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Overexpression of a new cellulose synthase gene (*PuCesA6*) from Ussuri poplar (*Populus ussuriensis*) exhibited a dwarf phenotype in transgenic tobacco

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Abstract

Cellulose is synthesized in plant cell walls by cellulose synthase (*CesA*) genes. The characterization of a new cellulose synthase gene (*PuCesA6*) (GenBank Accession No. HQ686077) from an economically important tree, Ussuri Poplar (*Populus ussuriensis*) is reported here. The predicted PuCesA6 protein is highly similar to *Populus tremuloides* PtrCesA6 (99%) expressing in all expanding cells depositing primary cell wall. The domain structures of PuCesA6 was predicted by multiple alignment analysis, which contained a N-terminal cysteine rich zinc binding domain, 8 putative transmembrane helices (TMH), a signature D, D, D, QxxRW motif, 5 alternating conserved regions (CR-P) and 2 hypervariable regions (HVR). Subcellular localization analysis showed that PuCesA6 protein was localized in the cytomembrane. Ectopic expression of PuCesA6 in tobacco significantly exhibited "dwarf" phenotype, with final aerial height less than 35 cm (approximately two-thirds height of wild type). The phenomenon could be explained by post-transcriptional gene silencing (PTGS) of the expression of the homologous genes in tobacco. Thus, identification of new *CesA* genes from poplar tree genomes is essential for enhancing knowledge of cellulose biosynthesis in trees that has many fundamental and commercial implications.

Keywords: "dwarf" phenotype; *PuCesA6*; *Populus ussuriensis*; Tobacco.
Abbreviations: BA_6-benzylaminopurine; NAA_naphthaleneacetic acid; TMH_transmembrane helices; CR-P_conserved region; HVR_hypervariable regions; PTGS_post-transcriptional gene silencing; ORF_open reading frame; Km- kanamycin.

Introduction

As an integral component of plant cell walls, cellulose is of great economic value due to its abundance and structural characteristics. In primary cell walls of trees, cellulose contributes to about 20–30% in dry weight. Cellulose microfibrils of primary walls control the size and shape of plant cells. The secondary cell walls with greater cellulose crystallinity, higher degree of polymerization and better organized cellulose microfibrils contain about 40–50% cellulose, and provide mechanical strength and rigidity to the entire plant (Mellerowicz et al., 2001; Green, 1994). Our current understanding of the molecular mechanism of cellulose biosynthesis in higher plants is mainly derived from studies in model herbaceous plants and fiber crops. Paradoxically, in contrast to cellulose abundance and its plethora of uses, we know little about the molecular processes involved in cellulose biosynthesis in tree plants.

The major wood component, cellulose, a linear polymer of β-1,4-glucan residues, is formed from UDP-glucose and this reaction is catalyzed by the enzyme cellulose synthase (*CesA*) (Delmer and Amor, 1995; Saxena etal., 1990). The first *CesA* gene was cloned from bacteria that produce extracelluar cellulose (Matthysse etal., 1995; Wong etal., 1990), followed by the identification of two putative *CesA* genes in cotton (Pear et al., 1996). Today, a large number of *CesA* as well as *CesA*-like (*Csl*) genes, forming a large superfamily, have been identified and sequenced from many different plants, such as *Arabidopsis*, rice and maize (Richmond and Somerville, 2000; Holland et al., 2000; Hazen et al., 2002; Tanaka et al., 2003). Several lines of evidence implicate the plant *CesA* genes in the process of cellulose synthesis. At least 10 *CesA* genes in the model plant were identified based on the molecular approaches coupled with availability of genome sequence information, which can be classified into six orthologous groups (Somerville, 2006). The mutant complementation analyses show that these six groups of isoforms have nonredundant functions in cellulose synthesis (Doblin et al., 2002). The first cellulose-deficient mutant, *rsw1* (*AtCesA1*) caused a temperature-sensitive radial cell expansion defect with reduced amounts of crystalline cellulose in *Arabidopsis* (Arioli et al., 1998). It is generally accepted that in *Arabidopsis* the CESA1, CESA3, and CESA6 or CESA6-like proteins are required for functional primary cell wall complexes (Arioli et al., 1998; Scheible et al., 2001; Fagard et al., 2000), whereas CESA4, CESA7, and CESA8 are required for functional secondary cell wall complexes (Taylor et al., 1999, 2000, 2003). Null mutants for CESA1 and CESA3 are gametophytic lethal (Persson et al., 2007), indicating the essential nature of the genes. In contrast, CESA6 null mutants show a relatively mild phenotype, which might be explained by the existence of CESA2, CESA5, and CESA9, which are closely related to CESA6 (Joshi, 2003). So far, similar direct functional evidence through mutant identification and complementation with wild type copy of defective *CesA* gene is not available for *AtCesA2*, *AtCesA5*, *AtCesA9* and *AtCesA10* genes in *Arabidopsis*, most probably due to their functional redundancy or overall lower level of gene expression and tissue-specificity.

Arabidopsis is a widely accepted model system for the study of plant biology as well as many aspects of tree biology. However, a major difference between trees and other plants is the wood forming capacity of trees. Since the process of wood formation is unique for trees, more specific model systems are needed for its detailed analysis (Taylor, 2002). Identification of

new and distinct tree *CesA* genes, defining the site and pattern of their expression during tree development and further exploring their functions will finally lead to their utilization towards improving cellulose production in economically important trees. In this study, we used a Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with gene specific primers designed based on the conserved regions of 10 complete coding sequences of *CesA* from NCBI database to isolate and characterise a full-length *CesA* cDNA isolated from *Populus ussuriensis* Kom., an ortholog of *PtrCesA6* from *Populus tremuloides*, as *CesA* genes involved in primary cell wall development in aspen trees.

