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RESEARCH PAPER

Introduction of the rice *CYP714D1* gene into *Populus* inhibits expression of its homologous genes and promotes growth, biomass production and xylem fibre length in transgenic trees

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Abstract

The rice (*Oryza sativa*) *OsCYP714D1* gene (also known as *EUI*) encodes a cytochrome P450 monooxygenase which functions as a gibberellin (GA)-deactivating enzyme, catalysing 16α, 17-epoxidation of non-13-hydroxylated GAs. To understand whether it would also reduce the production of active GAs and depress the growth rate in transgenic trees, we constitutively expressed *OsCYP714D1* in the aspen hybrid clone *Populus alba×P. berolinensis*. Unexpectedly, ectopic expression of *OsCYP714D1* in aspen positively regulated the biosynthesis of GAs, including the active GA_1 and GA_4, leading to promotion of the growth rate and biomass production in transgenic plants. Transgenic lines which showed significant expression of the introduced *OsCYP714D1* gene accumulated a higher GA level and produced more numerous and longer xylem fibres than did the wild-type plants. Quantitative real-time PCR indicated that transcription of most homologous *PtCYP714* genes was suppressed in these transgenic lines. Therefore, the promoted GA and biomass production in transgenic trees constitutively expressing *OsCYP714D1* is probably attributed to the down-regulated expression of the native *PtCYP714* homologues involved in the GA biosynthesis pathway, although their precise functions are yet to be further elucidated.

Key words: Aspen, biomass, gibberellin, *OsCYP714D1*, *Populus*, transgenic plants.

Introduction

In plants, gibberellins (GAs) play crucial roles in shoot elongation and reproductive development, including stem elongation, leaf expansion, seed germination, and flowering. Most genes involved in the biosynthesis and catabolism pathways of GAs have been identified, especially in the model plant species *Arabidopsis thaliana* and rice (*Oryza sativa*) (Hedden and Phillips, 2000; Olszewski *et al*., 2002; Sun and Gubler, 2004). Also, many mutants with altered GA metabolism or signalling pathways have been studied in *A. thaliana* (Koornneef and van der Veen, 1980; Vabanova *et al*., 2007; Ariizumi *et al*., 2008) and rice (Ueguchi-Tanaka *et al*., 2000; Ikeda *et al*., 2001; Sasaki *et al*., 2003; Sakamoto *et al*., 2004). These GA-related mutants have been used to identify the key components in GA metabolism and signalling pathways, and some of them have successfully contributed to the green revolution to confer useful agronomic traits in cereals (Peng *et al*., 1999; Sasaki *et al*., 2002; Spielmeyer *et al*., 2002).

Previous studies have shown that GAs are biosynthesized from geranylgeranyl diphosphate, and three types of enzymes, plastid-localized terpene cyclases, membrane-bound

cytochrome P450 monooxygenases (P450s), and soluble 2-oxoglutarate-dependent dioxygenases (2ODDs), are responsible for the conversion of geranylgeranyl diphosphate into bioactive GAs, such as GA_1 and GA_4 (Zhu *et al*., 2006). The bioactive GA_1 and GA_4, and their immediate precursors GA_{20} and GA_9, respectively, are further deactivated by GA 2-oxidases (GA2oxs) (Thomas *et al*., 1999; Yamaguchi and Kamiya, 2000; Olszewski *et al*., 2002). In *Arabidopsis*, AtGA2ox7 and AtGA2ox8 also catalyse the 2-oxidation of C_{20}-GAs (Schomburg *et al*., 2003; Lee and Zeevaart, 2005).

To date, a few loss-of-function mutants of the GA2ox gene family members have been recognized (Hedden and Phillips, 2000; Sakamoto *et al*., 2004). In pea (*Pisum sativum*), the loss-of-function mutation in the *PsGA2ox1* gene causes a tall phenotype (Lester *et al*., 1999). In rice, the recessive tall rice mutant *elongated uppermost internode* (*eui*) (Rutger and Carnahan, 1981), which demonstrates a rapid and enhanced elongation of internodes, has been used to improve the heading performance of male strerile cultivars genetically (Shen and He, 1989; He and Shen, 1991, 1994; Yang *et al*., 2002). Recently, the *EUI* gene was isolated. Map-based cloning reveals that *EUI* encodes a previously uncharacterized P450, CYP714D1, that acts as a GA-deactivating enzyme through 16α, 17-epoxidation of 13-hydroxylated GAs (Zhu *et al*., 2006).

In rice, 16α, 17-$(OH)_2$-GA_4 has been identified as a metabolite of GA_4 exogenously applied to seedlings (Kobayashi *et al*., 1993), and as an aglycone of a GA glucoside in anthers (Hasegawa *et al*., 1994). The 16α, 17-$(OH)_2$-GAs were also found in other plant species, such as *Pisum sativum* fruits (Santes *et al*., 1995), *Lupinus albus* seeds (Gaskin *et al*., 1992), developing *Malus domestica* seeds (Hedden *et al*., 1993), *Cibotiumglaucum* sporophytes (Yamane *et al*., 1988), *Prunus avium* seedlings (Blake *et al*., 2000), and *Populus trichocarpa* capsules (Pearce *et al*., 2002). However, whether these 16α, 17-$(OH)_2$-GAs are produced via 16α, 17-epoxy GAs by EUI-related enzymes is still unclear in these plant species, especially in the model woody plant *Populus trichocarpa*. In this work, the rice *OsCYP714D1* gene was constitutively expressed in aspen. It was found that ectopic expression of *OsCYP714D1* in transgenic aspen positively regulated the biosynthesis of GAs, leading to promotion of the growth rate and biomass production. Moreover, transcription of most homologous *PtCYP714* genes was suppressed in the fast-growing transgenic lines. These findings will aid in future attempts to engineer the growth trait by manipulating biosynthesis of GAs in aspen, possibly as well as in other plants.

Materials and methods

Plasmid construct and plant transformation

The plant vector was generously provided by Professor Zuhua He (Shanghai Institutes for Biological Sciences, CAS, China). The 1.9 kb rice *OsCYP714D1* (*OsEui*) full-length cDNA, driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter and terminated by the NOS terminator, was inserted into the *Eco*RI and *Hin*dIII sites in pCAMBIA1301 (http://www.cambia.org) (Zhu *et al*., 2006). The construct was introduced into the *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method (Wise *et al*., 2006) for aspen transformation.

