing Chemical Reagent Co., LTD) 15 g/L agar, 100 µg/mL ampicillin] at 37°C for 12 hours to achieve logarithmic growth phase. For protein expression, pre-cultures were inoculated (2% v/v) into 2× YT medium [10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0] containing 100 µg/mL ampicillin and grown at 37°C (200 rpm) to OD₆₀₀ = 0.5–0.8. Expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 28°C for 2–6 h.

2.3 Production purification

Post-induced cultures were centrifuged (5720 × *g*, 15 min, 4°C) and pellets were resuspended in 2% (w/v) SDS. The mixture was autoclaved at 121°C for 20 min to degrade lipids/nucleic acids, followed by centrifuged at 5423× *g* for 5 min. Pellets were washed thrice with deionized water to remove SDS. Subsequently, the precipitation was treated with 2% (w/v) NaOH (China National Medicines Corporation Ltd.) and autoclave again at 121°C for 20 min. After cooling, the mixture was centrifuged at 5423× *g* for 10 min to remove NaOH. Final pellets were washed thrice (9168× *g* for 10 min).

2.4 Western blot analysis of Cesa protein

Proteins were extracted using membrane and cytosol protein extraction kit (Shanghai Epizyme Biomedical Technology Co., Ltd PC202). Firstly, *Escherichia coli* pellets were collected by centrifugation (4°C, 5,000 ×*g*, 10 min) and resuspended in ice-cold PBS containing 0.2 mM EDTA and 0.1 mM PMSF. Cell lysis was performed by freeze-thaw cycling with lysozyme in a 1:10 (w/v) pellet-to-buffer (AIWB-012) ratio. The lysate was centrifuged (4°C, 8,000 ×*g*, 20 min), and the supernatant was further ultracentrifuged (4°C, 10,000 ×*g*, 60 min). The final pellet was membrane protein and resuspended in minimal ice-cold PBS and stored at – 80°C. Protein concentration was determined by BCA assay a kit (P0010). Serially diluted standards and samples (20 µL each) were mixed with 200 µL BCA working reagent in a 96-well plate, incubated at 37°C for 30 min, and measured at 562 nm using a microplate reader. A standard curve was generated for concentration calculation. For SDS-PAGE all reagent used is from Affinibody LifeScience Co., Ltd, 70 µg of protein per lane was denatured in 5 × SDS loading buffer

(AIWB-0025) at 37°C for 30 min and then resolved on 8% stacking/10% resolving non-stained gels (A kit of one step maker gel Cat#NSF10). Proteins were transferred to PVDF membranes (AIWB-0015P) at 300 mA for 30min using transfer buffer UniTMSDS-PAGE (AFRB-T500) Membranes were blocked with Minute Block (AIWB-004) for 10min at room temperature and subsequently incubated overnight at 4°C with primary antibodies diluted in antibody dilution buffer (AIWB-009). The following antibodies were used: His-Tag Monoclonal Antibody (Cat# AF12309, diluted 1:10,000, RRID: AB_2314622), diluted 1:3,000 and OmpA (35kDa) polyclonal antibody (Abmart Shanghai Co., Ltd. Cat# PH19226, diluted 1:10,000, RRID: AB_3091646) as a loading control. After three times TBST washes, membranes were incubated with HRP-conjugated Goat Anti-Mouse IgG (H + L) secondary antibody (Cat# AF12300M, diluted 1:10,000, RRID: AB_2769851) for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection kit (AIWB-006) and imaged Under darkroom conditions, an X-ray film was carefully overlaid on the membrane and exposed for optimized duration. 10-250kDa marker (AIWB-011plus).

2.5 Microstructure analysis of synthesized cellulose

Purified products were diluted (1:100,000 in pH 3 HCl), deposited on copper grids, stained with 2% (w/v) phosphotungstic acid (pH 7.0) for 30 sec, and imaged on a transmission electron microscope (JEM-1400, JEOL) at 80 kV.

2.6 Chemical groups analysis of synthesized cellulose

The chemical groups of the products were detected by infrared spectroscopy, and the crude products extracted by SDS/NaOH method were tested in liquid mode using a microinfrared rheological combination instrument [MARS60, Thermo Fishe Company, USA]: about 2 μ L of sample suspension was dropped on the test table, and then dried for detection. The measurement wavelength ranges from 500 to 4000cm⁻¹ and the number of scan times is 32.