Results

Cloning and analysis of the PuCesA6

Full-length cDNA of the gene encoding *PuCesA6* was cloned, the sequence was deposited in GenBank with the accession number of HQ686077. The open reading frame (ORF) of *PuCesA6* is 3264 bp in length from the ATG start codon to the TAA stop codon, encoding a predicted polypeptide of 1087 amino acids with a molecular weight of 122.51 kDa and a pI of 6.57. The predicted PuCesA6 polypeptide exhibited extremely high identity values at the amino acid level (99%) with CesA6 in *Populus tremuloides* (Fig. 1).

The predicted amino acid sequence of *PuCesA6* contained highly conservative features of plant cellulose synthase. Fig. 1. shows the schematic diagram of PuCesA6 protein. A N-terminal zinc finger domain was found to be highly conserved in all CesA proteins known to date (Joshi, 2003). The lacks of zinc binding domain assembled the CesA proteins as linear terminal complexes and obstructed the cell microfibril structures (Delmer, 1999). *PuCesA6* also contained 8 putative transmembrane helices (TMH, the first two TMH region of *PuCesA6* was towards the N-terminal of amino acid sequence, the other six TMH regions were toward the C-terminal of *PuCesA6* sequence) (Fig. 1, Fig. 2). This phenomenon suggested that the first two associate as the integral membrane protein (Richmond and Somerville 2000). Holland et al. (2000) reported many of the glycosyltransferases, including the plant and bacterial CesA proteins predicted to be anchored in the cell plasma membrane by transmembrane helices. The cytoplasmic loop between the second and third TMH regions of *PuCesA6* contained a conserved QxxRW motif (D, D, D, QxxRW) sequence (Fig. 1) that was predicted to be involved in substrate binding and catalytic activities of CesA enzymes (Vergara and Carpita, 2001; Beeckman et al., 2002). The presence of D, D, D, QxxRW motif in *PuCesA6* suggested that the *PuCesA6* associates with glycosyltransferases in catalyzing the biosynthesis of long-chain polysaccharides (Samuga and Joshi 2004). The predicted plant-conserved region (CR-P) and hypervariable regions (HVR-I and HVR-II) were also present (Fig. 2). The CR-P region was suggested to be implicated in the cellulose biosynthesis at "rosette complexes. As reported by Roberts et al. (2002), the CR-P region of plant CesA was accompanied with the origin of the rosette terminal complexes that consist of multiple catalytic subunits formed by *CesA* gene. The HVR region was reported to be able to define CesA proteins of *Oryza sativa*, *Zea mays* and *Arabidopsis thaliana* into different sub-classes (Vergara and Carpita, 2001).

A phylogenetic analysis of the deduced protein sequences of *PuCesA6* gene indicated interesting similarities with the corresponding *CesA* genes previously characterized in other plants (Fig. 3). *PuCesA6* was most similar to the *PtrCesA6* from *Populus tremuloides* and belonged to the clade with the *BlCesA6*, *EgCesA6*, *ZmCESA6*, *7* and *8*. Presence of a *PuCesA6*, a monocot like *CesA* in dicot tree species with conservation of sequence and expression pattern was interesting. Many of other clades contained members from plants of both monocot and dicot lineages, indicating that the divergences into at least some of these subclasses might have arisen relatively early in the evolution of these genes.

Subcellular localization analysis of PuCesA6 gene

The subcellular localization of the PuCesA6 protein was examined by introduction of the PuCesA6-GFP fusion protein into onion epidermal cells by particle bombardment. The control GFP fluorescence was observed in the cytoplasm and nucleus of transformed onion cells (Fig. 4A, B and C), whereas the PuCesA6-GFP was only detected in the cytomembrane (Fig. 4D, E and F). Other studies examining plant CesA6 localization had also revealed that CesA6 was a membrane protein. In the *Arabidopsis*, at least two of the three primary CesAs (CESA3 and CESA6) were functional when labeled with GFP and its derivatives. Both GFP-CESA3 and YFP-CESA6 were observed at the plasma membrane as discreet particles that moved along linear trajectories coincident with underlying cortical microtubules (Desprez et al., 2007; Paredez, 2006).

Construction of plant binary vector

In order to verify the function of *PuCesA6*, the plant binary vector pROKII-*PuCesA6* was constructed. The *PuCesA6* gene was inserted into the *Xba* I and *Kpn*I sites of the binary vector pROKII under the control of CaMV35S promoter and NOS terminator along with a kanamycin-resistance gene as a selectable maker. The entire diagram of construction and the structures of plasmid were shown in Fig. 5A. The recombinants were identified by PCR (Fig. 5B) and restriction endonuclease digestion analysis (Fig. 5C).

Transformation and identification of transgenic tobacco

To investigate the physiological function of *PuCesA6*, we performed plant transformation to obtain transgenic tobacco. The putative transgenic shoot buds were obtained 15 days after transferring to the fresh selection medium (Fig. 6A). At last, 19 independent plant lines regenerated on selection medium containing 50mg L^{-1} Km. Ten of nineteen transgenic tobaccos were determined by PCR using special primers for *NptII* gene, the positive control and transgenic tobacco all produced the expected band of 781bp (Fig. 6C). The sterilized seeds of T_1 were spread onto selection plates for homozygous transgenic tobacco (Fig. 6B), then transplanted seedlings into soil and moved to greenhouse for observation.

Three transgenic lines and one wild type tobacco were confirmed by Southern blot analysis. The plasmid pROKII-*PuCesA6* was added as positive control. Southern hybridization showed that all of other bands produced the expected band of 865 bp, except the wild type (Fig. 6D), which indicated that the *PuCesA6* gene had been integrated into the genome of the test plants.

Northern blot analysis also showed that three of transgenic lines displayed a distinct band of *PuCesA6* gene, while the wild type did not, which confirmed that the *PuCesA6* gene had been successfully expressed in the transgenic lines (Fig. 6E).