In this study, the Yinzhong (*P. alba*×*P. berolinensis*) hybrid clone, which is commercially grown in the north part of China, was used for plant transformation. Generally, *in vitro* grown plants were subcultured monthly by aseptically transferring shoot apices to fresh MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg l^{-1} naphthaleneacetic acid (NAA). Plantlets were grown in the tissue culture room with cool white fluorescent light (~200 μmol m^{-2} s^{-1}) under short day conditions (12 h light/12 h dark). The temperature was kept at 21–25 °C in the day time and at 15–18 °C at night. The *OsCYP714D1* gene was transformed into the hybrid aspen as described previously (Wang *et al*., 2011). Independently regenerated transgenic lines were propagated, potted, and grown in the greenhouse.

PCR, reverse transcription–PCR (RT–PCR), and quantitative real-time PCR

For PCR analyses, genomic DNA was isolated from fresh leaves (~500 mg for each sample) of 1-month-old tissue cultured wild-type (WT) and regenerated transgenic lines as described previously (Kang *et al*., 2010). Gene-specific primers (forward, 5'-CGCGGGCTTGCTTTGGGAGTGA-3'; and reverse, 5'-CCGCC GCAGACCTCGAGCACCT-3') and GC buffer (TaKaRa, Japan) were used to amplify a 463 bp PCR product.

For RT–PCR analyses, total RNA was isolated from shoots of 1-month-old tissue cultured WT and transgenic plants with the RNAiso Reagent (TaKaRa, Japan). After treatment with DNase I (Promega), 2 μg of total RNA was subjected to reverse transcription using the reverse transcriptase ReverTra Ace (TOYOBO, Japan) at 42 °C for 1 h. The resultant cDNA was then used for RT–PCR with gene-specific primers. GC buffer and gene-specific primers were the same as used for PCR analysis (38 cycles). The elongation factor gene *PtEF1β* was employed as an internal control (25 cycles). The primers for *PtEF1β* are: forward 5'-GACAAGAAGGCAGCGGAGGAGAG-3' and reverse 5'-CAA TGAGGGAATCCACTGACACAAG-3'.

Quantitative real-time RT–PCR was performed with the RotorGene 3000 system (Corbett Research) using the SYBR Green Realtime PCR Master Mix (TOYOBO, Japan) to monitor double-stranded DNA products. Data analysis was performed with Rotor-Gene software version 6.0, and relative amounts of mRNA were calculated based on the comparative threshold cycle method. The relative expression of each target gene was normalized using the housekeeping gene *PtEF1β*, and the expression value of the WT was set to 1. The primers used in this research are shown in Supplementary Table S1 available at *JXB* online.

β-Glucuronidase (GUS) activity analysis

Histochemical GUS staining was conducted as described previously (Gallagher, 1992). Briefly, leaf explants were incubated overnight at 37 °C in a reagent mix containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM each of potassium ferri- and ferrocyanide, 10 mM EDTA (pH 7.0), and 0.1% Triton X-100. After staining, the tissues were cleared of chlorophyll with 95% ethanol.

Southern blot analysis

For Southern blot analysis, 10 μg of genomic DNA isolated from the leaves of WT and transgenic plants was digested with *Eco*RI, electrophoresed on 0.8% agarose gels, and transferred onto Hybond N^+ nylon membranes with a Whatman Biometra® Vacuum-Blot System. The 463 bp PCR product of *OsCYP714D1* was used as a hybridization probe. Standard procedures for Southern blot analysis and probe labelling were conducted with a DIG DNA Labeling and Detection Kit1 (Roche, Germany) following the manufacturer's instructions.

Growth measurement and sample collection

Rooted WT and transgenic plants were transplanted into soil and maintained in the greenhouse. Plants were watered every 2 d and fertilized with a complete nutrient solution once a week. After 2 weeks, healthy plants (at least eight individuals for each line) were marked at the base internode. This was used as a reference point for measuring the growth of plants. The height was counted from the top to the reference point every week for a period of 6 weeks. The number of leaves and internodes of 8-week-old plants were counted, with the first internode being defined as that below the first leaf of at least 1 cm length in the apex. The average of internodes (10th, 11th, and 12th) with the upper leaves was used to measure the diameter of stems, and the length of internodes and petioles, respectively.

For GA content analysis, the upper leaves of the 11th internode were sampled and immediately frozen in liquid nitrogen. For anatomical studies, the 11th internode was excised and immediately fixed in FAA solution. The 18th internode was excised and chilled on ice immediately for subsequent fibre length measurements. All of the remaining part after sampling was separated into shoot (including stem and leaves) and root fractions, and used for determination of the fresh weight, and then, after drying at 65 °C for 5 d, for dry weight determination.

Anatomical characterization

Following conventional chemical fixation in FAA and dehydration in an ethanol series, samples (the 11th internode of each line) were embedded in paraffin. Transverse or longitudinal sections of 8 μm thickness were cut on a microtome (Leica, Germany). The paraffin-embedded sections were dried for 2 d at 42 °C and then dewaxed and re-hydrated as follows. The sections were immersed in dimethylbenzene for 5–10 min and this was repeated once with fresh dimethylbenzene. Then, the sections were immersed in 50% dimethylbenzene (diluted in absolute ethyl alcohol) followed by a gradient of ethyl alcohol (100, 95, 85, and 70%) to re-hydrate. For all of the solutions, the immersion time was 5–10 min. Following that, the sections were immediately dipped into 0.2% toluidine blue (0.2 g of toluidine blue dissolved in 60 ml of ethyl alcohol and 40 ml of distilled water) for 10 s and then washed in water twice (5–10 s each time). The dyed sections were used for microexamination. Images were captured under bright field using an ECLIPSE 80i microscope. Using the UTHSCSA Image Tool software, the radial width of the outer bark, phloem, cambium, and xylem, the thickness of the secondary cell wall in xylem and phloem fibre, and the length of outer bark cells were measured, and the numbers of xylem cells (for transverse sections) and outer bark cells (for longitudinal sections) were counted.

Fibre length measurements

For fibre length measurements, trimmed pieces of outer xylem from the 18th internode of 2-month-old plants grown in the greenhouse were prepared. The samples were macerated in a boiling solution of 10% hydrogen peroxide and 50% glacial acetic acid for 4–6 h, rinsed with distilled water three times, neutralized with sodium carbonate, and washed again in water. Finally, the fibres were separated from each other in water, and measured under an ECLIPSE 80i microscope. The lengths of at least 300 fibres per sample were measured.