2.7 Quantitative Determination of β -1,3-Glucan Content

To determine the β -1,3-glucan composition in in vitro synthesized products, fluorometric quantification was performed based on the specific fluorescence emission (λ_{ex} 400 nm) generated by callose-aniline blue complexes. Purified PtCesA1 and PtCesA8 products (~ 0.1 g wet weight each), alongside pQE vector controls, were pretreated with 98% ethanol (v/v) overnight at 4°C. Following ethanol immersion, samples were centrifuged at 10,000 $\times$ g for 10 min (TGL20MW, Hunan Herexi Instrument & Equipment Co., Ltd) to remove soluble contaminants. The resulting pellets were homogenized in 1 mL callose extraction buffer (Suzhou Keming Biotechnology Co., Ltd.) and incubated at 80°C for 20 min to enhance polysaccharide solubility.

β -1,3-Glucan quantification was conducted using a commercial callose assay kit (Suzhou Keming Biotechnology Co., Ltd., Cat# PZZ-1-Y) according to manufacturer protocols. A six-point calibration curve (0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) was established using chromatographically pure callose standards. Fluorescence intensity measurements were acquired with a multimode microplate reader

(Turner Biosystems, TBS-380, USA). All samples and standards were analyzed in triplicate, with background subtraction performed using extraction buffer blanks.

2.8 Integrated Experimental Methodology for β -1,4-Glucan Quantification

Purified PtCesA1, PtCesA8, and pQE control products (~ 0.1 g wet weight) were subdivided into experimental and control aliquots. For reference and comparison, 0.1 g cellulose nanofiber (CNF) treatment were used. Cellulase-treated samples (Celluclast, Novozymes) were hydrolyzed at 50°C for 1 h and residual glucose was measured via anthrone-sulfuric acid assay [27]. Briefly, samples after hydrolysis were combined with AN reagent and heated at 120°C for 30 min to degrade polysaccharides. After washing pellets were reacted with 3 mL anthrone-sulfuric acid reagent under identical heating conditions. Absorbance at 625 nm was measured using a L6 spectrophotometer (Shanghai Yoke Instrument Co., Ltd.), with glucose standards (0.00–0.20 mg/mL) establishing the calibration curve. β -1,4-glucan content was calculated by subtracting control group glucose levels from enzymatically hydrolyzed samples, leveraging the specific interaction between anthrone and glucose dehydration products formed in concentrated sulfuric acid.

2.9 Immunofluorescence Localization of PtCesA Proteins

Immunofluorescence localization of PtCesA1 and PtCesA8 in engineered *Escherichia coli* was performed following established protocols[28]. Briefly, induced cells expressing either PtCesA1 or PtCesA8 were harvested, resuspended in citrate-buffered saline (CBS: 40 mM sodium citrate, pH 5.0, 110 mM NaCl, 2.2 mM KCl), and filtered through a 50- μ m nylon mesh. Cells were fixed in 2% paraformaldehyde (PFA) at 4°C overnight, centrifuged (1,000 $\times$ *g*, 10 min, RT), and resuspended in 0.1 M glycine to quench residual PFA. Aliquots (100 μ L) were deposited onto poly-L-lysine-coated coverslips and sequentially treated with 1 mg/mL lysozyme in TE buffer (100 mM Tris-HCl, pH 6.7, 5 mM EDTA) at 37°C for 1 h and 1% NP-40 in PBS (10 mM phosphate buffer, pH 7.4, 136 mM NaCl, 2.7 mM KCl) at 30°C for 30 min. Following four PBS washes, non-specific sites were blocked with 1% BSA (RT, 1 h), incubated with anti-PtCesA primary antibody (4°C, overnight), and labeled with Alexa Fluor 488-conjugated secondary antibody (5 μ g/mL, 2 h, dark). Coverslips were washed, mounted with SlowFade™ Gold Antifade Mountant, and imaged on a Zeiss LSM 880 confocal microscope using standardized parameters (5% laser power, 800 gain, 1 Airy unit pinhole) to ensure consistency.

3 Results

3.1 Heterologous expression of PtCesA1 or PtCesA8

Western blot analysis demonstrated the successful heterologous expression of PtCesA1 and PtCesA8 in *Escherichia coli*. Induced cultures exhibited distinct protein banding patterns compared to non-induced controls (Fig. 1). Comparative analysis revealed distinct protein banding patterns between induced and non-induced cultures (Fig. 1A). While control samples (empty pQE vector) and uninduced cultures (A1-0,

A8-0) displayed no detectable bands, induction with Isopropyl- β -D-thiogalactopyranoside (IPTG) for 2 hours resulted in the appearance of novel bands in both PtCesA1 (A1-2) and PtCesA8 (A8-2) samples. Specifically, PtCesA8 exhibited a prominent band at 110 kDa, corresponding to the full-length protein, whereas PtCesA1 showed a predominant band at 122 kDa, consistent with its predicted molecular weight. Figure 1B demonstrates successful membrane protein extraction as evidenced by OmpA detection.