Altered the height of plant and the length of the fiber cells in transgenic tobacco

Six homozygous (T_3 generation) transgenic lines were selected

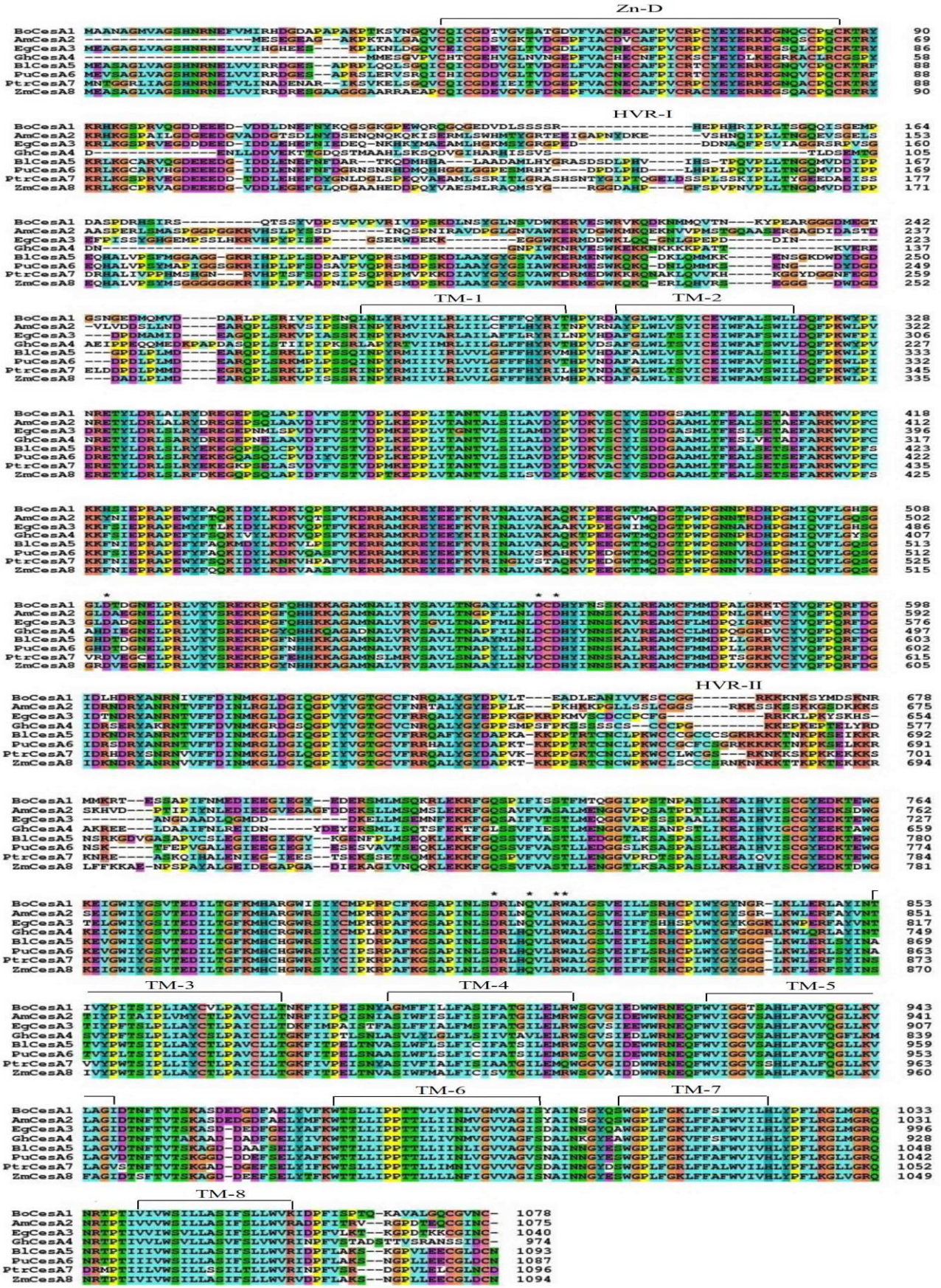

Fig 1. Alignment of deduced amino acid sequences. The zinc binding domain (Zn-D) and the transmembrane domains (TM) are indicated by brackets above the sequences. The highly conserved D, DxD, and D residues and the QxxRW motif are denoted by asterisks. Bo, *Bambusa oldhamii*; Am, *Acacia mangium*; Eg, *Eucalyptus globulus*; Gh, *Gossypium hirsutum*; Bl, *Betula luminifera*; Ptr, *Populus tremuloides*; Zm, *Zea mays*.

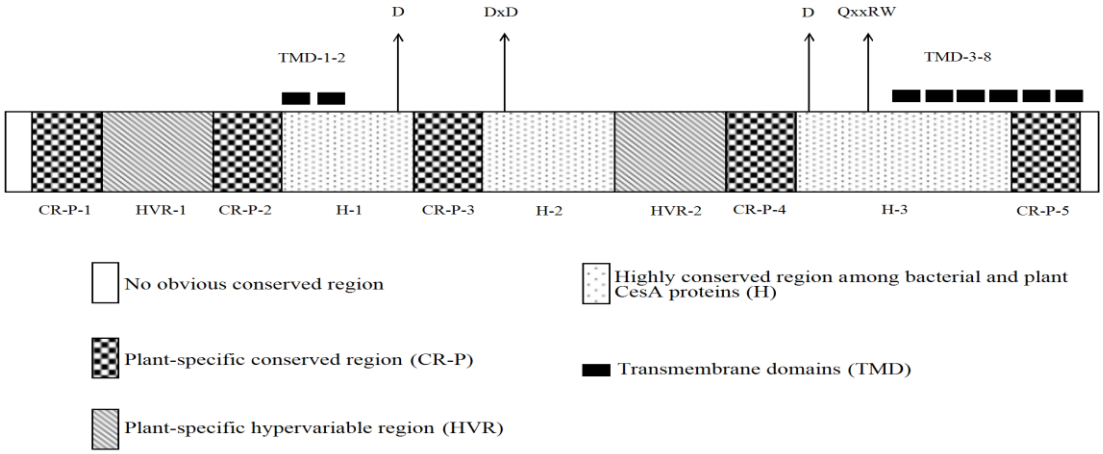

Fig 2. Domain structure of PuCesA6 protein showing various conseved regions.