GA and IAA content determination

Sampled leaves were homogenized in liquid nitrogen using a mortar and pestle, and then lyophilized. Samples of 2 g dry weight (DW) were purified and finally analysed at Wuhan University (China) as described previously (Chen *et al.*, 2011). [$^2H_2$]GA_1 (1 ng g^{-1}), [$^2H_2$]GA_4 (10 ng g^{-1}), [$^2H_2$]GA_9 (10 ng g^{-1}), [$^2H_2$]GA_{12} (30 ng g^{-1}), [$^2H_2$]GA_{53} (50 ng g^{-1}), and [$^2H_5$]indole acetic acid (IAA; 10 ng g^{-1}) were added to plant samples as internal standards and the recovery was 87.1–108.6%. Except for GA_1, which was detected at low concentration in three transgenic lines L4, L13, and L32, but not detected in the WT and line L23, significant differences in IAA, GA_{12}, GA_9, GA_4, and GA_{53} between transgenic plants and the WT were analysed using Student's *t*-test at $P < 0.05$. Values are means ±SD of three biological replicates of three individual plants from the WT or the same transgenic line.

Statistical analysis

For statistical analyses, the Student's *t*-test was used to generate every *P*-value. The tests were one-tailed. The data were normalized and all samples were normally distributed with homogeneity of variance.

Results

Constitutive expression of OsCYP714D1 *in* P. alba×P. berolinensis

A construct containing the open reading frame of *OsCYP714D1* (Fig. 1A) was introduced into the genome of the Yinzhong (*P. alba*×*P. berolinensis*) hybrid clone by *Agrobacterium tumefaciens*-mediated transformation. More than 20 independently regenerated lines were obtained and seven transgenic lines (L4, L6, L13, L23, L29, L32, and L33) were identified by PCR analyses. The expected 463 bp band was detected in all the checked transgenic lines (Fig. 1B). RT–PCR and GUS staining analyses further confirmed the expression of *OsCYP714D1* and *Gus* in transgenic plants (Fig. 1C, D). Transgenic plants were subsequently transferred to pots and grown in the greenhouse for further experiments.

Constitutive expression of OsCYP714D1 *significantly promoted the growth of transgenic plants*

Previous studies have shown that when *OsCYP714D1* was constitutively expressed in rice (driven by the 35S promoter), transgenic rice showed severe dwarfism and failed to set grain (Zhu *et al.*, 2006). To understand the functions of *OsCYP714D1* in poplar development, transgenic poplar plants constitutively expressing *OsCYP714D1* were generated. Among the seven independently derived and PCR-confirmed transgenic lines (Fig. 1B), four lines with different *OsCYP714D1* expression levels (lines L4, L13, L23, and L32) were chosen for the following studies (Fig. 1C). After 8 weeks growth in the greenhouse, significant growth phenotype differences were observed between WT and transgenic plants (Fig. 2A–D). The growth of three transgenic lines (L4, L13, and L32), which showed significant expression of *OsCYP714D1*, was faster than that of the WT in terms of plant height (Fig. 3A), leaf number (Fig. 3B), stem diameter (Fig. 3C), stem internode and leaf petiole length (Fig. 3D), and shoot fresh and dry weights (Fig. 3E, F), whereas little difference was found between the WT and transgenic line L23, which showed almost undetectable expression of *OsCYP714D1* (Figs 2, 3). To confirm that the *OsCYP714D1* gene was inserted into the aspen genome, Southern blot

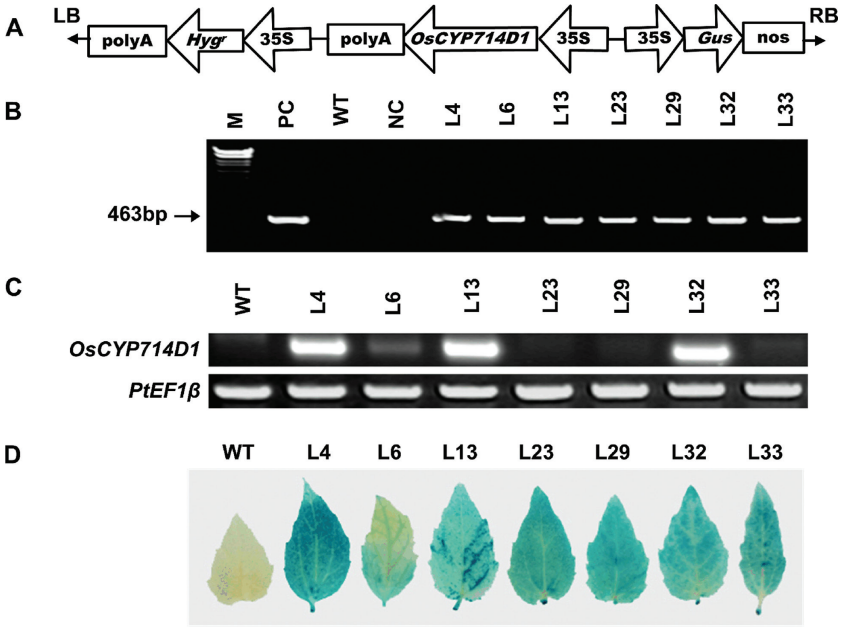

Fig. 1. Structure of the plant expression vector and confirmation of transgenic plants. (A) Schematic representation of T-DNA regions in the plant expression vector pCAMBIA1301-OsCYP714D1. LB, T-DNA left border repeat; 35S, CaMV 35S promoter; *Gus*, β-glucuronidase gene; nos, nopaline synthase gene terminator; *Hyg*, hygromycin phosphotransferase gene; RB, T-DNA right border repeat. (B) PCR confirmation of transgenic plants. A 463 bp PCR product was detected in all transgenic lines. M, λ-EcoT14 I digest DNA markers; PC, PCR product with pCAMBIA1301-OsCYP714D1 plasmid DNA as template; WT, PCR product with wild-type plant genomic DNA as template; NC, PCR product with double-distilled water as template; L4–L33, PCR products with genomic DNA from regenerated hygromycin-resistant Yinzhong yang plants as template. (C) RT–PCR confirmation of *OsCYP714D1* transcripts in the wild type (WT) and different lines (L4–L33). Total RNA was isolated from 1-month-old WT and different transgenic lines (L1–L16). The *PtEF1*β gene was used as an internal control. RT–PCR was performed with *OsCYP714D1*-specific primers or *PtEF1*β-specific primers. (D) Histochemical GUS staining of WT and transgenic (L4–L33) leaves of Yinzhong aspen. (This figure is available in colour at *JXB* online.)

analyses were performed. Most transgenic plants had 1–2 copies of the transgene (Fig. 2E).