Further validation by DIA proteomic analysis (Figure S1) revealed significant enrichment of soluble domain peptides (including partial PCR and CSR regions) but no detectable membrane-associated sequences. This observation suggests that the sample preparation process may have selectively extracted soluble proteins while failing to isolate membrane-bound fractions. Notably, the detected gene sequences aligned with those previously reported for *Populus tremula* $\times$ *tremuloides* CesA8, with corresponding regions highlighted in light blue and light green boxes for clarity [29]. In summary, these findings conclusively confirm the successful heterologous expression of both PtCesA1 and PtCesA8 in *E. coli*.

3.2 Characterization of synthesized cellulose

In order to explore the morphology and properties of the products synthesized in the experiment, Transmission electron microscopy (TEM), ATR-FTIR and cellulase treatment methods were respectively used to analyze. The results were shown as follows.

3.2.1 Microscopic morphology

TEM analysis of SDS/NaOH-purified samples revealed broadly similar architectures for PtCesA1 and PtCesA8, both distinct from the empty pQE vector control (Fig. 2). Acicular crystals (2–7 nm width) were present in all specimens (Fig. 2B–C, E–F, G–I, red arrows), though markedly scarce in controls. Both PtCesA isoforms primarily formed disordered needle-like networks (2–7 nm diameter; Fig. 2G–I), with Occasional flagellar remnants (20–30 nm diameter; Fig. 2D, G, H, bright yellow arrows) represented non-dominant features.

3.2.2 Chemical group analysis

ATR-FTIR was employed to characterize the chemical composition of heterologously expressed PtCesA1 and PtCesA8 products following 6-hour induction and sequential SDS/NaOH purification (Fig. 3A). Spectra confirmed cellulose-like chemical structures in both PtCesA1 and PtCesA8 products. Key absorption peaks included: 3277 cm^{-1} represents the stretching vibration peak of OH, 2921 cm^{-1} represents the stretching vibration peak of C-H, and 807 cm^{-1} represents the C1-H vibration absorption peak of heterocarbon, the $-\text{CH}_2$ bending vibration peak at 1440 cm^{-1} , the cellulose-CH bending vibration peak at 1396 cm^{-1} , and the C1-O-C4 stretching vibration peak at 1154 cm^{-1} [30]. The broadening of the O–H band (3340 cm^{-1}) indicates amorphous cellulose in PtCesA1 products. Minor impurity peaks (e.g.,

1636 cm⁻¹, amide I carbonyl stretch) suggested residual bacterial proteins, likely due to incomplete purification[31].

3.2.3 Glycosidic linkage analysis

To further analyze the product composition, the β -1,3-glucan and β -1,4-glucan contents in the samples were quantified. Previous studies have documented that certain *E.coli* strains possess inherent capabilities for extracellular polysaccharide biosynthesis[32]. Fluorometric quantification using aniline blue binding revealed minimal β -1,3-glucan content. Quantitative results for experimental groups (PtCesA1/PtCesA8) and the control group wild-type *E.coli* XL1-blue (empty pQE vector) revealed callose concentrations consistently within the range of 0.33–0.37 mg/mL (Fig. 3B) and indistinguishable from pQE controls ($p = 0.35$). This evidence suggests that the detected callose in product mixtures primarily originated from endogenous bacterial synthesis rather than in vitro production through PtCesA protein expression. These collective findings demonstrate that the heterologously expressed PtCesA products either lack or contain only trace amounts of glucose molecules linked by β -1,3-glycosidic bonds.

To validate the presence of β -1,4-glucan in the synthesized products, targeted enzymatic hydrolysis was performed using Celluclast, a cellulase specifically cleaving β -1,4-glycosidic bonds. Post-hydrolysis residues were quantified via the anthrone-sulfuric acid method, which measures total carbohydrate content through glucose-derived chromogenic reactions. Relative glucose levels were determined spectrophotometrically using sulfuric acid-hydrolyzed samples (Fig. 3C).

Experimental data revealed a significant reduction in residual glucose content for both PtCesA1 (31.0% $\pm$ 4.7%) and PtCesA8 (36.2% $\pm$ 3.3%) expression products following Celluclast treatment, demonstrating enzymatic degradation of β -1,4-linked glucan chains. In contrast, empty vector (pQE) controls showed no statistically significant difference in glucose levels before and after treatment ($p = 0.93$), reduction in residual glucose content 8.0% $\pm$ 3.4%. endogenous β -1,4-glucan production from *E. coli* polysaccharides such as lipopolysaccharides or capsular polymers were effectively excluded. The CNF process significantly reduced the residual glucose content. The reduction reached 94.0%, with a standard deviation of 6.3% (mean $\pm$ SD). Based on the above analysis, it can be confirmed that the heterologous expression products contain cellulose and do not contain callose.