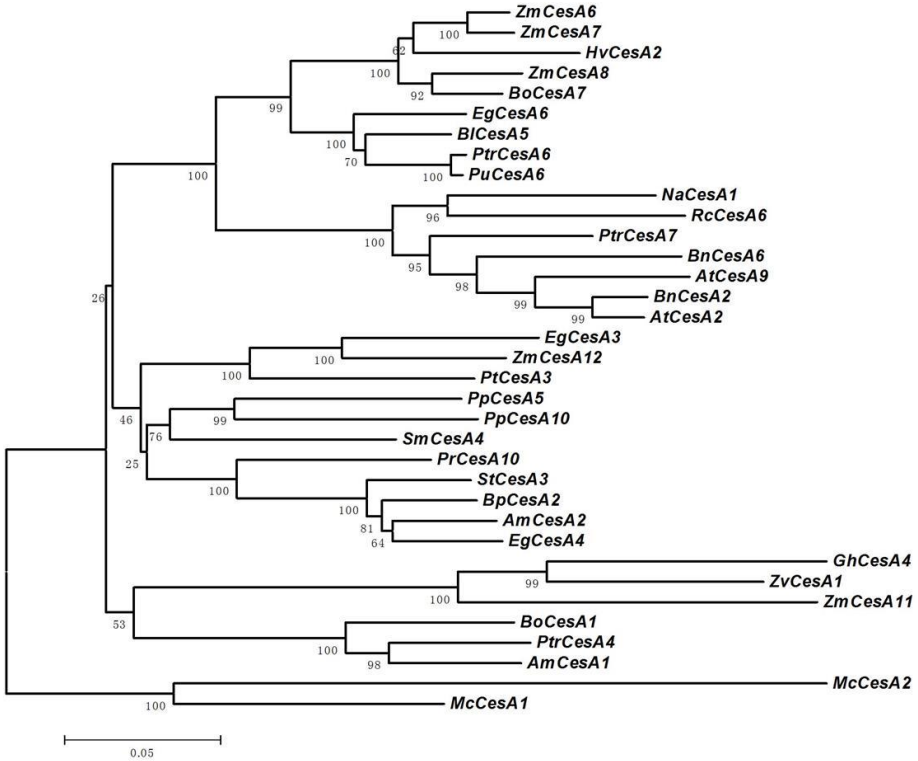

Fig 3. Cladogram of the amino acid sequences of the predicted protein encoded by PuCesA6 and full-length CesA sequences from other species. Am, *Acacia mangium*; At, *Arabidopsis thaliana*; Bl, *Betula luminifera*; Bn, *Brassica napus*; Bo, *Bambusa oldhamii*; Bp, *Betula platyphylla*; Br, *Brassica rapa*; Eg, *Eucalyptus globulus*; Gh, *Gossypium hirsutum*; Hv, *Hordeum vulgare*; Mc, *Mesotaenium caldariorum*; Na, *Nicotiana alata*; Pp, *Physcomitrella patens subsp. Patens*; Pr, *Pinus radiata*; Pt, *Pinus taeda*; Ptr, *Populus tremuloides*; Rc, *Ricinus communis*; Sm, *Selaginella moellendorffii*; St, *Solanum tuberosum*; Zm, *Zea mays*; Zv, *Zinnia violacea.*

for the phenotype analyses. Under long-day conditions (with a 16 h light/8 h dark cycle), all plant flowered 65 days after sowing. All the transgenic tobacco plants showed "dwarf" phenotype, with average aerial height of 39.5 cm, especially the line 1 with final aerial height of 35 cm, while more than 9 wild type plants showed average aerial height over 55 cm (Fig. 7 A). The wild type plants were approximately 1.2- to 1.5-fold higher than those in the plants in the control group ($P \leq 0.01$) (Fig. 7 A, B). There was no significant variation in either flower or fruit morphology, or fertility levels, between transgenic and wild type plants except that the length between stem nodes of the transgenic plants, which was shorter than that of wildtype.

HNO_3 maceration was used to observe the fibers of plant stems. Here, the length of fiber cells was measured under a microscope. In statistical significance testing, it showed that the length of the fiber cell had no significant differences between transgenic individuals and wild-type individuals ($P>0.05$). The length of the wild type fiber cells was 2.86mm on average, whereas the average was 2.42 mm in transgenic fiber cells

