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Overexpression of UDP-glucose dehydrogenase from *Larix gmelinii* enhances growth and cold tolerance in transgenic *Arabidopsis thaliana*

N.N. LI[1], L. CHEN[1], X.H. LI[2], Q. LI[1], W.B. ZHANG[2], K. TAKECHI[3], H. TAKANO[3], and X.F. LIN[1]*

College of Life Sciences, Inner Mongolia University, Hohhot 010021, P.R. China[1]
College of Forestry, Inner Mongolia Agricultural University, Hohhot 010019, P.R.China[2]
Graduate School of Science and Technology, Kumamoto University, Kurokami, Kumamoto 860-8555, Japan[3]

Abstract

Uridine diphosphate glucose dehydrogenase (UGDH) plays an important role in biosynthesis of hemicellulose by catalyzing oxidation of UDP-glucose (UDP-Glc) to UDP-glucuronate (UDP-GlcA), a key sugar nucleotide involved in biosynthesis of the plant cell wall. In this study, a *UGDH* ortholog referred to as *LgUGDH* was isolated from *Larix gmelinii* using PCR and rapid amplification of cDNA ends techniques. Real-time PCR shows that the *LgUGDH* gene was expressed primarily in larch stems in addition to its roots and leaves, and Southern blot analysis indicates that UGDH is encoded by two paralogous genes in *L. gmelinii*. Overexpression of *LgUGDH* increased the content of soluble sugars and hemicelluloses and enhanced vegetative growth and cold tolerance in transgenic *Arabidopsis thaliana*. These results reveal that *L. gmelinii* UGDH participates in sucrose/polysaccharide metabolism and cell wall biosynthesis and may be a good candidate gene for enhancing plant growth, cold tolerance, and hemicellulose content.

Additional key words: hemicellulose, real time PCR, RACE, sugars, UDP-glucuronate.

Introduction

Uridine diphosphate glucose dehydrogenase (UGDH) plays an important role in biosynthesis of hemicellulose through multistep irreversible sugar interconversion involving NAD^+-linked oxidation of UDP-glucose (UDP-Glc) to UDP-glucuronate (UDP-GlcA) (Nelsetuen and Kirkwood 1971, Amino *et al*. 1985, Gibeaut 2000). Alternatively, UDP-GlcA can also be formed by a more complex reaction *via* the myo-inositol oxygenation pathway (Loewus *et al*. 1973, Roberts and Loewus 1973, Sasaki and Taylor 1984). Both of these pathways may exist in plants and their importance is dependent on plant species and tissue (Witt 1992, Seitz *et al*. 2000).

Uridine diphosphate-GlcA is a direct precursor of many sugar nucleotides including UDP-galacturonate, UDP-D-xylose, UDP-arabinose, and UDP-apiose, which are glycosyl donor substrates for hemicellulose and pectin biosyntheses (Gibeaut and Carpita 1994, Reiter and Vanzin 2001, Johansson *et al*. 2002). Hemicellulose is a key factor in secondary cell wall biosynthesis providing approximately a half of biomass of the primary cell wall to strengthen cell wall structure (Delmer *et al*. 1988, Witt 1992, Zablackis *et al*. 1995, Gibeaut 2000, Seifert 2004, Karkonen and Fry 2006, Sato *et al*. 2013). Uridine diphosphate glucose dehydrogenase, which might be rate-limiting in synthesis of hemicellulose, plays an indispensible role in all plant tissues and organs at all stages of development (Dalessandro and Northcote 1977a,b, Robertson *et al*. 1995, Gibeaut 2000).

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Abbreviations: CaMV - cauliflower mosaic virus; CTAB - cetyltrimethylammonium bromide; LSD - least significant difference; MS - Murashige and Skoog; NAD - nicotinamide adenine dinucleotide; *NPT II* - neomycin phosphotransferase II gene; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription polymerase chain reaction; UDP-Glc - uridine diphosphate glucose; UDP-GlcA - uridine diphosphate glucuronate; UGDH - uridine diphosphate glucose dehydrogenase; UGPase - uridine diphosphate glucose pyrophosphorylase; WT - wild type.

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* Corresponding author: fax: (+86) 471 4992867 8020; e-mail: linxiaofei04@hotmail.com

Genes for UGDH have been identified in various plants including *Glycine max* (Tenhaken and Thulke 1996, Stewart and Copeland 1998), *Arabidopsis thaliana* (Seitz *et al*. 2000), *Populus tremula* × *P. tremuloides* (Johansson *et al*. 2002), *Dunaliella salina* (He *et al*. 2005), *Zea mays* (Karkonen *et al*. 2005), *Boehmeria nivea* (Liu *et al*. 2008), *Eucalyptus grandis* (Labate *et al*. 2010), *Gossypium hirsutum* (Pang *et al*. 2010), *Hordeum vulgare* (Matsumoto *et al*. 2011), and *Prunus persica* (Sato *et al*. 2013). The molecular characteristics and expression features of these *UGDH* orthologs have been investigated. The plant UGDH has been identified as a cytosolic protein lacking a transmembrane domain, whereas it has been reported to be membrane-associated in some non-plant species including *Cryptococcus neoformans* and *Escherichia coli* (Rigg and Barett 1998, Griffith *et al*. 2004).