3.3 Spatiotemporal organization of PtCesA proteins

Immunofluorescence labeling of engineered strains revealed isoform-specific assembly dynamics (Fig. 4).

Post-treatment with lysozyme/EDTA/detergent effectively exposed antigenic epitopes for antibody recognition. PtCesA1 localized to linear terminal complexes (TCs) at cell peripheries, resembling *Acetobacter*-like configurations. In contrast, PtCesA8 exhibited spatial heterogeneity: only a subset of cells formed ordered TCs (red arrowheads), while most displayed disorganized distributions. This suggests that PtCesA8 trimers may dissociate during membrane treatment or antibody labeling, destabilizing higher-order complexes.

4 Discussion

Our heterologous expression of *Populus trichocarpa* Cesa isoforms, PtCesA1 and PtCesA8, in *Escherichia coli* resulted in the synthesis of cellulose-like polymers with structural features distinct from native plant or bacterial cellulose. Proteomic and biochemical analyses confirmed that both isoforms independently catalyzed the formation of β -1,4-glucan, validating their intrinsic cellulose synthase activity. While PtCesA1 and PtCesA8 were successfully expressed, the observed filamentous architectures support functional assembly of these isoforms in *E. coli*.

Heterologous expression of cellulose synthases in *Escherichia coli* yields distinct products depending on enzyme origin. Expression of plant-derived PtCesA1 and PtCesA8 generates morphologically dissimilar outputs compared to bacterial BcsA-BcsB complexes named [33]. Unlike the needle-like assemblies' characteristic of Cesa1/Cesa8 products, CESEC yields short crystalline platelets. This divergence stems from fundamentally distinct synthesis mechanisms. However, neither Cesa1- nor Cesa8-derived products replicate native plant cellulose microfibrils. This discrepancy likely arises from the absence of essential plant-specific machinery in the bacterial host—including the microtubule-guided synthesis apparatus and accessory proteins required for microfibril assembly and crystallization. Furthermore, alkaline treatment during bacterial product purification may contribute to fiber shortening[30].

ATR-FTIR spectra and cellulase hydrolysis assays unequivocally demonstrated the cellulose-like nature of the synthesized products. However, X-ray diffraction (XRD) and selected-area electron diffraction (SAED) failed to resolve canonical cellulose I or II crystallinity. Instead, the observed intermediate crystalline structure may reflect alkaline-induced polymorphic transitions during SDS/NaOH purification, as previously reported for alkali-treated cellulose (21, 22). Generally, 2%SDS and 2%NaOH do not convert cellulose I into cellulose II. This aligns with our TEM findings of PtCesA8-derived crystalline domains (2–7 nm) resembling alkali-stabilized cellulose II-like structures. Although TEM revealed partially ordered fibrils, XRD/SAED data are required to conclusively determine cellulose polymorphism. We acknowledge this limitation and are currently optimizing protocols for XRD analysis of nanoscale cellulose.

Despite these advances, limitations persist. Low product yields and residual protein impurities (evidenced by 1636 cm^{-1} FTIR peaks, indicative of amide I bonds from bacterial contaminants) highlight inefficiencies in purification workflows. Notably, our research results differ from previous expressions of subunits such as plant or bacterial Cesa in proteoliposomes [34]. The partial crystallinity achieved here suggests that *E. coli* may uniquely harbor lipid microenvironments or chaperones that facilitate Cesa self-organization, as proposed for bacterial cellulose synthase macrocomplex assembly. To test this hypothesis, future work should integrate lipidomic profiling of *E. coli* membranes during Cesa expression with cryo-electron tomography to resolve isoform-specific assembly mechanisms. Such efforts could bridge the evolutionary divide between plant and bacterial cellulose synthesis, enabling the design of bioengineered cellulose with programmable architectures for sustainable materials applications.

Declarations

Author Contributions

Shijing Sun: Conceptualization, investigation, Writing - Original Draft, Project administration; **Huasha Liang** and **Kairen Zhang:** Investigation, Formal analysis, Data Curation Validation, Writing - Original Draft; **Peiqi Yang** and **Renjie Li:** Investigation; **Zhongyuan Zhao** and **Tongming Yin:** Writing - Review & Editing, Supervision.

Notes

The authors declare no competing financial interest.