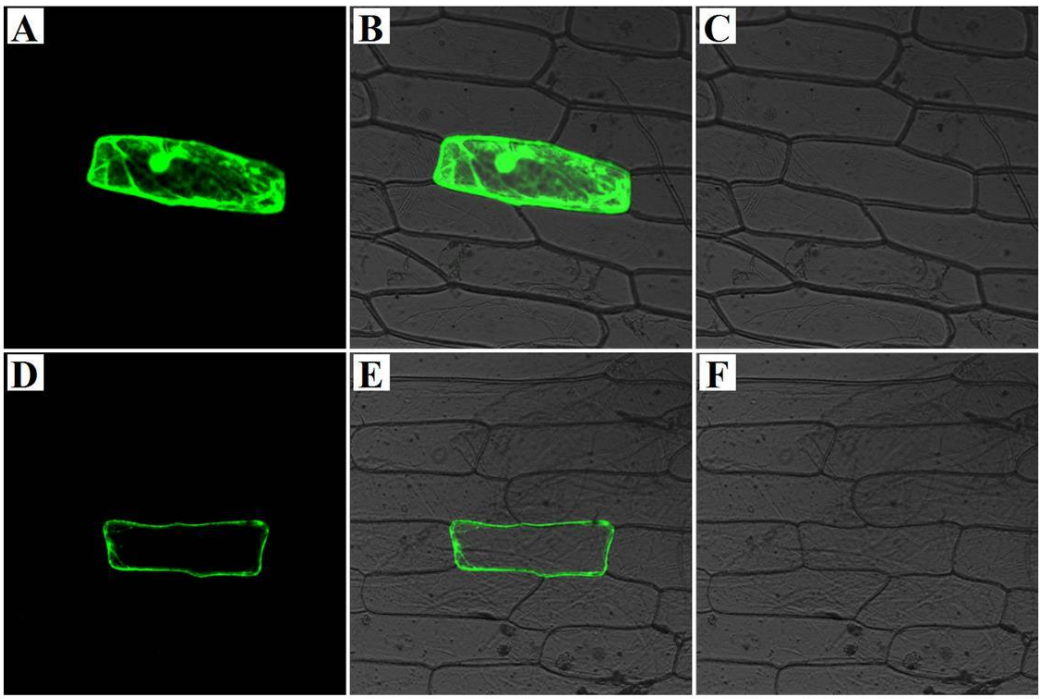

Fig 4. Subcellular localization analysis of the *PuCesA6* gene. The PuCesA6-GFP fusion and GFP alone were each expressed transiently under the control of the CaMV35S promoter in onion epidermal cells and observed under a confocal microscope. The photographs were taken in a dark field for green fluorescence localization (**A**, **D**), in a bright field to examine cell morphology (**B**, **E**) and in combination (**C**, **F**). (**A**, **B**, **C**) The cell was transiently expressing the GFP control, (**D**, **E**, **F**) The cell was expressing the PuCesA6-GFP fusion.

(line1, 3 and 5). Besides, the length of fiber cell from transgenic tobacco was significantly shorter (just 80%) than that of wild tobacco by using statistic method ($P \leq 0.01$) (Fig. 7C).

Discussion

Although the function of cellulose synthase genes have been identified clearly in *Arabidopsis*, rice and maize, the molecular mechanism of cellulose biosynthesis is unclear, especially the mode of cellulose biosynthesis in tree plants is weak. Studies using tree species have also revealed valuable information about *CesA* genes involved in secondary cell wall development, which includes *PcCesA1* from hybrid poplar (Wang and Loopstra, 1998), *PtrCesA1* (Wu et al., 2000), *PtrCesA2* (Samuga and Joshi, 2002), *PtrCesA3* (Joshi, 2003; Kalluri, 2003), *PtrCesA4* and *PtrCesA5* (Kalluri and Joshi, 2003), *PtrCesA6* and *PtrCesA7* (Samuga and Joshi, 2004) from aspen. Genetic studies indicate at least three *Arabidopsis* CESA isoforms (CESA1, CESA3, and CESA6 or CESA6-like proteins) are required for primary cell wall synthesis (Desprez et al., 2007; Persson et al., 2007). The CESA1, CESA3 and CESA6 three genes are also co-regulated at the mRNA level (Scheible et al., 2001). Lesions in CESA1 (*rsw1*), CESA3 (*cev1*), or CESA6 (*prc1*) lead to a deficiency in elongation in dark-grown seedlings (Fagard et al., 2000; Ellis et al., 2002; Arioli et al., 1998). CESA5 and CESA2 are partially redundant with CESA6 and most probably compete with CESA6 for the same binding site in the complex with the analysis of single, double, and triple mutants (Desprez et al., 2007). *PtrCesA6* plays roles in primary wall development that identified by situ localization of *PtrCesA6* to cells of root, leaf and shoot apex (Samuga and Joshi, 2004). Interestingly, expression profile of *PtrCesA6* is similar to three maize *CesA* genes, *ZmCesA6-8* from maize (Holland et al., 2000; Dhugga, 2001). The plant binary vector pROKII-*PuCesA6* was constructed for the identification of the function of *PuCesA6*. As the results of previous studies indicated, three phenotypes may appear in transgenic tobacco with the total length of sense strand expressing vector. I: *PuCesA6* works alone, activated to overexpress in the cellulose synthesis of plants, thereby promoting the growth of plants; II: *PuCesA6* is one of the subunits of cellulose synthase complex, overexpression in plants will neither increases the number of cellulose complex nor promotes cellulose biosynthesis; III: antisense inhibition phenotype appears in the sense transgenic plants, the phenomenon can be explained by post-transcriptional gene silencing (PTGS) in common transcription in transgenic study. This phenomenon was first discovered by Napoli et al. (1990). They attempted to overexpress chalcone synthase (CHS) in pigmented petunia petals to obtain the deep colors flower. Unexpectedly, the introduced gene created a block in anthocyanin biosynthesis, and produced white flowers and patterned flowers with white or pale nonclonal sectors on a wild-type pigmented background (Napoli et al., 1990). Subsequently, in the study of *par-1* gene, wild-type worms were injected with the *par-1* antisense or sense RNA; however, the results suggested *par-1* gene was inactivated (Guo et al., 1995). PTGS is a sequence specific RNA down-regulation mechanism that targets the trigger RNA molecules as well as the RNA molecules that share a certain sequence homology with the trigger. Until 1998, double-stranded RNA was identified as a major component of the PTGS process in plants (Fire et al., 1998; Smith et al., 2000).