Reiter and Vanzin (2001) identified four *UGDH* isoforms from the genome database of *A. thaliana*; they show a high similarity to each other. Klinghammer and Tenhaken (2007) found the fifth *UGDH* pseudogene located on chromosome III of *A. thaliana*; it shows a weak similarity to the other isoforms. Four *UGDH* genes were identified in *P. trichocarpa* by searching the genome database, and two *UGDH* isoforms were detected in *E. grandis* by Southern blot analysis (Labate *et al*. 2010). Two highly homologous *UGDH* genes were identified in *Z. mays* by searching the *GnpSeq Genoplante* database (Karkonen *et al*. 2005). Genes for UGDH from *Nicotiana tabacum* were strongly expressed in roots and the youngest stem internodes (Bindschedler *et al*. 2005), which was similar to the expression pattern observed in *A. thaliana* (Seitz *et al*. 2000). Maximum expression was observed in stems of *B. nivea* plants in addition to its leaves and roots (Liu *et al*. 2008). Tissue-specific expression of *P. tremula* × *P. tremuloides UGDH* was predominantly in developing xylem and young leaves. Expression of *P. tremula* × *P. tremuloides UGDH* was strongly induced by sucrose, sorbitol, and PEG-8000, but UGDH protein content was unaffected. These results suggest that UGDH in *P. tremula* × *P. tremuloides* plays an important role in osmoregulation, which is regulated at post-transcriptional or translation levels (Johansson *et al*. 2002).

Larix gmelinii is a deciduous tree of the *Pinaceae* family; it comprises a large proportion of coniferous forests in China and Mongolia. It can be found in coniferous forests in temperate and boreal zones over the world and shows cold tolerance. It has a high economic value.

Sequences of *UGDH* were isolated from various plants including gymnosperms *Larix kaempferi* and *Picea sitchensis*. In this study, we report that the *LgUGDH* gene from *L. gmelinii* affected vegetative growth, biosynthesis of hemicellulose, and cold tolerance when heterologously expressed in *A. thaliana*. The results could also provide new insights into the function of UGDH in plants.

Materials and methods

Plants: Mature seeds of *Larix gmelinii* (Rupr.) Kuzen. were provided by the *Ganhe Forestry Bureau Seed Orchard*, Inner Mongolia and were stored in plastic bags at 4 °C for 6 - 12 months. The seeds were sown in pots with a wet mixture of soil and *Vermiculite* (1:1, m/m), and both DNA and RNA were extracted from four-week-old plants.

Seeds of *Arabidopsis thaliana* L. ecotype Nossen were sterilized with 70 % (v/v) ethanol and a 2 % (m/v) NaClO solution and then sown on Murashige and Skoog (1962; MS) medium. After vernalization at a temperature of 4 °C for 3 d, the seeds were cultured at 22 °C, a 16-h photoperiod, and irradiance of 45 μmol m^{-2} s^{-1}. Ten-day-old *A. thaliana* seedlings were transplanted into pots containing soil and grown under the same conditions as described above.

Isolation of *LgUGDH* cDNA: The total RNA was extracted from four-week-old *L. gmelinii* plants using the cetyltrimethylammonium bromide (CTAB) method (Chang *et al*. 1993). To isolate *LgUGDH* cDNA from *L. gmelinii*, degenerate primers were designed based on UGDH orthologs from *Picea sitchensis* (ABR17780.1), *E. grandis* (ABP04019.1), *Oryza sativa* (AAS07200.1), *Paulownia tomentosa* (AAR32717.1), *Cinnamomum osmophloeum* (AAR84297.1), *Glycine max* (AAB58398.1), *G. hirsutum* (ADB24766.1), and *A. thaliana* (BAB02581.1). The primers were degUGDH/F1, degUGDH/F2, degUGDH/R1, and degUGDH/R2, which correspond to the highly conserved amino acid sequences GAGYVGG, GAGKAA, KFDWDHP, and DTRETPA, respectively (see Table 1 Suppl.). To isolate the *LgUGDH* cDNA fragment, reverse transcription (RT) PCR was performed using a RT-PCR kit (*Takara*, Dalian, China) and the degenerate primers. First-strand cDNA was synthesized from 1 μg of the total RNA at 42 °C for 30 min with 1 mm^3 of an oligo dT-adaptor primer. The first PCR reaction required 2.5 mm^3 of 10× *Ex Taq* buffer (*Takara*), 2 mm^3 of 2.5 mM dNTPs, 0.2 mm^3 of 2.5 units of *Takara Ex Taq HS* DNA polymerase (*Takara*), 1 mm^3 of 25 μM primers (degUGDH/F1 and degUGDH/R1), and 1 mm^3 of cDNA in a 25-mm^3 volume with the following conditions: 35 cycles of 94 °C for 30 s, 48 °C for 60 s, and 72 °C for 1 min, followed by 72 °C for 5 min as a final extension (a standard PCR protocol). The second PCR was carried out using the primers degUGDH/F2 and degUGDH/R2 under the same conditions. A 3'-end fragment of the

LgUGDH gene was amplified by 3'-rapid amplification of cDNA ends (RACE) PCR using the standard PCR setup with the gene-specific primers 3'RACE-F1 and 3'RACE-F2, and the primer M13M4, and annealing at 56 °C for 30 s. First-strand cDNAs for 5'-RACE were synthesized using 1 μg of the total RNA with a *SMART RACE* cDNA amplification kit (*Clontech*, Mountain View, CA, USA) according to the manufacturer's instructions. A 5'-RACE fragment of the *LgUGDH* gene was amplified by 5'-RACE PCR using the gene-specific primers 5'RACE-R1 and 5'RACE-R2, and the primers *Universal Primer Mix A* (*UPM*) and *Nested Universal Primer A* (*NUP*) of the *SMART RACE* cDNA amplification kit.

The full-length *LgUGDH* cDNA fragment containing the total coding region was obtained using the standard PCR setup with the primer sets UGDH-full-F and UGDH-full-R, and annealing at 53 °C for 45 s. All of the above PCR products were subjected to electrophoresis, extracted from the agarose gel, and then inserted into the pEASY-T1 vector using a pEASY-T1 cloning kit (*TransGen Biotech*, Beijing, China) according to the manufacturer's instructions. Sequencing DNA was performed by the Beijing Genomics Institute (BGI, China) and analyzed using *BLAST*.