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Figures

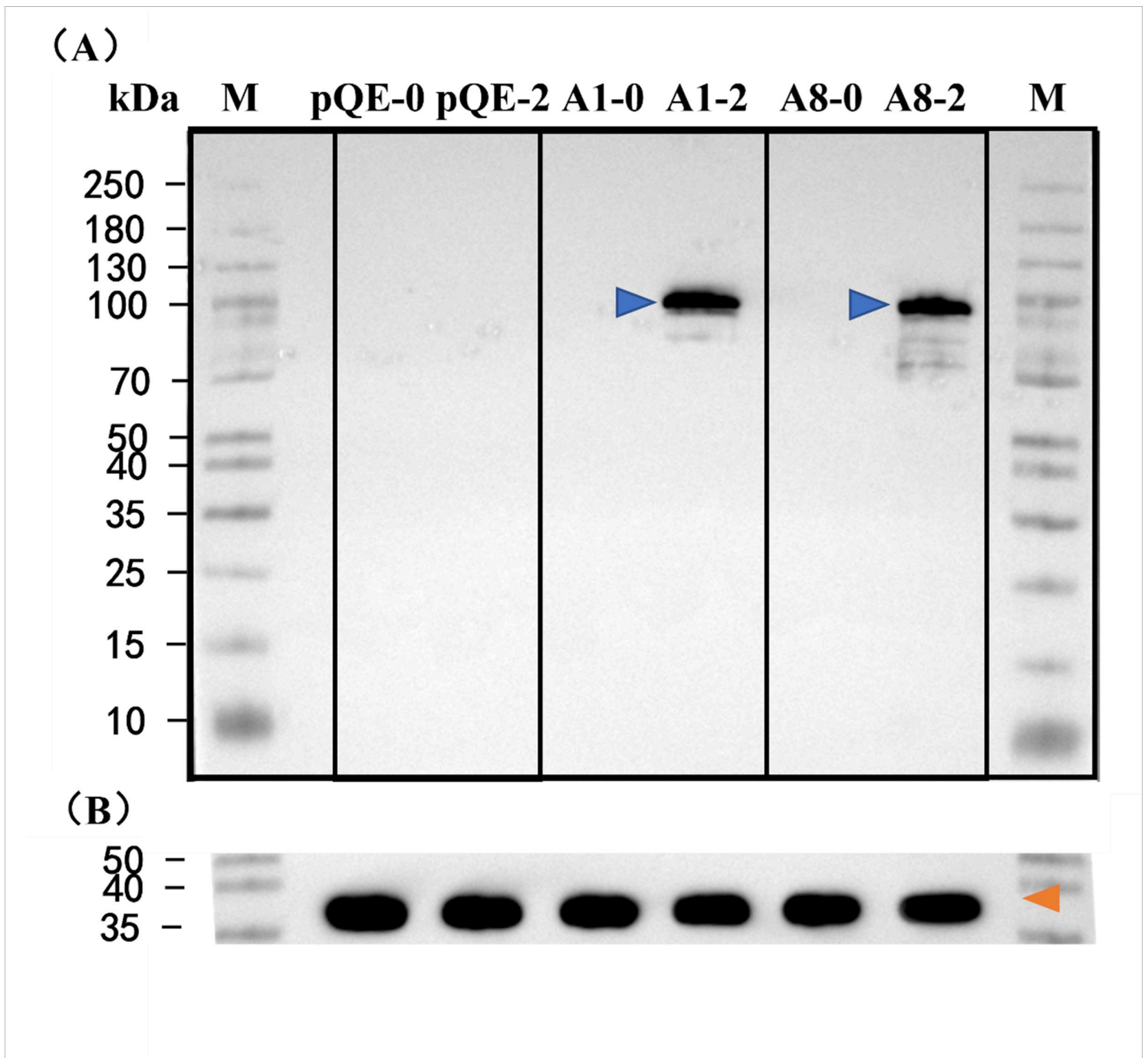


Figure 1

Heterologous expression analysis of recombinant PtCesA1 and PtCesA8 in *E. coli* XL1-Blue. (A) Western blot of protein extracts probed with anti-His tag antibody. Lane details: M – Tri-color prestained protein marker (10–250 kDa). pQE: empty vector control, A1-0/A8-0 – uninduced controls; A1-2/A8-2 – cultures harvested 2 h post-induction with 0.5 mM IPTG. Blue arrows indicate predicted full-length proteins: ~122 kDa for PtCesA1 and ~110 kDa for PtCesA8. (B) Parallel blot probed with anti-OmpA antibody showing consistent loading (~35 kDa). Orange arrow indicate OmpA signals.

Further validation by DIA proteomic analysis (Figure S1) revealed significant enrichment of soluble domain peptides (including partial PCR and CSR regions) but no detectable membrane-associated

sequences. This observation suggests that the sample preparation process may have selectively extracted soluble proteins while failing to isolate membrane-bound fractions. Notably, the detected gene sequences aligned with those previously reported for *Populus tremula* × *tremuloides* CesA8, with corresponding regions highlighted in light blue and light green boxes for clarity [29]. In summary, these findings conclusively confirm the successful heterologous expression of both PtCesA1 and PtCesA8 in *E. coli*.