Constitutive expression of OsCYP714D1 *increased xylem cell number, fibre cell wall thickness, and xylem fibre cell length*

A detailed anatomical study of the three transgenic lines L4, L13, and L32 was performed. Compared with the WT control, the transgenic lines showed not only enhanced radial width of the stem and xylem, but also thickened phloem fibre cell walls and an elongated longitudinal length of the outer bark cells (Supplementary Fig S1 at *JXB* online). The observed differences were further confirmed by measurements by microscopy (Fig. 4). The stem diameter growth was primarily due to the widened xylem since there was no obvious difference between the WT control and transgenic lines in the cambium zone, phloem, and outer bark (Fig. 4A). Moreover, the widened xylem was mainly caused by increased xylem cell number (Fig. 4B). The difference in growth in height in the transgenic plants, compared with the WT control, was primarily caused by the increased lengths of the xylem fibre cells (Fig. 4C) and the collenchyma and parenchyma cells (Fig. 4E). In contrast to the changes in the xylem, the main difference in the phloem of the transgenic plants when compared with the WT control was the thickened phloem fibre cell walls (Fig. 4D).

GA accumulation was higher in transgenic plants

GA content was determined in the leaves of actively growing WT and transgenic plants. Transgenic plants showed high levels of the early 13-hydroxylated C_{19} GAs (GA_{53} and GA_1) and the non-13-hydroxylated C_{19} GAs (GA_{12}, GA_9, and GA_4). The levels of the biologically active GA_4 in transgenic lines L4, L13, and L32 were pronouncedly higher than in the control plants. The level of GA_1 was relatively low in transgenic lines, but was undetectable in the control plants (Fig. 5).

Expression of most GA pathway genes was changed in transgenic plants

The expression of most genes involved in GA biosynthesis and catabolism pathways was examined by quantitative real-time PCR. Four GA biosynthesis genes (three GA20oxs, *PtGA20ox1*, *PtGA20ox3*, *PtGA20ox4*; and one GA3ox, *PtGA3ox1*), two GA2ox GA catabolism genes (*PtGA2ox1* and *PtGA2ox2*), one GA receptor gene (*PtGID1*), and two GA suppressor genes (*PtRGL1-1* and *PtGAI2*) involved in GA signalling were analysed (Fig. 6). For the genes involved in GA synthesis, *PtGA20ox3* and *PtGA3ox1* were down-regulated in transgenic lines L4, L13, and L32 compared with the controls. However, the *PtGA20ox4* gene was slightly up-regulated in all four transgenic lines. No distinct change

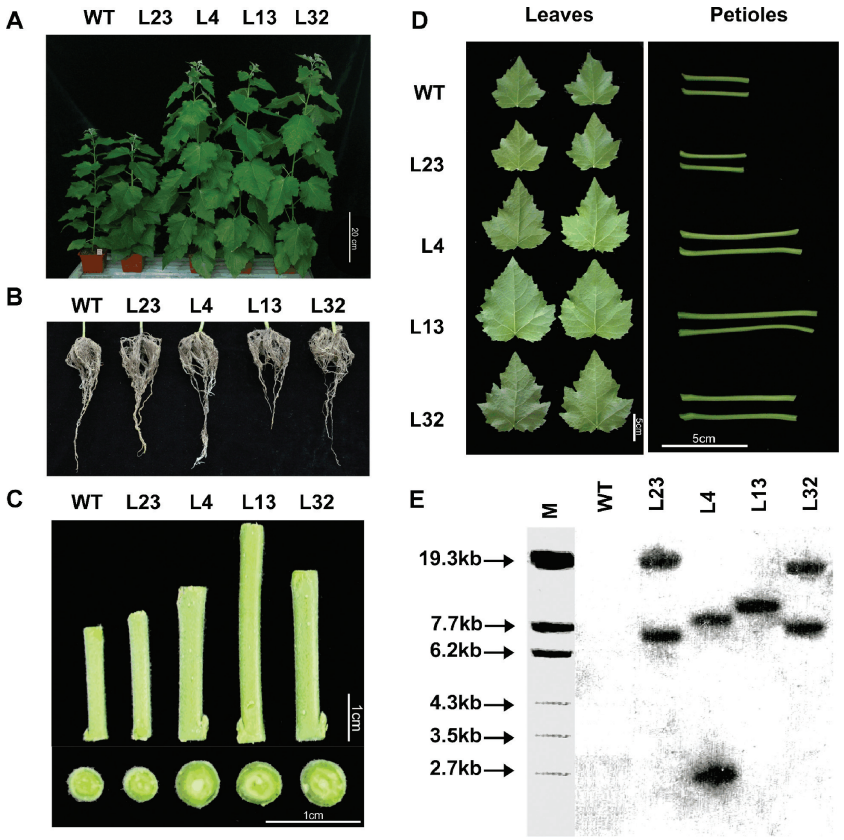

Fig. 2. Phenotypes and Southern blot analyses of 2-month-old wild-type and transgenic plants constitutively expressing *OsCYP714D1.* WT, wild type; L23, a negative control transgenic line with very low expression of *OsCYP714D1*; L4, L13, and L32, three independent transgenic lines with high expression of *OsCYP714D1.* (A) Shoot growth phenotypes. (B) Root phenotypes. (C) The 12th internodes count from the apex. (D) Leaves and petioles of the 12th internodes. (E) Southern blot analyses. Genomic DNA was digested with *Eco*RI, electrophoresed, and probed with a DIG-labelled 463 bp PCR product of the *OsCYP714D1* gene. The number of bands reflects the number of transgene insertions. DNA molecular weight markers are shown on the left. (This figure is available in colour at *JXB* online.)

was observed in the expression of *PtGA20ox1*. For the genes involved in GA catabolism, *PtGA2ox2* was slightly down-regulated in transgenic lines L4, L13, and L32, in contrast to the negative control L23 in which the gene was up-regulated compared with the wild type. *PtGA2ox1* showed no obvious changes between the WT and transgenic plants, except for L32, in which the expression of *PtGA2ox1* was strongly increased. As a key GA receptor gene, *PtGID1* expression was increased in L4 and L32 but not in L13. As for the two suppressor genes, down-regulated expression was observed in L4 and L13 for *PtRGL1-1*, but only in L13 for *PtGAI2*.

Expression of PtCYP714 homologues was down-regulated in transgenic plants

To gain insight into the gene expression changes that might be associated with the increased GA accumulation, altered GA pathway gene expression, and the dramatically increased growth of transgenic plants, the expression levels of the native homologous genes of *OsCYP714D1* in *Populus* were analysed. The database of *P. thichocarpa* Jamboree Gene Models at http://genome.jgi-psf.org/pages/blast.jsf?db=Poptr1_1 was blasted with the protein sequence of OsCYP714D1. About 100 hits were found, and the six with the highest homology were named PtCYP714A3, PtCYP714E2, PtCYP714E4, PtCYP714E5, PtCYP714E6, and PtCYP714F1 according to The P450 Homepage (http://drnelson.uthsc.edu) (Supplementary Fig. S2 at *JXB* online). Since the location of PtCYP714E5 and PtCYP714E6 is adjacent and their transcripts share 99% homology, PtCYP714E6 is regarded as a duplicate of PtCYP714E5. Therefore, *PtCYP714A3*, *PtCYP714E2*, *PtCYP714E4*, *PtCYP714E5*, and *PtCYP714F1* were selected for further analyses. It was observed that the transcriptions of most homologous *PtCYP714* genes were down-regulated in these transgenic lines (Fig. 7).