Phylogenetic analysis was performed using the mature protein sequences of UGDHs from *O. sativa*, *N. tabacum*, *A. thaliana*, *Z. mays*, *G. max*, *E. grandis*, *P. tomentosa*, *P. trichocarpa*, *P. tremula* × *P. tremuloides*, *P. sitchensis*, *G. hirsutum*, *H. vulgare*, *L. kaempferi*, *Selaginella moellendorffii*, and *Physcomitrella patens*. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 1.24542784 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 29 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 462 positions in the final dataset. Evolutionary analyses were conducted in *MEGA5*.

Southern blot analysis: The total genomic DNA was isolated from four-week-old *L. gmelinii* plants using the CTAB method (Murray and Thompson 1980). A probe of about 0.5 kb, corresponding to the coding region of the *LgUGDH* cDNA, was labeled using a PCR *DIG Probe* synthesis kit (*Roche Molecular Biochemicals*, Indianapolis, USA) with the PCR primers UGDH-Full-F and 5'RACE-R2. Reverse transcription PCR and genomic PCR were performed with the UGDH-Full-F and 5'RACE-R2 primers to confirm that introns were not present in the probe region. For Southern blotting, 10 μg of the total genomic DNA were digested with restriction enzymes (20 units of *Eco*RI or *Bam*HI), subjected to electrophoresis, transferred to membranes, and hybridized with the *LgUGDH* probe. The hybridized products were detected with a nonradioactive digoxigenin luminescence detection system (*Boehringer*, Mannheim, Germany).

Genetic transformation of *Arabidopsis*: The plasmid pBI101-*LgUGDH* was constructed with the pBI101-35::Gus-Hm vector containing an intronless version of the GUS gene from pIG121-Hm (Akama *et al*. 1992). A fragment of the hygromycin phosphotransferase gene with a cauliflower mosaic virus (CaMV) 35S promoter as well as the *GUS* gene with a nopaline synthase terminator were excised from pBI101-35::Gus-Hm by digestion with *Bam*HI. The *LgUGDH* open reading frame was amplified with LgUGDH-*Bam*HI-F and LgUGDH-*Bam*HI-R which contain a *Bam*HI site. The PCR product was digested with *Bam*HI and inserted between the CaMV 35S promoter and the nopaline synthase terminator of the digested pBI101-35::Gus-Hm vector to generate pBI101-*LgUGDH* (Fig. 3*A*).

The pBI101-*LgUGDH* construct was introduced into the *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method, and transformation of *A. thaliana* plants was performed according to the simplified in-plant infiltration method described by Kim *et al*. (1999). Transgenic lines were obtained by screening on MS medium with kanamycin (40 mg dm^{-3}) and confirmed by PCR performed using the standard PCR setup with the vector-specific primers: NPTII-F and NPTII-R, and annealing at 55 °C for 30 s. The selected kanamycin-resistant individuals were self-pollinated to establish strains homozygous for the transgene. All analyses involved homozygous lines. Physical characteristics, such as area, length, and width of the fifth leaf from wild-type (WT) and transgenic *A. thaliana* plants, were measured with an *LI-3000C* portable area meter (*Li-Cor*, Lincoln, NE, USA). To investigate the cold tolerance of transgenic *A. thaliana* overexpressing *LgUGDH*, WT and transgenic plants cultured on MS medium for 7 d were transferred to 4 °C, and fresh mass of WT and transgenic plants were recorded at 1, 3, 5, and 7 d after culture in 4 °C.

Real-time PCR: To investigate *LgUGDH* expression, real-time PCR was performed with the *LgUGDH*-specific primers (LgUGDH-SP-F and LgUGDH-SP-R). The *LgActin* gene (AB523401, LgActin-F, and LgActin-R, Zhu *et al*. 2011) was used as an internal control. The total RNA (2 μg) was reverse transcribed into first-strand cDNA with *PrimeScript*^{TM} *RT Master Mix* (*Takara*). The cDNA diluted five times was used as a template in each well for real-time PCR analysis. The cDNA was amplified by *SYBR Premix Ex Taq*^{TM} *II* (*Takara*) in a *Rotor-gene Q* real time PCR platform (*Qiagen*, Hilden, Germany) under the following conditions: 95 °C

denaturation for 30 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Before proceeding with real-time PCR, we routinely verified that the primers for *LgUGDH* and *LgActin* had a similar slope with high correlation coefficients by constructing a standard curve (R^2 = 0.99). The threshold cycle (CT) values of the triplicate real-time PCRs were averaged, and the fold changes of transcription levels of the target gene (*LgUGDH*) relative to the reference gene (*LgActin*) were analyzed by the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). All experiments were repeated three times for cDNA prepared from three batches of plants.

Reverse transcription PCR: To investigate *LgUGDH* expression in transgenic *A. thaliana* plants, RT-PCR was performed with the *LgUGDH*-specific primers UGDH-full-F and 5'RACE-R2. The *AtActin* 2 gene (the primers Atactin2-F and Atactin2-R, Lin *et al.* 2008) was used as an internal control. The RNA was prepared from three-week-old transgenic *A. thaliana* plants using the CTAB method (Chang *et al.* 1993). The cDNA was synthesized from 1 μg of the total RNA at 42 °C for 30 min with 1 mm^3 of an oligo-dT adapter primer using an RT-PCR kit (*Takara*). The PCR was performed using the standard PCR setup at 56 °C for 30 s.

