The *EgMUR3* xyloglucan galactosyltransferase from *Eucalyptus grandis* complements the *mur3* cell wall phenotype in *Arabidopsis thaliana*

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Abstract Xyloglucan is the major hemicellulosic polymer found in the primary cell walls of dicots. Xyloglucan tethers cellulose microfibrils conferring rigidity and strength for maintenance of cell integrity, and it is thought that its metabolism contributes to cell elongation and thus plant growth. Here, we have cloned and characterized a *Eucalyptus grandis* gene ortholog of the *Arabidopsis thaliana* *MUR3* gene (xyloglucan galactosyltransferase), thus termed *EgMUR3*. *EgMUR3* represents an intronless sequence of 1,854 bp predicted to encode a protein of 617 amino acid residues. It exhibits 73% identity and 82% similarity to the *A. thaliana* *MUR3* gene. To demonstrate that this gene encodes a functional enzyme, the putative ORF was cloned into a binary vector under the control of a constitutive promoter and transformed into the *A. thaliana* *mur3* mutant. The effect of the genetic complementation was investigated by xyloglucan oligosaccharide fingerprinting of wall material. The results confirmed that *EgMUR3* represents indeed a xyloglucan galactosyltransferase of *E. grandis* able to use endogenous substrate(s) in *A. thaliana*,

suggesting that both species share common steps in xyloglucan biosynthesis.

Keywords Cell walls · *Eucalyptus grandis* · Xyloglucan · MALDI-TOF MS · Transformation

Introduction

Eucalypts are among the fastest growing trees and are suitable candidates as biomass resource for bioenergy and pulping purposes. Breeding targets have been mainly biomass yield gains, fiber quality, biotic and abiotic stress tolerance, flowering control, and herbicide resistance. The domestication of the *Eucalyptus* genus is just in its infancy, mainly because classic genetic breeding of forest trees is hampered by the long juvenile stage and many traits of interest can be only assessed at rotation age (Boerjan 2005). Molecular and classic breeding are two approaches taken in parallel toward the designing of elite trees. Biotechnology exploit the advantage of speeding up the investigation of gene function by transgene transference to model plants, while classic breeding offers the opportunity of identifying new genetic elements controlling plant traits through the exploration of the natural diversity.

Comprehension of cell wall metabolism is the direction toward altering biomass for biofuel and pulping purposes in this economically important tree. In spite of literature describing protocols for *Eucalyptus* spp. transformation (Serrano et al. 1996; Moralejo et al. 1998; Gonzalez et al. 2002), transcriptional analysis of gene expression (Paux et al. 2005; Foucart et al. 2006; Navarro et al. 2009), gene structure, and diversity (McKinnon et al. 2005; Sassaki et al. 2005), there is a lack of report on functional analysis of

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cell wall-related genes in eucalypt species in comparison to the other important forestry species (Chen et al. 2001; Sibout et al. 2002; Baucher et al. 2003). Much of the research being conducted in this area is under confidentiality policies adopted by private companies. Recently, a paper reported that the overexpression of a gene encoding the *Eucalyptus camaldulensis* HD-Zip class II transcription factor increased fiber length and growth in transgenic tobacco plants (Sonoda et al. 2009).

All plant cells are encased in a wall, which consists of complex heteroglycans and polyphenolics. The major load-bearing structure in the dicot primary wall, i.e., the wall of elongating cells, is a cellulose–xyloglucan network, whose metabolism plays a major role in cell elongation (Somerville et al. 2004). Xyloglucan (XyG) coats and permeates cellulose microfibrils (Pauly et al. 1999a), limiting their aggregation and connecting them via tethers that directly or indirectly regulate the mechanical properties of the wall. XyG consists of β -1,4 linked glucosyl residues, which interact tightly and non-covalently with the cellulose microfibrils. Unlike cellulose, XyG contains quite diverse side-chain structures that render the polymer soluble in aqueous solutions. XyG structure and the adopted nomenclature is described by Fry et al. (1993) and Hoffman et al. (2005). The precise distribution and function of the individual side-chain moieties are not known. However, it is thought that the galactosyl residue is needed for solubility of the polymer (de Lima and Buckeridge 2001). Furthermore, fucose residues are thought to flatten the XyG backbone, allowing for an enhanced interaction with cellulose microfibrils (Levy et al. 1997).

With the help of forward and reverse genetic approaches, *Arabidopsis* mutants have been obtained that shed light on the biosynthesis of XyG. The first and best-known large-screening for cell wall mutants was conducted by Reiter and colleagues by the characterization of mutants with an altered wall-sugar composition (Reiter et al. 1997). Among these so-called *mur*-mutants, mutants exhibiting a decrease in fucose were represented by *mur1*, *mur2*, and *mur3*. The XyG structure in all of those mutants was altered (Lerouxel et al. 2002). MUR1 represents a GDP-mannose dehydratase, an enzyme needed for the biosynthesis of the precursor GDP-fucose (Zabackis et al. 1996). MUR2 represents an XyG fucosyltransferase (Perrin et al. 1999). MUR3 encodes for a galactosyltransferase, which galactosylates only the third xylose residue in the XXXG core leading to XXLG (Madson et al. 2003). In addition to a substantial reduction in galactosyl moieties, and hence a lack of the fucosyltransferase acceptor substrate site, *mur3* XyG also lacks fucosyl residues. Different phenotypes related to different MUR3 alleles have been described. The occurrence of a stop codon after the transmembrane domain (TMD) in another *mur3* allele leads to a truncated protein,

which caused disorganization of the endomembrane system (Tamura et al. 2005). Despite a significantly altered XyG structure, *mur3* plants do not exhibit any apparent change in morphology and/or growth behavior under regular growth conditions (Reiter et al. 1997). However, a change in tensile strength of etiolated hypocotyls was observed (Pena et al. 2004).