Discussion

As a large group of tetracyclic diterpenes, GAs can be deactivated by conjugation (Schneider and Schliemann, 1994), epoxidation (Zhu *et al.*, 2006), methylation (Varbanova *et al.*, 2007), and 2-oxidation. Map-based cloning has revealed that the *EUI* gene encodes a previously uncharacterized P450, CYP714D1, which catalyses 16α, 17-epoxidation of non-13-hydroxylated GAs in rice. At the heading stage, the *eui* mutant exhibited an extremely elongated uppermost

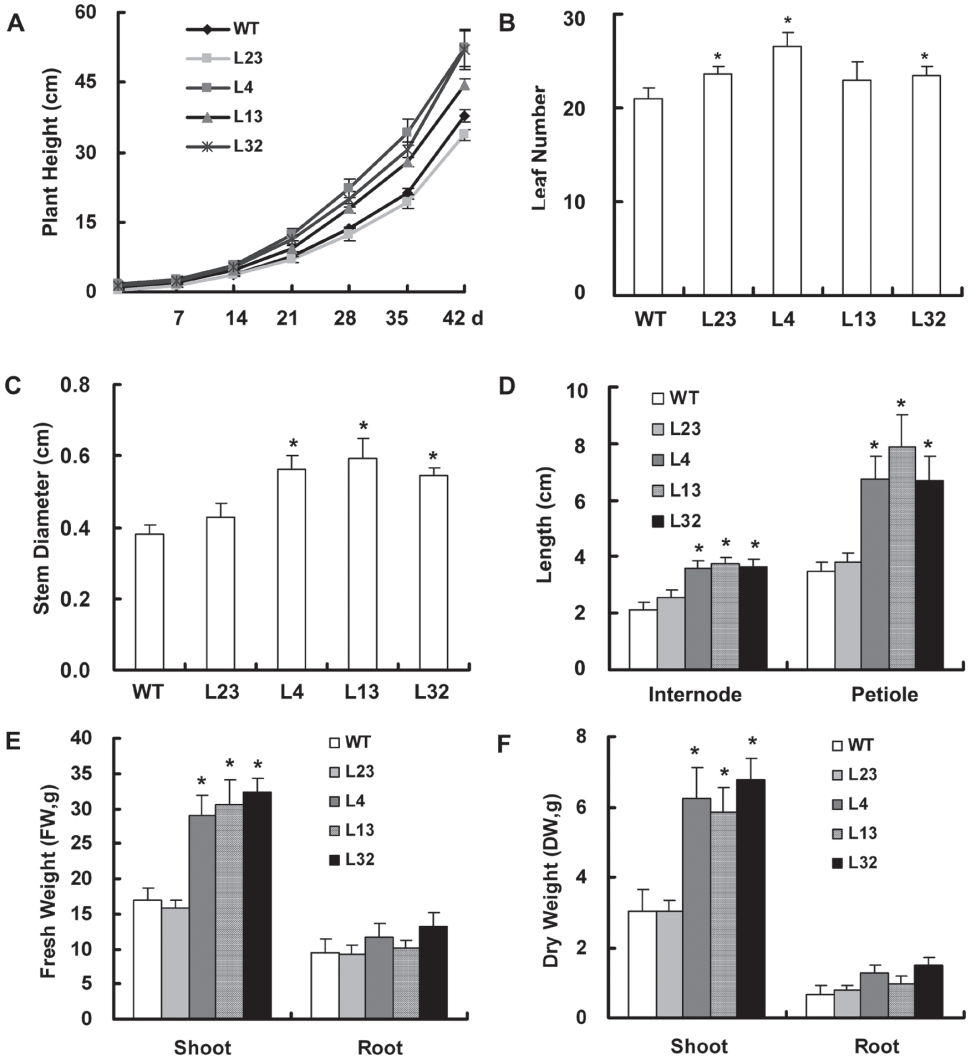

Fig. 3. Enhanced growth of transgenic plants constitutively expressing *OsCYP714D1*. (A) Cumulative shoot elongation of various transgenic *OsCYP714D1*-expressing lines. (B–D) Leaf number, stem diameter, internode and petiole length, and fresh and dry weight comparison of wild-type and transgenic lines. After generation from tissue culture and potting, plants were grown in the greenhouse for 6 weeks (at time zero). The 10th, 11th, and 12th internodes and petioles counting from the apex were used. Fresh weights were measured immediately after harvest. For dry weight analyses, the materials were dried out at 65 °C for 5 d. Shoot weights include stems and leaves only. WT, wild-type control; L23, negative control; L4, L13, L32, three *OsCYP714D1* transgenic lines. Values are means ±SD of 10 individual plants with three independent biological replicates. * indicates significant differences in comparison with the WT at $P < 0.05$ (Student's *t*-test).

internode and accumulated extremely high levels of bioactive GAs in the uppermost internode. In contrast, transgenic rice overexpressing *OsCYP714D1* exhibited a severely dwarfed phenotype (Zhu *et al*., 2006). Unlike the transgenic rice, transgenic aspen constitutively expressing *OsCYP714D1* showed an extremely fast growing phenotype (Figs 2, 3).

Modification of GA biosynthesis has been successfully applied to crop breeding (Sakamoto *et al*., 2003). GA20ox has been well established as a key enzyme in the biosynthesis of the plant hormone GA, and in the regulation of GA-controlled plant growth (Hedden and Kamiya, 1997). GA20ox-overexpressing plants showed an improved growth rate and biomass, and produced high levels of the 13-hydroxylated C_{19} GAs (GA_{20}, GA_1, and GA_8) and the non-13-hydroxylated C_{19} GAs (GA_9, GA_4, and GA_{34}) in both internodes and leaves (Eriksson *et al*., 2000). Overexpression of *PtGID1*, a GA receptor gene, in transgenic poplars also led to rapid growth, and increased elongation and xylogenesis (Mauriat and Moritz, 2009). Since OsCYP714D1 is involved in the deactivation of GAs (GA_4, GA_9, and GA_{12}) in rice (Zhu *et al*., 2006), it was thus speculated that the fast growing phenotypes of *OsCYP714D1* transgenic plants could also be attributed to the increased GA production. To this end, the GA contents in both WT and *OsCYP714D1* transgenic lines were measured. Similarly, compared with the WT control, *OsCYP714D1* transgenic plants accumulated high levels of the early 13-hydroxylated GAs (GA_{53} and GA_1) and the non-13-hydroxylated GAs (GA_{12}, GA_9, and GA_4) (Fig. 5).