Activity of UGDH: Tissue extraction and UGDH activity assays were performed as described by Roberts and Cetorelli (1973) with some modifications (Robertson *et al.* 1996). The fifth rosette leaves from 40-d-old WT and transgenic *A. thaliana* plants were used for protein extraction (Li *et al.* 2014), and UGDH activity was measured by enzymatically determining reduction of $NADP^+$. Absorbance at 340 nm was determined using a *TU-1810* spectrophotometer (*Purkinje Instrument Co*., Beijing, China). Activity of UGDH was calculated based on an assumption that 2 mol of NADH were formed per 1 mol of oxidized UDP-Glc.

Content of sugars, lignin, cellulose, and hemicellulose were measured in the fifth rosette leaves of 40-d-old *A. thaliana* plants 3 h after starting irradiation. The samples were ground into powder and extracted twice with 80 % (v/v) ethanol at 80 °C for 30 min and further washed with 50 % (v/v) ethanol at 80 °C for 30 min. After centrifugation (24 000 *g*, 4 °C, 30 min), sucrose, glucose, and fructose in the supernatant were measured by enzymatically determining reduction of $NADP^+$ at 340 nm after successively adding coupling enzymes glucose 6-P-dehydrogenase, hexokinase, phosphoglucose isomerase, and invertase (Sekin 1978). The residual pellets were incubated at 60 °C for 3 h with α-amylase and amyloglucosidase, and the liberated glucose represented the starch content. Lignin content was measured using a modified micro-Klason method (Huntley *et al.* 2003), cellulose content was measured according to the H_2SO_4 digestion method (Updegraff 1969), and hemicelluloses were sequentially extracted following the standard method of Van Soest (1963) and Van Soest and Wine (1967) modified for the use of small sample volumes and starch-rich material (Schädel *et al.* 2010).

Statistical analysis: All data are expressed as means ± standard deviations (SDs). Statistical differences were assessed by one-way analysis of variance (*ANOVA*) using Fisher's least significant difference (LSD) test. The significance of differences between means was identified at a probability value of 0.05. All the analyses were conducted using the *SPSS 18.0* software (*SAS Institute*, Chicago, USA).

Results

To isolate the *LgUGDH* cDNA, two degenerate primer sets were designed based on UGDH orthologs of plants. The use of a nested RT-PCR method and the degenerate primer sets resulted in the isolation of a 750-bp fragment. Primers for RACE were then generated and 3' and 5' RACE were performed to isolate a full-length 1909-bp *LgUGDH* cDNA (DDBJ, accession No. LC005487), which encodes a protein of 480 amino acids containing all the conserved features of UGDHs (Fig. 1 Suppl.). Analysis by *BLAST* shows that the deduced LgUGDH amino-acid sequence shared over 85 % identity with UGDH proteins from *A. thaliana*, *G. max*, *E. grandis*, *P. tomentosa*, *C. osmophloeum*, and *O. sativa*. Phylogenetic analysis reveals that LgUGDH was in a sister group to the UGDH clade of gymnosperms including *L. kaempferi* and *P. sitchensis* (Fig. 2 Suppl.). These results suggest that the *LgUGDH* gene has features and functions similar to those of *L. kaempferi* and *P. sitchensis*.

Southern hybridization was performed to investigate the copy number of the *UGDH* gene in the *L. gmelinii* genome (Fig. 1*A*). The larch genomic DNA was digested with *Eco*RI or *Bam*HI restriction enzymes, which have no corresponding restriction sites in the *LgUGDH* cDNA probe region. The digested genomic DNA was hybridized with an *LgUGDH* probe (Fig. 1*A*). The absence of an intron in the probe region was confirmed by RT-PCR and genomic-PCR (Fig. 1*B*). Two bands were detected in each genomic DNA sample suggesting that the *LgUGDH* gene probably has an additional paralog in the *L. gmelinii* genome. To examine tissue-specific expression of *LgUGDH*, RNA was extracted from roots, stems, and

leaves of four-week-old *L. gmelinii* seedlings, and real-time PCR was performed with the *LgUGDH*-specific primers LgUGDH-SP-F and LgUGDH-SP-R. The results show that the *LgUGDH* gene was expressed in all the larch plant organs with a higher expression in stems than in leaves and roots (Fig. 2).

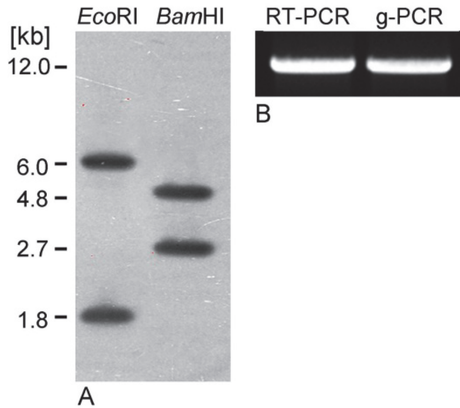

Fig. 1. Southern blot analysis of *LgUGDH*. *A* - Genomic Southern analysis showing the hybridization pattern of the *LgUGDH* gene. Genomic DNA was digested with restriction enzymes *Eco*RI or *Bam*HI and hybridized with a *LgUGDH* probe. *B* - RT-PCR and genomic (g) PCR analysis for proving the probe did not span an intron.