In this study, we identified a *Eucalyptus grandis* MUR3 ortholog here termed *EgMUR3*. *EgMUR3* was overexpressed in the *Arabidopsis thaliana mur3* mutant to assess its functionality. *EgMUR3* was confirmed to be a functional xyloglucan galactosyltransferase expressed mainly in elongating stem and young leaves of young *Eucalyptus urograndis* plants.

Materials and methods

Plant lines and growth conditions

Seeds of the *mur3* mutant were obtained from the Arabidopsis Biological Resource Center at the Ohio State University (Columbus). The mutation was in an Ecotype Columbia (Col-0) background. After a cold treatment by incubation of the seeds at 4°C in darkness for 48 h, in order to break seed dormancy, *A. thaliana* Col-0 and *mur3* were grown in the greenhouse of the Max-Planck Institute for Molecular Plant Physiology, Golm, Germany, with the following temperature/humidity parameters, 20°C/80% from 6 to 22 h, 18°C/50% from 22 to 6 h. Light intensity was 120 mmol m⁻²s⁻¹ until seed harvesting. Long-day condition for flowering induction consisted of 16 h light/8 h dark alternating cycles at 22°C, 120 mmol m⁻²s⁻¹ of light intensity and 60% humidity. Short-day conditions for producing leaf material for RNA extraction and cell wall preparation consisted of 8 h light/16 h dark at 22°C, under 120 mmol m⁻²s⁻¹ light intensity and 60% humidity.

Cloning of *EgMUR3*

A BAC library of *E. grandis* maintained at the Genomics Laboratory (BIOAGRO/UFV, Brazil) was used to clone *EgMUR3*. BACs were pooled and screened by PCR for genes involved in cell wall metabolism with primers designed according to EST data obtained from a private EST database (at <http://www.lge.ibi.unicamp.br/eucalyptus>). A positive BAC clone (87E04) of approximately 180 kb containing a putative ortholog of the MUR3 gene of *A. thaliana* was identified using the following primer pairs: *egmur* for 5'-GGCCACATTGTCCTTGTTTT-3' and *egmur* rev 5'-GGGAGTTTCCAAATGGCTCT-3'. A shotgun library of this BAC was constructed in order to obtain the complete sequence of *EgMUR3*. The library was

constructed after physical shearing of the insert fragment released from the BAC clone 87E04 using the TOPO® Shotgun Subcloning kit (Invitrogen). Sheared fragments were ligated into a pCR®4Blunt-TOPO® plasmid creating the EUGR_SG_006 subclone library containing inserts of approximately 1.5 kb. Large-scale sequencing of this library was performed (MegaBACE 500 sequencer, Amersham Pharmacia) using the universal primers SP6 (5'-ATTTAGGT GACTATAG-3') and T7 (5'-TAATACGACTCACTA TAGGG3'), which border the pCR®4Blunt-TOPO® cloning site, resulting in the complete sequence of the 180-kb genomic insert. The raw data was converted to FASTA query sequences and subjected in batches to BLAST X analysis. One of the shotgun sequences showed a high sequence homology to the *A. thaliana* MUR3 gene. Plasmid DNA of subclones of the EUGR_SG_006 library displaying sequence overlap to the *A. thaliana* MUR3 ortholog was isolated, and the insert between SP6 and T7 primers was fully sequenced in order to obtain the full *EgMUR3* gene sequence. Reads were processed with Phred (>20) for base calling, cross_match for removing vector sequences, and Phrap and Consed for assembly (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998). The final contig was analyzed using GENSCAN (<http://genes.mit.edu/GENSCAN.html>) to determine the full *EgMUR3* sequence.

Sequence analysis

Peptide properties were predicted by ProtParam (<http://www.expasy.org/cgi-bin/protparam>). The Conserved Domains Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) was searched for putative catalytic domains. Analysis of *EgMUR3* amino acid sequence for transmembrane domain was performed with TMHMM v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0>) and TopPred (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>). Hydrophobic cluster analysis (Callebaut et al. 1997; Gaboriaud et al. 1987) was performed at <http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA>.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA v. 4 (Tamura et al. 2007) and box-shading with GeneDoc (Nicholas et al. 1997). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree had the sum of branch length=5.95201462. Next to the branches, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown (Felsenstein 1985). The tree was 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 Dayhoff matrix-based method (Schwarz and Dayhoff 1979) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 33 positions in the final dataset.