In this study, the phenotype of transgenic tobacco may correspond to the PTGS. The length of fiber cell was significantly shorter than that of wild tobacco, which suggested that the endogenous homologous gene *NtCesA6* in the tobacco was inhibited by the overexpression of exogenous *PuCesA6* gene, which affected the cellulose biosynthesis in tobacco and

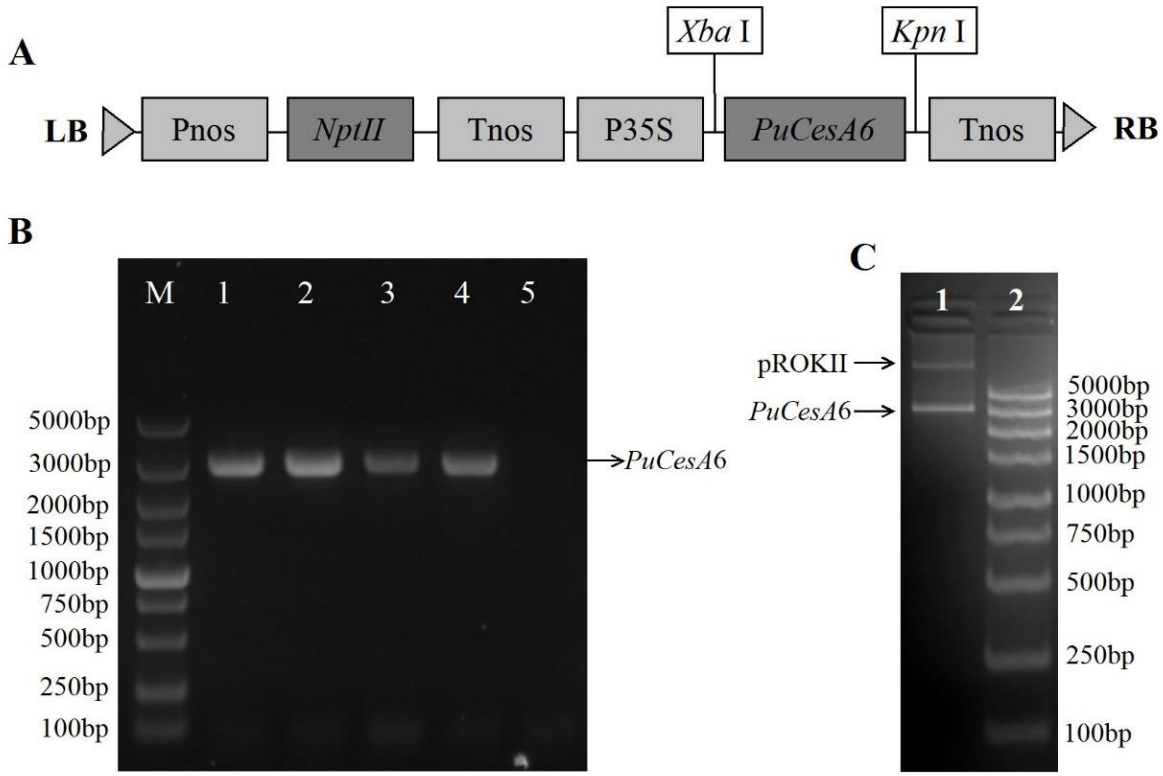

Fig 5. Reconstruction and confirmation of plant binary vector. **(A)** Schematic diagram of T-DNA region of the binary vector pROKII. LB, left border; Pnos, nopalina synthase promoter; *NptII*, kanamycin resistance gene; Tnos, nopalina synthase terminator; P35S, CaMV35S promoter; RB, right border; **(B)** 0.8% (w/v) agarose gel electrophoresis analysis of PCR products of recombinants. lane M, DNA marker DL5000; lane1-4, PCR products of 4 independent recombinants; lane5, negative control; **(C)** restriction endonuclease digestion analysis of recombinant. lane1, the digested recombinant with Xba I and Kpn I; lane2, DNA marker DL5000.

inhibited the growth of the transgenic tobacco. Immunolocalization studies indicate that the CesA proteins indeed constitute a part of the rosette complex (Kimura et al., 1999; Taylor et al., 2003). It can be deduced *PuCesA6* gene played an important part in the reassembly of a subunit of cellulose synthase terminal complexes ('rosettes'), overexpression of exogenous gene broke the cellulose synthase complex composition evironment, which affected the final cellulose biosynthesis.

Materials and Methods

Plant material and growth conditions

The biennial *Populus ussuriensis* Kom. was obtained from Mao'ershan experiment forest farm in Heilongjiang Province, China. The young leaves of *Populus ussuriensis* were immediately frozen in liquid nitrogen and stored at -80°**C** before the isolation of total RNA. The transgenic and wild-type tobacco (*Nicotiana tabacum* L.) seedlings were grown in pots containing a mixture of turf peat and sand (1:1 v/v) in a greenhouse under controlled conditions of 60-75% relative humidity and an average temperature of 22±2°**C**. Cool white fluorescent lights supplied photons at 200 µmol m^{-2} s^{-1}.

Cloning and analysis of PuCesA6 gene

The open reading frame (ORF) of *PuCesA6* was cloned from cDNA of poplar leaves using RT-PCR under the following conditions: predenaturing at 94°C for 4 min; 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min; and a final elongation at 72°C for 7 min. The primers used to amplify *PuCesA6* were as follows: forward primer, 5'-ATGGAAGTGAGTGCAGGTTTGGTGGC -3'and reverse primer, 5'-TTAATTACAGTCCAGTCCACATT-CCTC-3'. The deduced PuCesA6 protein was characterized using Expasy tools (http://www.expasy.org/tools). Sequence homology search of *PuCesA6* against the GenBank non-redundant nucleotide sequences was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment analysis was carried out to predict the plant special domain structures of PuCesA6, such as zinc binding domain, U-motifs, plant conserved region (CR-P), hypervariable regions (HVR) and D, D, D, QxxRW motif using the default parameters of the ClustalW algorithm (http://www.genome.jp/ tools/clustalw/). Phylogenetic analyses were performed using MEGA version 4.1 (Tamura et al., 2007).