To further analyze features and functions of *LgUGDH*, the plasmid pBI101-*LgUGDH* containing the *LgUGDH* cDNA driven by the CaMV35S promoter was constructed and introduced into *A. thaliana via Agrobacterium*-mediated transformation (Fig. 3*A*). Expression of *LgUGDH* transgene was confirmed by RT-PCR in four homozygous transgenic lines produced by self-pollination (Fig. 3*B*). Activity of UGDH was

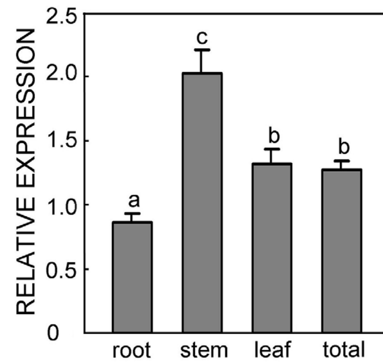

Fig. 2. Real-time PCR was used to measure the relative expression of *LgUGDH* in leaves, stems, roots, and total plants of 40-d-old *L. gmelinii*. Different letters indicate significant differences in Fisher's LSD test ($P < 0.05$); means ± SDs were calculated from three independent experiments.

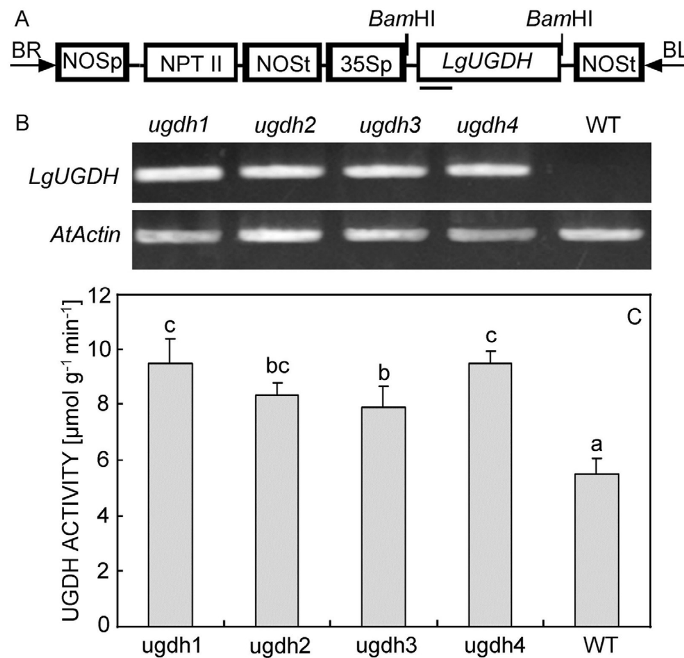

Fig. 3. Molecular identification of *LgUGDH*-transformed *Arabidopsis thaliana* lines. *A* - T-DNA region of the vector pBI101-*LgUGDH* used to produce transgenic *LgUGDH* plants (35Sp - cauliflower mosaic virus 35S promoter, *NOSp* - nopaline synthase promoter, *NOSt* - nopaline synthase terminator, *NPT II* - neomycin phosphotransferase II gene, BR - right border, BL - left border; the location of the probe used for Southern hybridization is indicated by a *black line*). *B* - Reverse transcription PCR analysis to confirm expression of the *LgUGDH* transgene in various transgenic lines; *AtActin* gene was used as an internal control. *C* - uridine diphosphate glucose dehydrogenase (UGDH) activities of WT and transgenic lines (*ugdh1 - 4*); means ± SDs were calculated from five plants per line; different letters indicate significant differences ($P < 0.05$).

Fig. 4. Effects of *LgUGDH* overexpression on transgenic *Arabidopsis thaliana*. *A*-*D* - WT (*above the red line*) and transgenic (*below the red line*) plants cultivated on Murashige and Skoog medium for 3 (*A*), 7 (*B*), 10 (*C*), and 14 d (*D*) after sowing. *E* - WT and transgenic plants (*ugdh1 - 4*) cultivated on vertical plates showing the difference in growth and development of roots. Three-week-old transgenic plants (*F*) showed a better growth than WT plants (*G*). Four-week-old transgenic plants (*H*) showed a faster growth rate than WT plants (*I*). Transgenic plants (*left*) were considerably taller than WT plants (*right*) at five weeks after sowing (*J*). Compared with the WT (*K*, *L*, *O*, and *P*), parenchyma cell walls in the fifth leaf (*K*-*N*) and stem (*O*-*R*) of the *LgUGDH*-transformed lines (*M*, *N*, *Q*, and *R*) were thicker. The regions contained in the *black boxes* indicated *by* the *arrows* in *K*, *M*, *O*, and *Q* are enlarged in *L*, *N*, *P*, and *R*, respectively.

measured in these transgenic lines using the fifth rosette leaf of 40-d-old plants to confirm the expression of LgUGDH at the translation level (Fig. 3*C*). Compared to the WT, the activity of UGDH increased to a varying extent but always significantly in the four *LgUGDH* transgenic lines (Fig. 3*C*).

Overexpression of *LgUGDH* in transgenic *A. thaliana* plants resulted in distinct phenotypes at different developmental stages as compared to the WT. No significant difference in germination was observed between the WT and transgenic plants, whereas the transgenic plants displayed a faster growth rate than the WT plants (Fig. 4*A-E*). After transplanting into soil, the transgenic plants were taller and grew faster than the WT plants (Fig. 4*F-J*). These results show that overexpression of the *LgUGDH* enhanced vegetative growth in the transgenic plants. To precisely analyze phenotypic differences between the WT and transgenic plants, morphological characteristics of 40-d-old plants including the area, length, and width of the fifth rosette leaf, the number of cauline branches, internode distances, and plant height were measured, as well as root length of 14-d-old plants (Fig. 3 Suppl.). All of the transgenic lines were taller than the WT plants after 40 d of growth. No significant differences in leaf length or cauline branch number were noted, but significant differences in