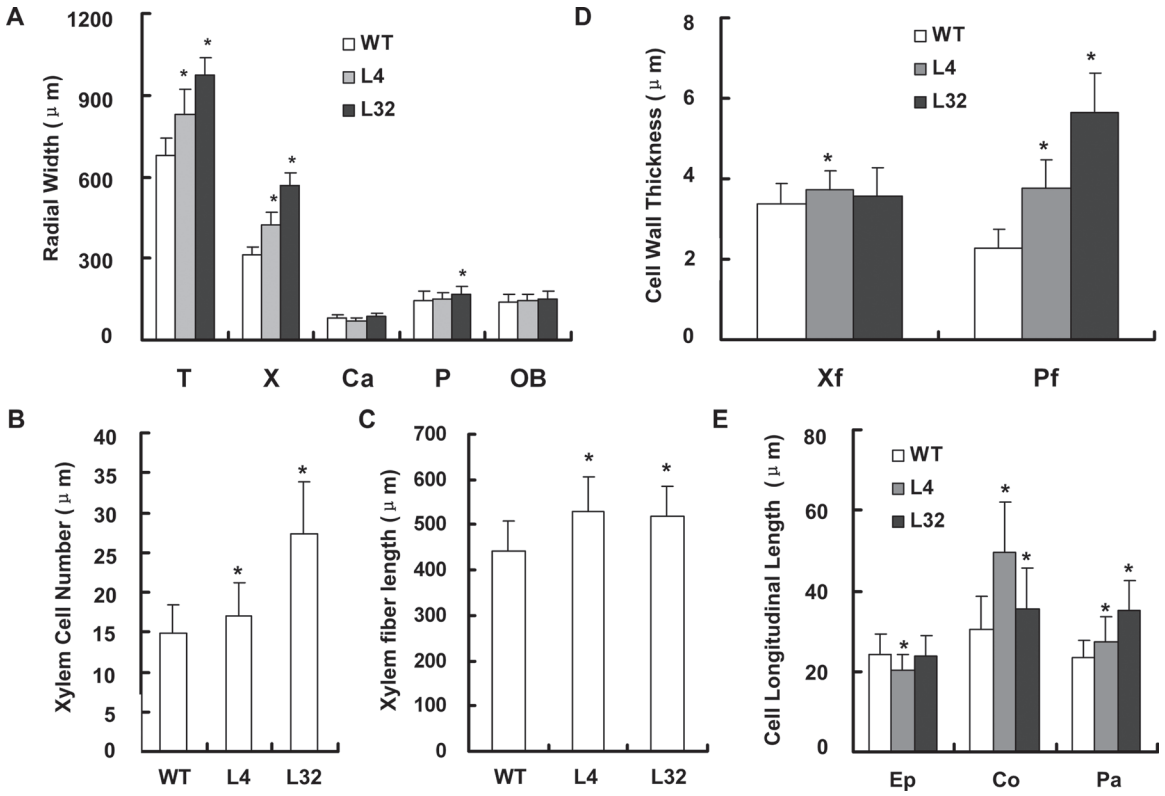

Fig. 4. Effects of *OsCYP714D1* expression on stem radial growth, xylem cell number, fibre cell wall thickness, and outer bark cell and xylem fibre cell length of 2-month-old wild-type (WT) and *OsCYP714D1* transgenic plants (lines L4 and L32). (A–E) Average values with standard deviations for radial width (A), xylem cell number (B), xylem fibre length (C), cell wall thickness of xylem fibres (Xf) and phloem fibres (Pf) (D), and cell length of outer bark (E). Values shown are means ±SD of at least 10 tissues (for radial width and xylem cell number) or 30 cells (for cell wall thickness, cell length of outer bark) or 300 cells (for xylem fibre length) of WT and transgenic plants (L4 and L32). T, total value of xylem, cambium, phloem, and outer bark; OB, outer bark; P, phloem; Ca, cambium; X, xylem; Xf, xylem fibre; Pf, phloem fibre; Ep, epidermis; Co, collenchyma; Pa, parenchyma. * indicates significant differences in comparison with the WT at $P < 0.001$ (Student's *t*-test).

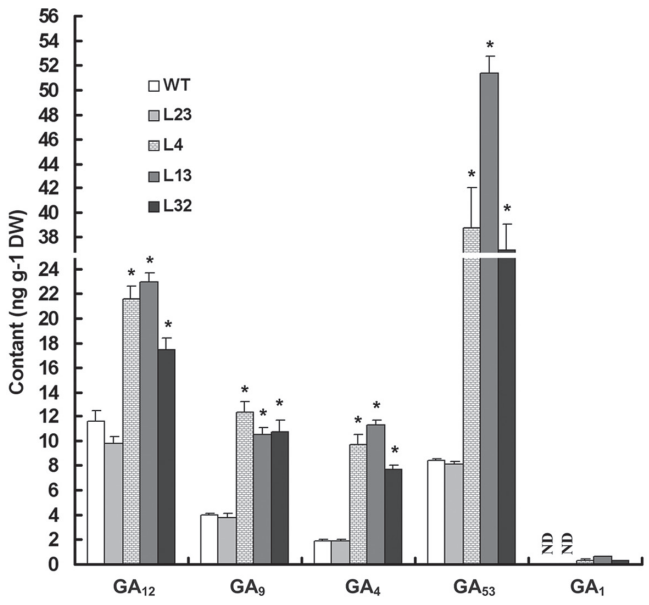

Fig. 5. Endogenous GA levels in the leaves of 2-month-old wild-type and *OsCYP714D1* transgenic plants. WT, wild type; L23, a negative control transgenic line with very low expression of *OsCYP714D1*; L4, L13, and L32, three independent transgenic lines with high expression of *OsCYP714D1*. Values are means ±SD of three biological replicates of three individual plants from the WT or the same transgenic line. ND, not detected. * indicates significant differences in comparison with the WT at $P < 0.05$ (Student's *t*-test).