Subcellular localization analysis of PuCesA6 gene

The *PuCesA6* gene was cloned into the vector pTH2 to generate the *PuCesA6-GFP* fusion gene driven by the CaMV35S promoter as described by Niwa (2003). The primers were designed as follows, forward primer, 5'-ATCGTCGACATGGGGCGCAAGAAAGTGCTG-3' and reverse primer, 5'-ATCCCATGGCTGGAAGCAAACTAAG-TGTTTCTG-3' (the underlined bases indicate the restriction sites for *Sal* I and *Nco* I, respectively). The PCR cycle profile was: predenaturation of 94°C for 4 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 3 min; a final extension of 72°C for 7 min. The *PuCesA6-GFP* construct was transformed into onion epidermal cells by particle bombardment (Bio-Rad

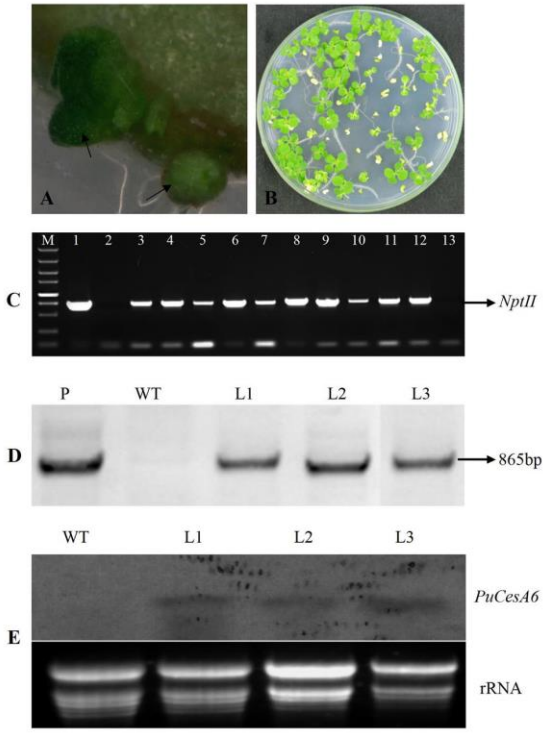

Fig 6. The confirmation of transgenic tobacco. **(A)** The putative transgenic shoot buds. Arrows indicated putative transgenic buds; **(B)** The sterilized seeds of T1 were screened onto selection plates (50mg L^{-1} Km); **(C)** Ten transgenic tobaccos were determined by PCR using special primers for *NptII* gene. lane M, DNA marker DL5000 (5000bp, 3000bp, 2000bp, 1500bp, 1000bp, 750bp, 500bp, 250bp, 100bp); lane1, pROKII vector containing the *PuCesA6* gene used as a positive control; lane2, wild type tobacco; lane3-12, PCR products of 10 independent transgenic tobaccos; lane13, negative control; **(D)** Southern blot analysis of transgenic and wild-type tobacco plants. The CaMV 35S promoter sequence was used as probe for Southern analysis. Genomic DNA was digested with *Hind*III and *Kpn* I and hybridized with the CaMV35S promoter probe. **(E)** Northern blot analysis of transgenic and wild-type tobacco plants, RNA was hybridized with the *PuCesA6* cDNA probe. P, positive control; WT, wild type; L1-L3, 3 independent transgenic tobaccos.

PDS-1000/He System, USA). PuCesA6-GFP fusion protein transient expression was observed using Zeiss Confocal Microscopy (Zeiss, Germany).

Construction of binary vector and plant transformation

To obtain the *PuCesA6* genes, polymerase chain reaction (PCR) amplification was carried out using the cDNA of poplar as the template with the following two primers, forward primer: 5'-GCTTCTAGAGAGATGGAAGTGAGTGCAGGTTTGGT GGC-3' and reverse primer: 5'-ATCGGTACCTTAATTACA-GTCCAGTCCACATTCCTC-3'. *Xba* I and *Kpn* I sites (underlined) were introduced to the end of the primers for convenience of plasmid construction. The 3.3 kb PCR product was cloned into pMD18-T vector (TaKaRa, Japan) to form the sub-cloning vector pMD18-T-*PuCesA6*. Plasmid pMD18-T-*PuCesA6* was digested with *Xba* I and *Kpn* I,then the gained 3.3 kb fragment was inserted into pROKII at *Xba* I and *Kpn* I, to form pROKII-*PuCesA6*. The recombinants were identified by PCR and restriction endonuclease digestion analysis. At last the resulting binary vectors, pROKII-*PuCesA6* was transferred into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw transformation method (Chen et al., 1994).

Leaf discs of tobacco were transformed by the *Agrobacterium*-mediated method as follows: Tobacco leaf disks were pre-cultured in the differentiation medium (MS medium + 20g L^{-1} sucrose + 0.1 mg L^{-1} 1-Naphthylacetic acid, NAA (Sigma,USA) + 0.5 mg L^{-1} N6-Benzyladenine, 6-BA (Sigma,USA) + 8g L^{-1} agar) for 2 days. Then, inoculated with *Agrobacterium* suspension for 3-5 min. Leaf disks were co-cultivated for two days in differentiation medium containing 200μM acetosyringone without antibiotics, then transferred to MS differentiation selection media containing 40 mg L^{-1} kanamycin (Km) and 500 mg L^{-1} Cefotaxime Sodium at 25°C in a 16 h light/8 h dark photoperiod at an intensity of ~2000 lux. After 15 days of culture, the explants with putative transgenic shoot buds were transferred to selection medium with 50 mg L^{-1} kanamycin and 500 mg L^{-1} Cefotaxime Sodium. Before the transgenic plants were transferred to soil, they were confirmed by PCR using specific primer pairs (NptII-F, 5'-AACAAGATGGATTGCACGCAGGTTCTCCGG-3'; NptII-R, 5'-GAACTCGTCAAGAAGGCGATAGAAGGC-GAT-3') for the *NptII* gene. The seeds were harvested, sterilized and plated on a selection medium (MS medium + 20g L^{-1} sucrose 8g L^{-1} agar) with 50 mg L^{-1} kanamycin to screen for homologous transformants.