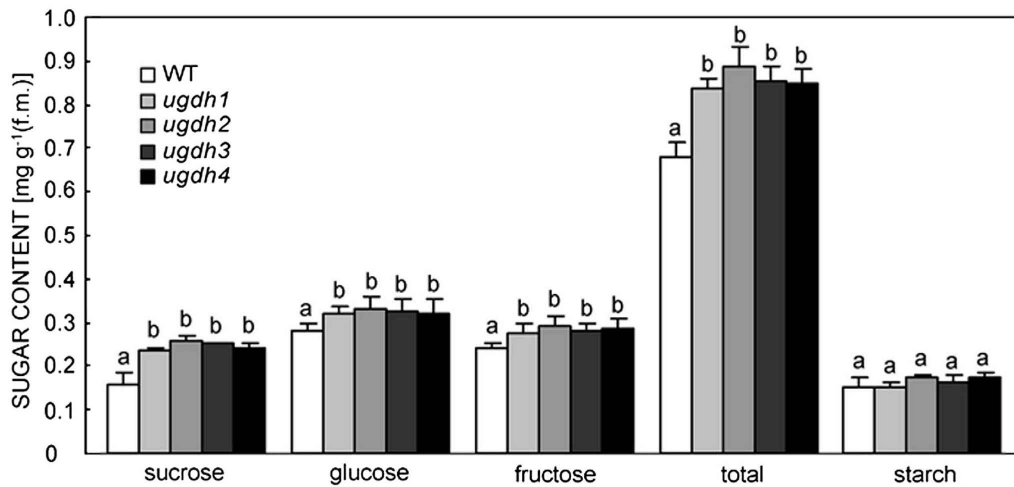

Fig. 5. Content of sucrose, glucose, fructose, total soluble sugars, and starch in the fifth rosette leaf of 40-d-old WT and transgenic *Arabidopsis thaliana*. Means ± SDs, $n = 5$; different letters indicate significant differences at $P < 0.05$.

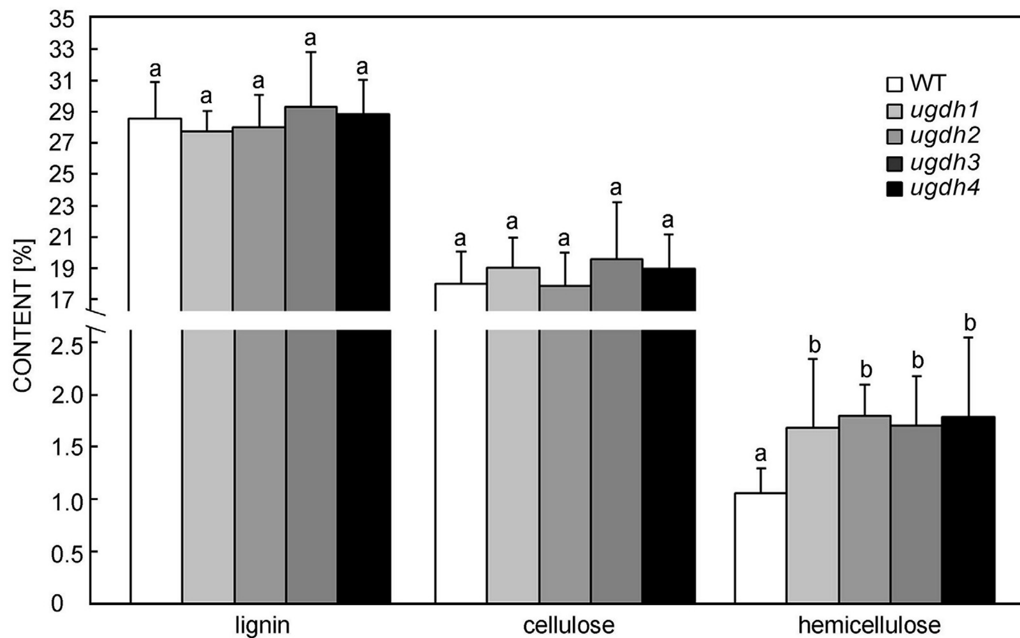

Fig. 6. Content of lignin, cellulose, and hemicellulose in stems of 40-d-old WT and transgenic *Arabidopsis thaliana*. Means ± SDs, $n = 5$; different letters indicate significant differences at $P < 0.05$.

internode distance, leaf width, leaf area, and root length between the WT and *LgUGDH* transgenic plants were detected. Content of sucrose, fructose, glucose, starch, lignin, cellulose, and hemicellulose were measured in the fifth rosette leaf of 40-d-old the WT and transgenic *A. thaliana* plants. The content of sucrose, glucose, fructose, and total soluble sugars increased significantly in the four *LgUGDH* transgenic lines compared to the WT, whereas there was no significant difference in starch content (Fig. 5). Hemicellulose content in stems of the four transgenic lines was significantly higher than in the WT, but no significant difference in lignin or cellulose amount was found between the stems of the WT and transgenic plants (Fig. 6). Thicker cell walls were noted in parenchyma cells of leaf veins and stems of the transgenic lines compared to the WT plants (Fig. 4*K-R*). These results reveal that the *LgUGDH* gene played a role in controlling biosynthesis of secondary cell walls.