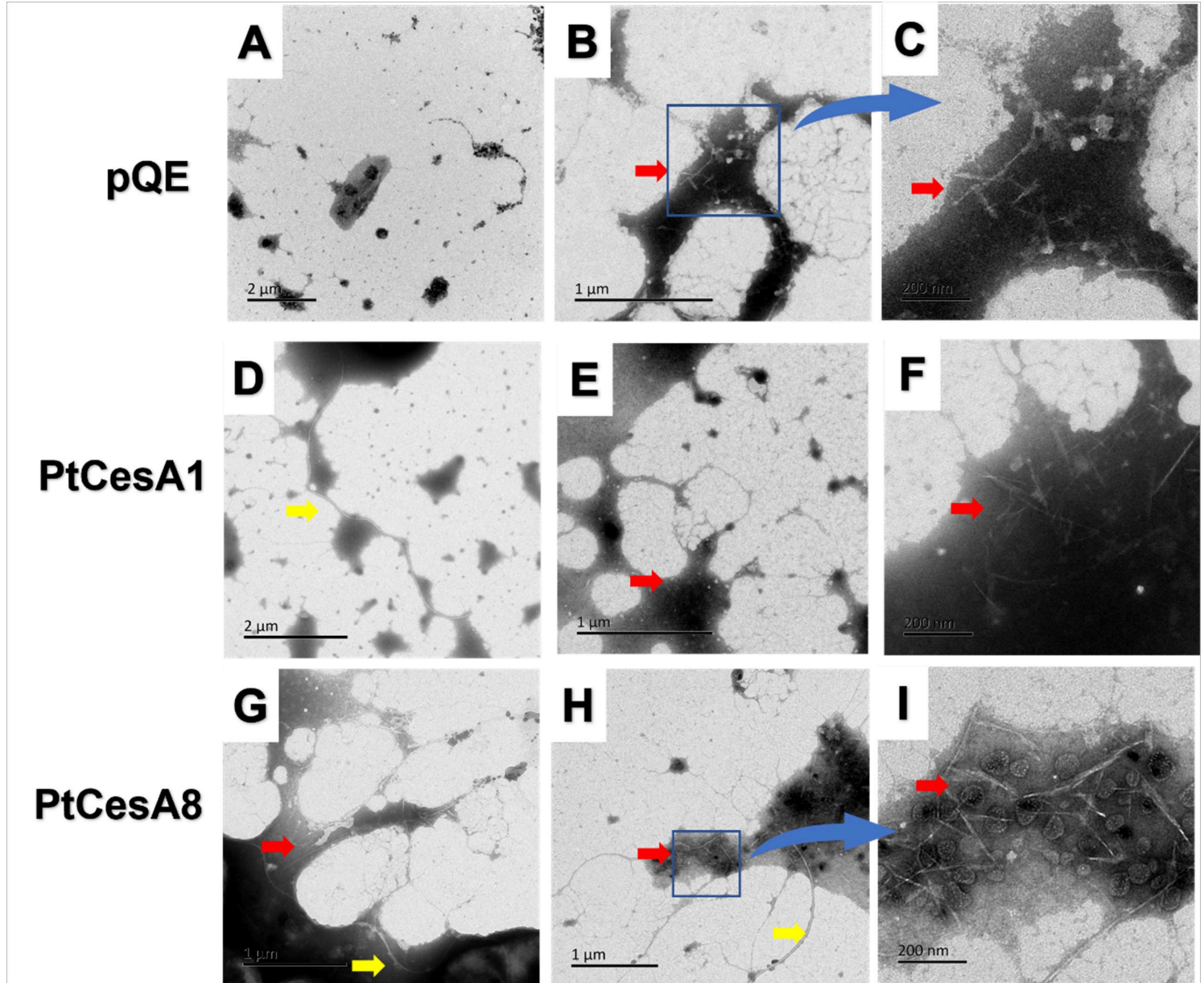
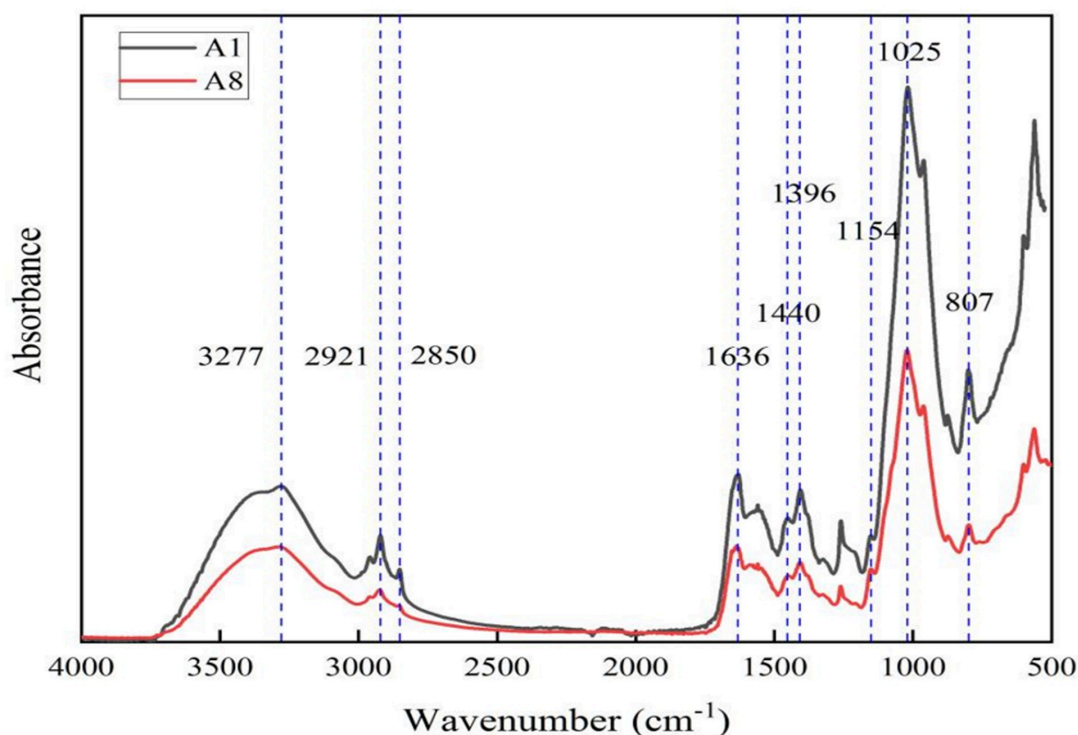