Southern and Northern blot analysis

For Southern blot analysis, genomic DNA was extracted from young leaves using the CTAB method (Doyle and Doyle, 1987). A sample of 20 μg of tobacco genomic DNA was digested with *Xba* I and *Hind* III, which do not cut within the CaMV35S promoter sequence. The digested DNA was transferred to a Hybond-N^+ nylon membrane and hybridized with CaMV35S promoter sequence labeled using $^{[DIG]}$-dUTP by DIG DNA Labeling Mix (Roche, Switzerland). Hybridization was carried out as described by DIG Nucleic Acid Detection Kit (Roche, Switzerland). For Northern analysis, 10 μg of total RNA of tobacco was separated on 1% agarose denaturing formaldehyde gel and transferred to a Hybond-N^+ nylon membrane. Then the membrane was hybridized with *PuCesA6* cDNA labeled using $^{[DIG]}$-dUTP according to the supplier's manual.

Separation and measurement of fiber cell

For observation of the fiber cell, the xylem in stem of 6-week-old transgenic (line1, 3 and 5) and wild-type tobacco were harvested in tube and heated at 80°C in water for 1h. Then the water was replaced by 30% HNO_3 and a pinch $KClO_3$. When the treated materials became soft, the liquid was removed. The wood pulp was gained by the mechanical shock after being washed several times with distilled water to remove residual HNO_3. The fiber cell was observed and measured using a microscopy (Liu et al., 2010).

Measurements and statistical analysis

The T_3 transgenic and wild-type tobacco seedlings were grown in a greenhouse under controlled conditions of 16 h light/8 h dark. 2 months later, flower buds were visible in the transgenic and wild type tobacco. Then, the plant switched from vegetative to reproductive growth, and the height of the plant was changed a little. Six homozygous transgenic lines (line1~6) were selected for analyses of plant height (three technical and three biological replicates for each line). All data were analyzed

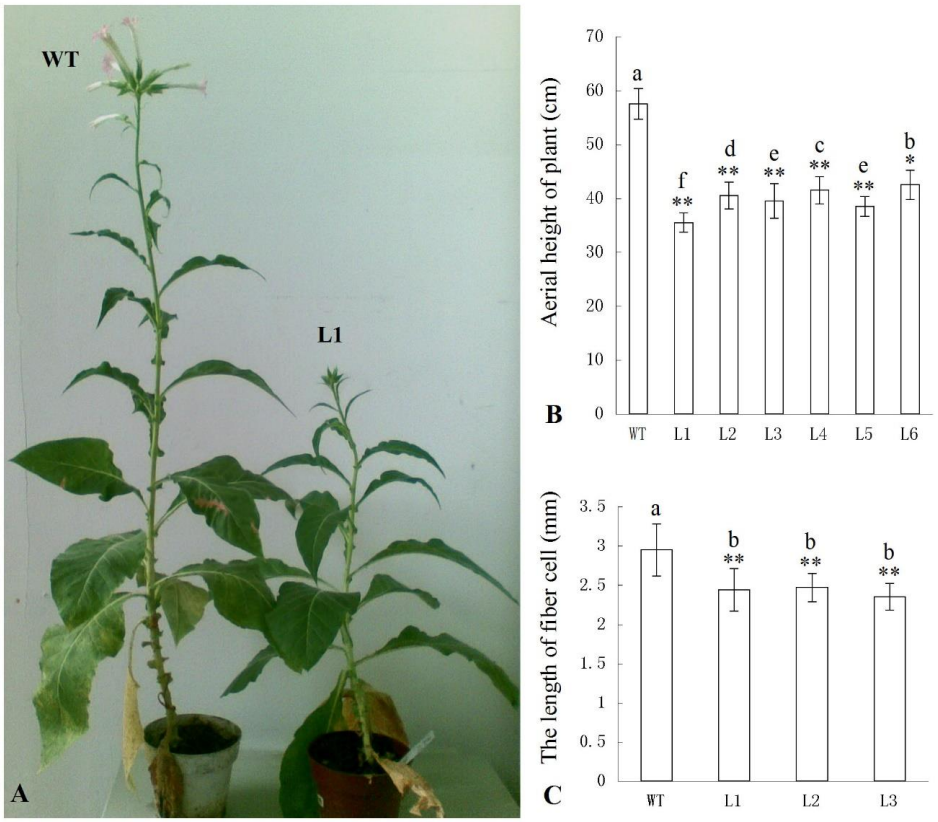

Fig 7. The morphological observation and measurement of wild type and transgenic tobacco. **(A)** The height difference between wild type and transgenic tobacco. WT, wild type; L1, one independent transgenic tobacco; **(B)** Aerial height of wild type and transgenic tobaccos; **(C)** The length of fiber cell from wild type and transgenic tobaccos. Each transgenic line showed significant differences compared with wildtype by SPSS 11.5 analysis (Student's t-test, p≤0.05 and 0.01); Values are expressed as means (n=3 samples for each test); error bars denote SD. ** p ≤0.01 for t test; * p ≤0.05 for t test. Different *letters* above columns indicate significance differences (P ≤0.05) between the mean values, which were determined based on LSD test. WT, wildtype; L1-L6, independent transgenic tobaccos.

using LSD test by SPSS 11.5, and statistical difference was compared based on Student's t test, taking P ≤0.05 (*), P ≤0.01 (**), as significant.

Conclusions

Cellulose is of great economic value as an integral component of plant cell walls. Cellulose synthase (*CesA*) represents enzymes involved in cellulose biosynthesis. Here, we cloned and summarized the poplar *CesA6* gene (No. HQ686077) from *Populus ussuriensis*. The domain structures of PuCesA6 were predicted by multiple alignment analysis, which contained many plant special domains. PuCesA6 protein was localized in the cytomembrane by particle bombardment. Ectopic expression of *PuCesA6* in tobacco significantly exhibited "dwarf" phenotype. Finally, identification of new *CesA* genes from poplar tree genomes may contribute to a better understanding of cellulose biosynthesis in trees.

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