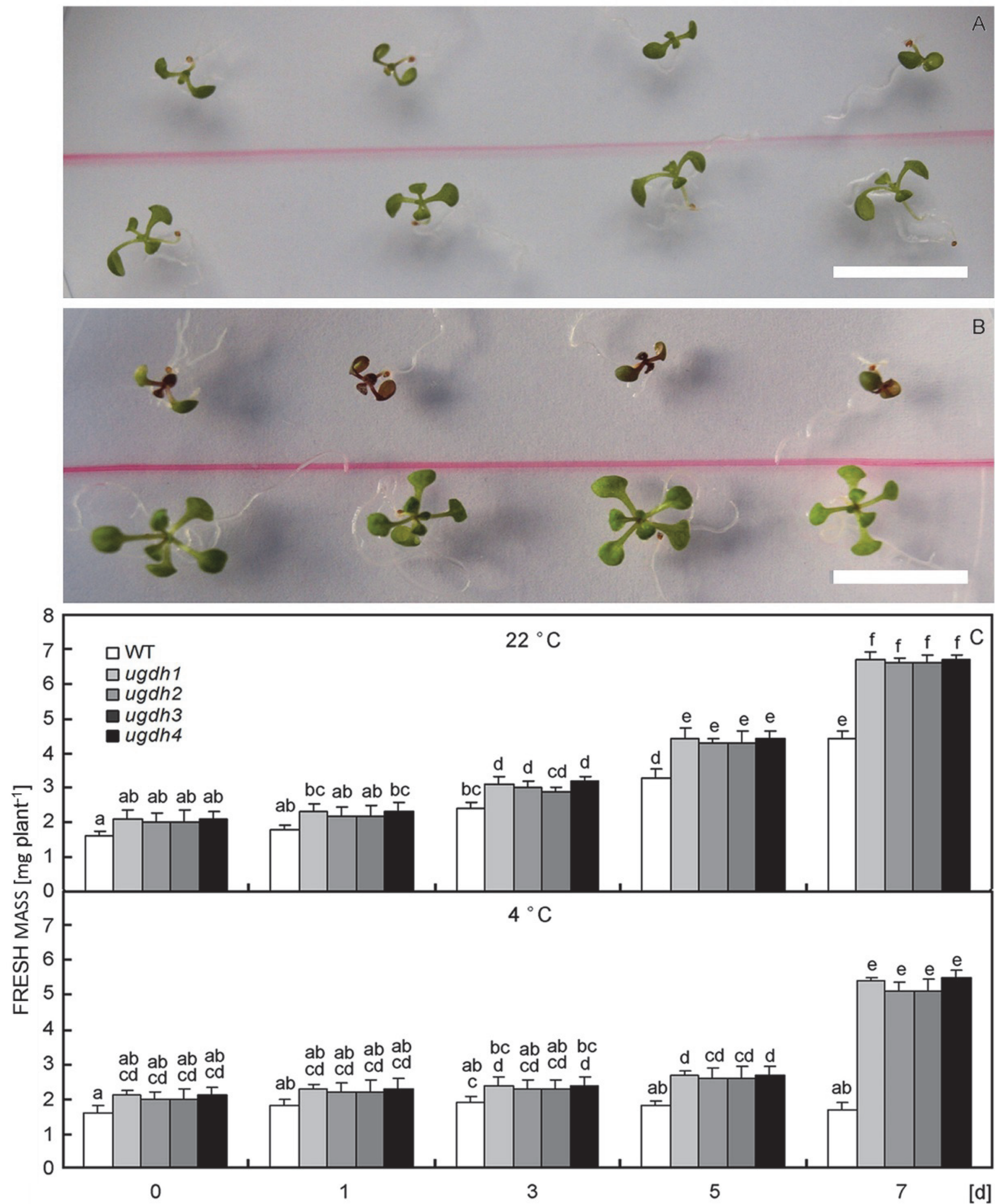

Fig. 7. Cold-tolerance analysis of *LgUGDH* transgenic plants. Images of seven-d-old WT, *above the red line*) and transgenic plants (*below the red line*) cultivated on Murashige and Skoog medium at 22 °C (*A*), and 4 °C (*B*) for 7 d. Fresh masses of the WT and transgenic lines cultured at 22 °C and 4 °C for 0, 1, 3, 5, and 7 d (*C*). Means ± SDs, *n* = 5; different letters indicate significant differences at $P < 0.05$.

To investigate cold-tolerance of the transgenic *A. thaliana*, 7-d-old seedlings were cultured under 4 °C for 1, 3, 5, or 7 d, and fresh mass was measured (Fig. 7). During 0 - 5 d of culture in 4 °C, fresh mass of the WT plants did not vary significantly, but fresh mass increased in the four *LgUGDH* transgenic lines (Fig. 7*C*). Damage caused by the low temperature began to appear in the WT plants after 5 d, and the WT plants began to turn brown after 7 d of culture at 4 °C. The transgenic plants showed a slow growth, and fresh mass continued to increase up to 7 d of culture at 4 °C (Fig. 7). However, the growth rate of the transgenic plants cultured at 4 °C (Fig. 7*B*) was lower than of the transgenic plants cultured at 22 °C (Fig. 4*D*). These results reveal that overexpression of *LgUGDH* enhanced cold-tolerance in the transgenic *A. thaliana*. To examine expression of *LgUGDH* under the cold stress conditions, 4-week-old *L. gmelinii* seedlings were grown at 4 °C for 0, 1, 6, 12, or 24 h, and the expression levels of *LgUGDH* were measured. Real-time PCR results show that expression of *LgUGDH* slightly decreased at 1 h, and then subsequently increased to a maximum at 12 h after culture in 4 °C (Fig. 4 Suppl.). These results indicate that the *LgUGDH* gene could play an important role in adaptation of *L. gmelinii* plants to low temperature.