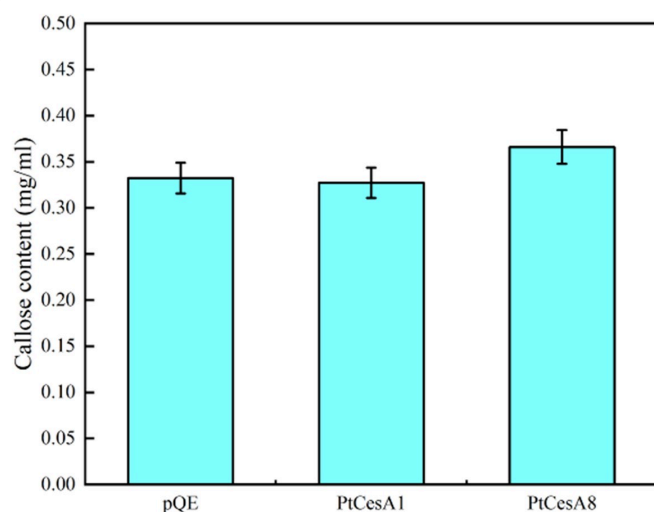
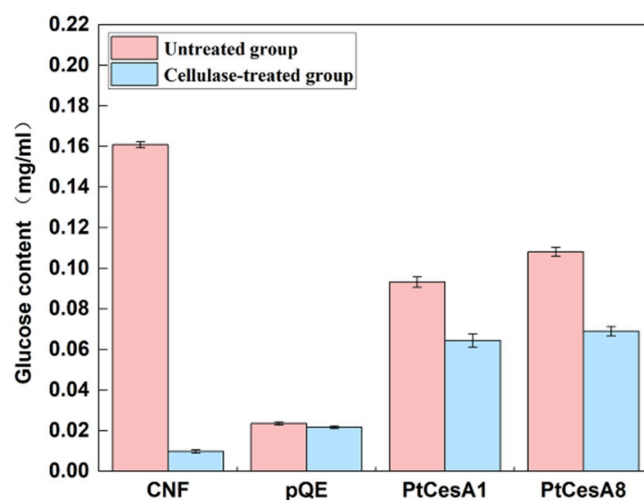