To clarify the possible reasons for the increased GA accumulation in *OsCYP714D1*-overexpressing plants, the transcripts of GA pathway genes were investigated (Fig. 6). Complex variations in expression were observed among these genes. Two GA oxidase genes (*GA20ox3* and *GA3ox1*) involved in GA biosynthesis were down-regulated. This could be a consequence of feedback down-regulation by the increased GA accumulation in *OsCYP714D1*-overexpressing plants. On the other hand, the expression of another GA20ox gene (*GA20ox4*) was increased in all the transgenic plants, including the negative control line L23, which implies that although *GA20ox4* might have a function or regulation mechanism different from *GA20ox3*, it is not the key factor that increases GA accumulation in transgenic plants. Similarly, different changes in expression were observed with *GA2ox1*

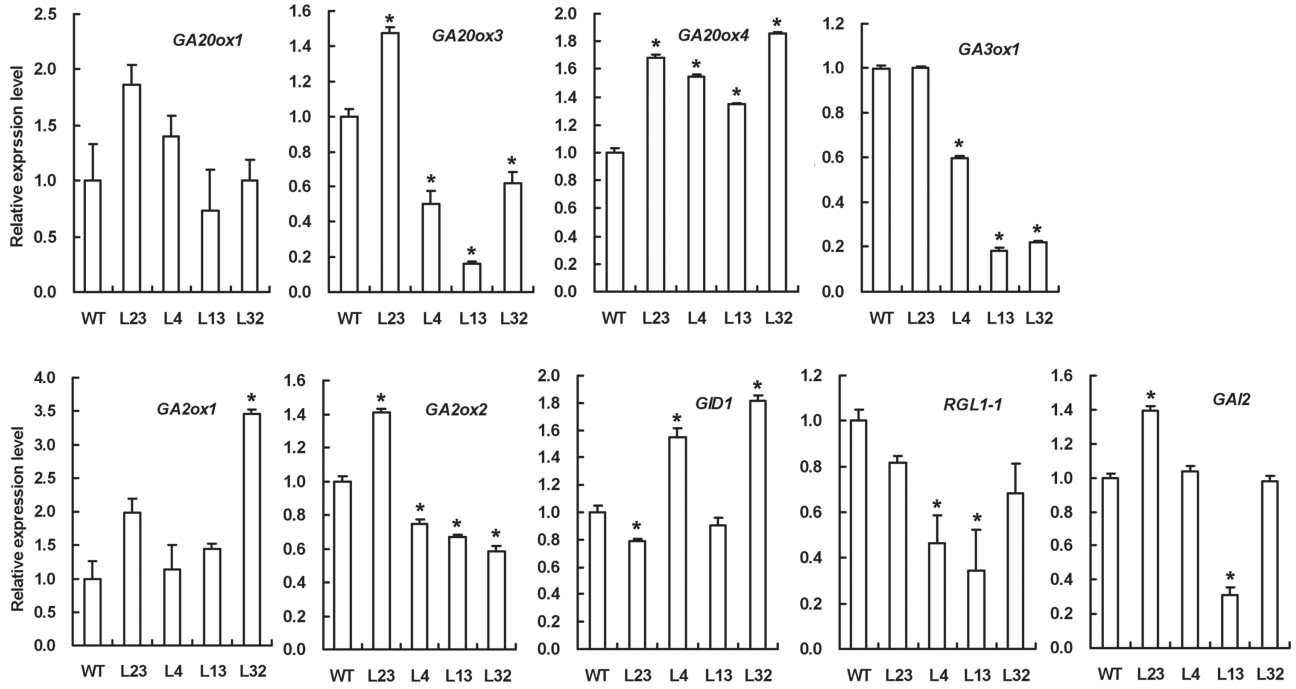

Fig. 6. Quantitative real-time PCR analyses of GA pathway genes in wild-type and *OsCYP714D1* transgenic plants. WT, wild type; L23, a negative control transgenic line with very low expression of *OsCYP714D1*; L4, L13, and L32, three independent transgenic lines with high expression of *OsCYP714D1.* Total RNA was extracted from leaves of 2-month-old WT and transgenic plants grown in the greenhouse, and reverse transcribed to cDNA for quantitative PCR amplification. Transcript levels were normalized using the housekeeping gene *PtEF1*β, and the expression value of *PtEF1*β was set to 1. Error bars represent the SD (*n*=3). *PtGA20ox1*, *PtGA20ox3,* and *PtGA20ox4*, PtGA 20-oxidase genes; *PtGA3ox1*, PtGA 3-oxidase gene; *PtGA2ox1* and *PtGA2ox2*, PtGA 2-oxidase genes; *PtGID1*, a soluble GA receptor gene; *PtRGL1-1* (a homologue of the *Arabidopsis RGL* gene) and *PtGAI2* (a homologue of the *Arabidopsis GAI* gene), putative DELLA proteins. The GenBank accession numbers are as follows: *PtGA20ox1* (AJ001326.2), *PtGA20ox3* (XM_002306627.1), *PtGA20ox4* (XM_002306626.1), *PtGA2ox1* (AY392094.1), *PtGA3ox1* (AY433958), *PtGA2ox2* (BU877509); *PtGID1* (XM_002328371.1), *PtRGL1-1* (eugene3.00040654), and *PtGAI2* (eugene3.00101036).

and *GA2ox2*, two GA2ox genes involved in GA catabolism (Busov *et al*., 2003). The slight down-regulation of *GA2ox2* in *OsCYP714D1*-overexpressing lines might be caused by the feedback of *OsCYP714D1*. No significant difference in *GA2ox1* expression was observed between the WT and the transgenic plants, except for L32, in which the expression of *GA2ox1* was significantly enhanced. This result might be attributed to the different insertion sites and copy numbers of the exogenous gene among the different transgenic lines. Similar results were also observed in the other analysed genes such as *GID1* and *GAI2*. The soluble GA receptor (GID1) and DELLA proteins are the key mediators of GA response pathways. DELLA proteins act as negative regulators and are degraded in response to GA treatment (Ueguchi-Tanaka *et al*., 2007). The expression of *PtGID1* and two putative DELLA protein genes, *PtGRL1-1* and *PtGAI2*, was also detected. The increased GA accumulation up-regulated the expression of *PtGID1* in transgenic lines L4 and L32, and, at the same time, down-regulated the expression of *PtRGL1-1* in L4 and L13, as well as of *PtGAI2* in L13.