Discussion

The plant cell wall consists of several polysaccharides including cellulose, hemicellulose, and callose. Uridine diphosphate glucose pyrophosphorylase, which is an important regulatory enzyme in cellulose and callose biosynthesis, catalyzes reversible production of glucose-1-phosphate and conversion of uridine triphosphate to UDP-Glc and pyrophosphate (Winter and Huber 2000, Kleczkowski *et al*. 2004). Uridine diphosphate glucose dehydrogenase catalyzes oxidation of UDPG to UDP-GlcA, a key sugar nucleotide involved in biosynthesis of hemicellulose. In this study, we cloned an *L. gmelinii* cDNA encoding a UGDH isoform (*LgUGDH*). The *LgUGDH* gene was highly expressed in stems, less in leaves, and least in roots. In *N. tabacum*, *NtUGDH* is expressed strongly in roots and the youngest internodes of stems and little in buds and leaves (Bindschedler *et al*. 2005), which is similar to the expression pattern observed in *A. thaliana* (Seitz *et al*. 2000). However, tissue-specific expression of *P. tremula* × *P. tremuloides UGDH* is predominant in developing xylem and young leaves, with some expression in mature and apical leaves (Johansson *et al*. 2002). Similar results were reported in ramie, where the maximum expression is observed in stems, intermediate expression in phloem and leaves, and the lowest expression in roots (Liu *et al*. 2008). These results suggest that the isoforms of *UGDH* might be preferentially involved in secondary growth of cell wall fibre. Multiple *UGDH* isoforms were indentified in genomes of some plants such as *A. thaliana* (Reiter and Vanzin 2001), *Z. mays* (Karkonen *et al*. 2005), and *E. grandis* (Labate *et al*. 2010). In this work, Southern blot analysis indicates the existence of two copies of *UGDH* in the *L. gmelinii* genome.

Heterologous overexpression of *LgUGDH* under control of a single CaMV 35S promoter significantly enhanced vegetative growth of the transgenic *A. thaliana* plants. However, no phenotypic differences compared with the WT were found in the antisense lines of *N. tabacum* (Bindschedler *et al*. 2007) and Mu-element insertion mutants (*ugdh-A1* and *ugdh-B1*) of *Z. mays* (Karkonen *et al*. 2005) although only low levels of UGDH expression were detected in the mutant lines of the two plant species. These facts raised two possibilities that there might exist a threshold level for UGDH in controlling secondary cell wall biosynthesis, and/or the myo-inositol oxygenation pathway might provide UDP-GlcA for biosynthesis of hemicelluloses under inhibition of UGDH expression. Therefore, a partial inhibition of UGDH expression either does not affect or only slightly affect growth and development of plants, whereas an excessive accumulation of UGDH plays a role in controlling the strength of sink tissues, which significantly enhances vegetative growth of plants.

The *Z. mays* knockout mutant *ugdh-A1* reduces pentosan biosynthesis suggesting that UGDH is a critical factor in sugar metabolism (Karkonen *et al*. 2005). In this study, content of soluble sugars, including sucrose, glucose, and fructose, were significantly higher in the rosette leaves of *LgUGDH* transgenic *A. thaliana* plants, indicating that *LgUGDH* may modify carbon allocation in favor of soluble sugars. However, no difference in starch content was found between the WT and *LgUGDH* transgenic plants indicating that the accumulated sugar was probably not stored in leaves in the form of starch, but formed a pool of available soluble sugars to accelerate metabolism and enhance vegetative growth. These results are similar to overexpression of the *L. gmelinii* UDP-glucose pyrophosphorylase (*LgUGPase*) gene in transgenic *A. thaliana* reported previously (Li *et al*. 2014).

Hemicellulose content of the plants overexpressing *LgUGDH* increased significantly compared to the WT plants. Thicker cell walls were observed in parenchyma cells in leaf veins and stems of the *LgUGDH*-transformed lines compared to the WT plants, confirming previous reports that UGDH is involved in cell wall biosynthesis and that UGDH activity may be related to activity of the hemicellulose synthase complex in the cell wall. These findings suggest that *LgUGDH* plays an important role in allocation of carbon, especially in hemicellulose

biosynthesis, and that *LgUGDH* may be a good candidate gene to accelerate fibre cell development and enhance vegetative growth in plants.

Hemicellulose, an important factor in secondary cell wall biosynthesis, represents a half of the primary cell wall biomass (Delmer *et al*. 1988, Witt 1992, Zablackis *et al*. 1995, Gibeaut 2000, Seifert 2004, Karkonen and Fry 2006, Sato *et al*. 2013). On the other hand, soluble sugars improve the efficiency of osmotic regulation under stress conditions. In *P. tremula* × *P. tremuloides*, expression of *UGDH* is strongly induced by osmotic stress (Johansson *et al*., 2002). Increases in content of hemicellulose and soluble sugars indicate that overexpression of *LgUGDH* contributes to plant adaptation to stresses. Adaptability to various stresses including salt, cold, and PEG-simulated drought was investigated, and a stronger cold-tolerance was confirmed in transgenic plants suggesting that *LgUGDH* may be a good candidate gene for improvement of cold resistance.

Previous research showed that overexpression of another *L. gmelinii* enzyme involved in cell wall polysaccharide production, *LgUGPase*, increase cellulose content in transgenic *A. thaliana*, but hemicellulose content remains unchanged (Li *et al*. 2014). In this work, overexpression of *LgUGDH* in *A. thaliana* increased content of hemicellulose, but cellulose content was unaffected. These results suggest that UGPase is a key enzyme in cellulose biosynthesis, whereas UGDH is a rate-limiting enzyme in hemicellulose biosynthesis. These results are consistent with a previous work, which showed the critical role of UGDH in regulation of polysaccharide biosynthesis in plants (De Luca *et al*. 1976, Robertson *et al*. 1995, Wegrowski *et al*. 1998).

This is the first study to report that UGDH overexpression increases hemicellulose content and enhances vegetative growth and cold tolerance in transgenic plants. Co-expression of *LgUGDH* and *LgUGPase* may thus be useful for improving growth, adaptability, and fibre content in forest trees.

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