Figure 2

Negative-stain TEM analysis of cellulose synthase complexes synthesized by *PtCesA1* and *PtCesA8*. All samples were harvested 6 hours post-induction and washed with SDS/NaOH. (A–C) Samples from vector-only control (pQE). (D–F) Samples from *PtCesA1*. (G–I) Samples from *PtCesA8*. Red arrows indicate synthesized cellulose synthase complexes. Bright yellow arrows denote flagellar-like structures. Blue boxes show regions magnified in subsequent panels. Scale bars as shown.

A**B****C****Figure 3**

Characterization analysis of the synthesized products by PtCesA1 and PtCesA8. (A) ATR-FTIR spectra of the synthesized products by PtCesA1 and PtCesA8 purified with SDS/NaOH. Up and down layer are samples from PtCesA1 and PtCesA8 separately. (B) Callose content of PtCesA1, PtCesA8 and pQE (WT). (C) Quantitative comparison of various products before and after treatment with cellulase. Data represent mean $\pm$ SD ($n = 3$ biological replicates). Statistical significance was determined by one-way ANOVA ($p < 0.05$).

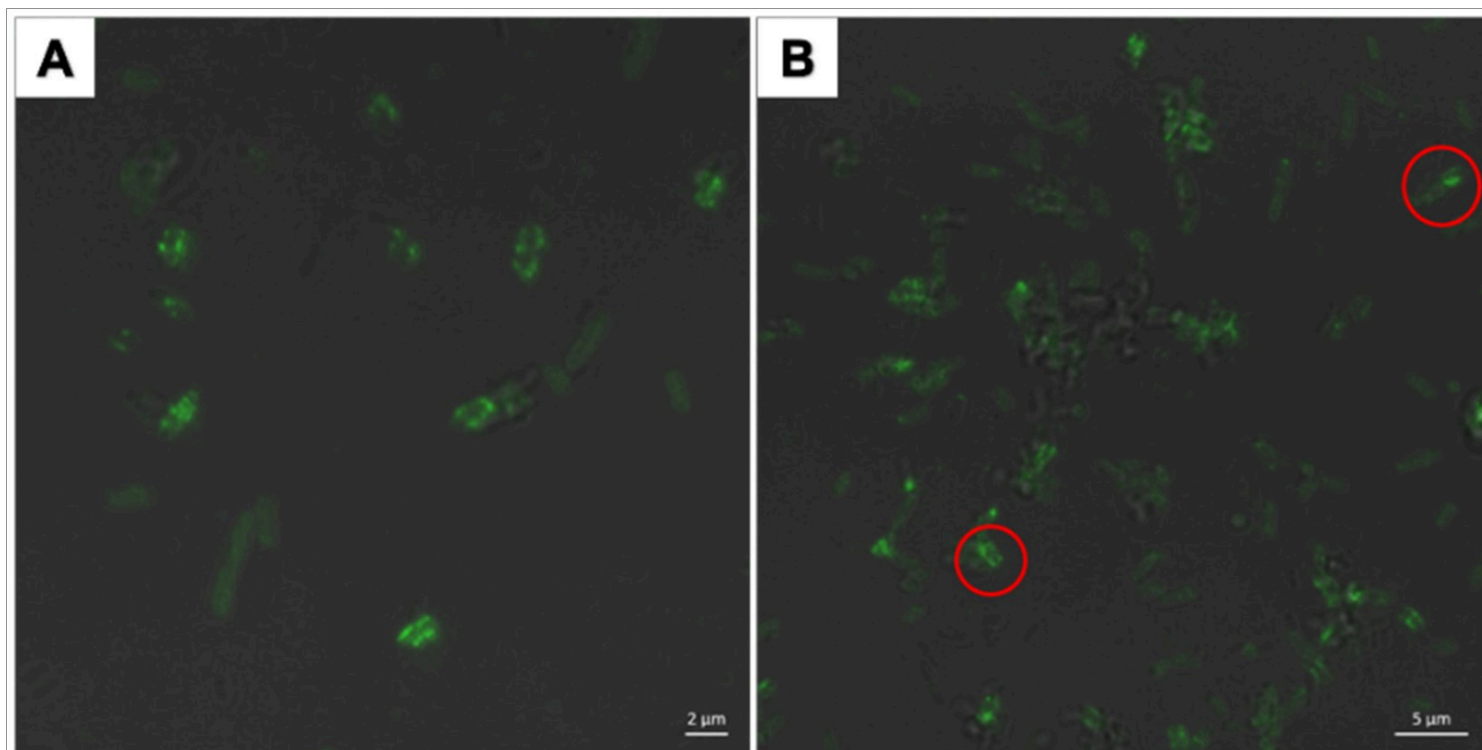


Figure 4

Fluorescence micrographs with immunolabeling of the engineering bacteria with PtCesA1 and PtCesA8 (shown in A and B) by the antibody against CesA protein, merged on the phase-contrast image. Pretreatment of the cell is lysozyme treatment followed by EDTA treatment and detergent treatment.

Supplementary Files

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- [floatimage6.jpeg](#)
- [floatimage1.png](#)