Although the expression levels of most GA pathway genes changed differently in the WT and transgenic plants, it seems that the differences were more likely to be the consequence but not the cause of the increased GA production in the *OsCYP714D1*-overexpressing plants. In *P. deltoides* hybrids and other *Populus* species, different GA members of both the early 13-hydroxylation and non-early 3- or 13-hydroxylation pathways of GA biosynthesis have been detected (Bate *et al*., 1988; Rood *et al*., 1988; Zanewich and Rood, 1994; Eriksson *et al*., 2000; Pearce *et al*., 2002). Therefore, different GA metabolites and different predominant biosynthesis pathways may exist in different *Populus* species. The unexpected fast growing phenotype and high levels of GA accumulation could be caused by the different function of OsCYP714D1 in aspen. OsCYP714D1 may use different substrate(s) in aspen which lead to a higher GA content. Indeed, transgenic plants overexpressing the *Arabidopsis Eui* gene (*AtCYP714A1* and *AtCYP714A2*) also showed different phenotypes from plants overexpressing *OsCYP714D1* when treated with exogenous GAs (Zhang *et al*., 2011). In addition, the CYP714 proteins in aspen share very high homology with OsCYP714D1 and AtCYP714s (Supplementary Fig. S2 at *JXB* online). Therefore, one of the other possibilities is that constitutive expression of *OsCYP714D1* suppressed the expression of *PtCYP714* genes in the transgenic aspen. The down-regulated expression of most *PtCYP714* genes in transgenic plants seems to support this hypothesis (Fig. 7). The down-regulated expression of putative orthologous *Populus CYP714* genes

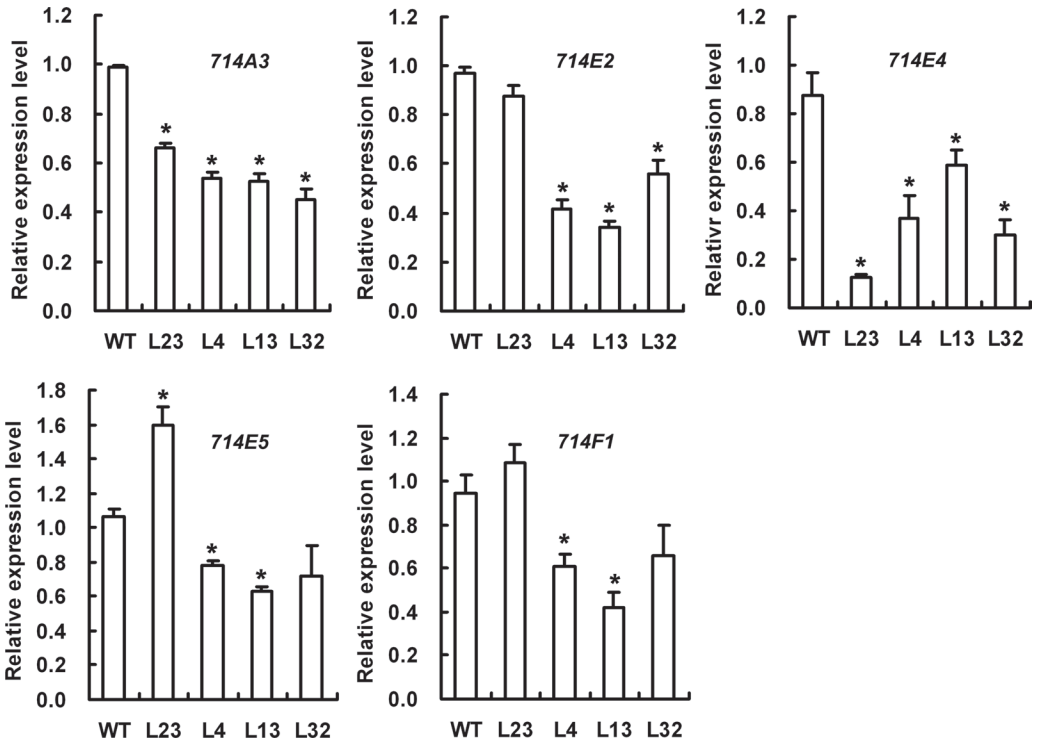

Fig. 7. Quantitative real-time PCR analyses of *OsCYP714D1* homologues in wild-type and *OsCYP714D1* transgenic plants. WT, wild type; L23, a negative control transgenic line with very low expression of *OsCYP714D1*; L4, L13, and L32, three independent transgenic lines with high expression of *OsCYP714D1.* Total RNA was extracted from leaves of 2-month-old WT and transgenic plants grown in the greenhouse, and reverse transcribed to cDNA for quantitative PCR amplification. Transcript levels were normalized using the housekeeping gene *PtEF1*β, and the expression value of *PtEF1*β was set to 1. Error bars represent the SD (*n*=3). The GenBank accession numbers and gene models for the poplar *OsCYP714D1* homologue genes are as follows: *PtCYP714A3* (gw1.125.153.1), *PtCYP714E2* (gw1.40.215.1), *PtCYP714E4* (fgenesh4_pm.C_LG_XIII 00485), *PtCYP714E5* (gw1.VIII.1518.1), and *PtCYP714F1* (fgenesh4_ pg.C_LG_X001058).

may also be a consequence of GA production. Further work on the functional characterization of these CYP714 family members in aspen will help to elucidate whether GA 16α, 17-epoxidation is a common GA deactivation reaction in the GA metabolism pathway in woody plant species.

It has been well documented that GAs are required for xylem fibre cell differentiation (Wareing, 1958; Digby and Wareing, 1966). They profoundly affect the development of secondary xylem fibres as well as both longitudinal and radial growth in hardwood species (Little and Pharis, 1995; Ridoutt *et al*., 1996) and conifers (Wang *et al*., 1995). In the present experiments, overexpression of *OsCYP714D1* in transgenic poplars increased GA contents, and, as a result, increased xylem cell number, and xylem fibre and cortex cell length in the stems. In addition, GA and IAA combination, not GA alone, could affect primary phloem fibre differentiation, including the thickness of phloem fibre cells in *Coleus blumei* (Aloni, 1979). It has also been suggested that a cross-talk between GA and auxin (IAA) exists during the growth and development of poplars (Björklund *et al*., 2007; Gou *et al*., 2010; Mauriat *et al*., 2011). Thus, to clarify the reason for the phloem fibre cell wall thickness in transgenic poplars (Supplementary Fig. S1G–I at *JXB* online), the endogenous IAA content was measured (Supplementary Fig. S3). As expected, the IAA content increased distinctly in the transgenic plants compared with the WT control. This might be attributed to the increased auxin polar transportation in transgenic plants (Björklund *et al*., 2007). Taken together, constitutive expression of the *OsCYP714D1* gene increased GA and IAA production, and consequently promoted the growth of transgenic plants. The substantially increased growth and biomass reported here provide a promising strategy for increasing wood production in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Cellular morphology of the stems of 2-month-old wild-type and *OsCYP714D1* transgenic plants (lines L4 and L32).

Figure S2. The encoded sequences of *PtCYP714A3*, *PtCYP714E2*, *PtCYP714E4*, *PtCYP714E5*, and *PtCYP714F1* from *Populus trichocarpa* genotype Nisqually-1 were aligned with *OsCYP714D1* from rice, and *AtCYP714A1* (At5g24910) and *AtCYP714A2* (At5g24900) from *Arabidopsis*.

Figure S3. Endogenous IAA levels in the leaves of 2-month-old wild-type and *OsCYP714D1* transgenic plants.

Table S1. Gene-specific primers used in this study.

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