Construction of pBinAr-*EgMUR3* and plant transformation

Primers containing adaptamers for *KpnI* and *SalI* (*egmur3_kpn_for* 5'-GGTACCATGAGACGCCGTTCTCGTGGCGA-3' and *egmur3_sal_rev* 5'-GTCGACTCATGACTGTCTCTCTGCTCGTT-3') were used to amplify the *EgMUR3* coding sequence from the BAC clone 87E04. A high-fidelity PCR with *Pfu* Turbo DNA polymerase (Invitrogen) was facilitated according to the manufacturer's recommendations. About 20 ng of BAC DNA were used as template in the PCR. A gradient procedure was used, starting from 65°C to 55°C (−1°C/cycle). Other steps consisted of 25 amplification cycles with primer annealing temperature set to 56°C. After the last cycle, 0.5 µl of Taq DNA polymerase was added to the mixture and an additional amplification cycle was performed during 10 min.

The PCR product was directly cloned into pCR®II-TOPO® vector (Invitrogen) according to manufacturer's instructions. The ligation reaction was transformed into one-shot competent bacteria (Invitrogen). Ampicillin-resistant colonies were picked, and plasmid DNA was extracted by alkaline lysis method (Sambrook and Russell 2001). Plasmidial DNA was analyzed by digestion with restriction enzymes, and a positive clone was selected and fully sequenced with *egmur3_kpn_for* and *egmur3_sal_rev* primers to confirm for the absence of mutations in the PCR-amplified *EgMUR3* ORF. The 1.8-kb insert corresponding to the *EgMUR3* ORF was excised from pCR®II-TOPO®-*EgMUR3* with *KpnI* and *SalI* restriction enzymes, gel-purified using the Nucleospin Extract II kit (Machery-Nagel), and ligated into the *KpnI*–*SalI* restriction sites in the MCS (multiple cloning site) of pBinAr (Hofgen and Willmitzer 1990), a pBIN19 derivative (Bevan 1984) containing the 35S promoter from the cauli-flower mosaic virus.

The pBinAr-*EgMUR3* construct was transformed and amplified into *Escherichia coli* DH5α. The pBinAr-*EgMUR3* plasmid was then purified and transformed into *Agrobacterium tumefaciens* strain GV 3101 (pMP90) by electroporation (Mersereau et al. 1990). After confirmation that the *A. tumefaciens* strain contained the proper *EgMUR3* gene, pBinAr-*EgMUR3* and an empty vector control (pBinAr) were transformed into *A. thaliana mur3* mutant by *A. tumefaciens*-mediated transformation (Bechtold and Pelletier 1998; Clough and Bent 1998). Infiltrated plants were grown for production of seeds. Sterilized seeds from infiltrated plants

were placed on Petri dishes with 1% (w/v) sucrose AMOZ media (11.0 g L⁻¹ MS medium, 2.5 g L⁻¹ MES, 7 g L⁻¹ bacto-agar, pH 5.7) plus antibiotics (50 µg mL⁻¹ kanamycin, 250 µg mL⁻¹ β-bactyl) for the selection of transformant plants. Plants were grown in long-day phytotron as described before.

To confirm the transgenic status of antibiotic resistant plants, total RNA was extracted from leaf material of 5-week-old plants using the RNeasy Plant Mini Kit (Qiagen). For cDNA synthesis, contaminant genomic DNA was removed with the DNA-free™ DNase Treatment & Removal Reagents (Ambion®). The cDNA synthesis was performed with Bioscript reverse transcriptase (Bioline USA Inc.) according to manufacturer's recommendations.

EgMUR3 message was detected by PCR with *EgMUR3* specific primers (egmur3RT_for 5'-CCATTGCGGTTAG GACTGAG-3' and egmur3RT_rev 5'-TGCTCTTAGTTCC CACCACTT-3') using the cDNA library as template. The constitutive APT gene (adenine phosphoribosyltransferase 1, At1g27450) was amplified as internal control with the primers: APT_for 5'-TCCCAGAATCGCTAAGATTGCC-3' and APT_rev 5'-CCTTTCCCTTAAGCTCTG-3'. The RT-PCR was performed with the recombinant Taq DNA polymerase from Invitrogen according to manufacturer's recommendations. Extension time was 1.5 min, and annealing temperature was 55°C. To confirm the specificity of primers used to amplify *EgMUR3*, a control PCR using *EgMUR3* primers and cDNA preparation from WT as template was conducted. *MUR3* gene primers were mur3RT_for 5'-GCACCAGTAGCCAATTCTAG-3' and mur3RT_rev 5'-GTCGCATACCAACCTTCATC-3'. In all RT-PCR analysis performed, 27 cycles were used.

Differential expression analysis of *EgMUR3* by RT-qPCR

E. urograndis plants were grown in pots with soil for 90 days under regular irrigation regime. Samples of roots, apical portion of stem, and expanding leaves were collected and pooled (two pools of three plants) for RNA extraction. Total RNA was extracted (Wang et al. 2008), and total RNA concentration was measured spectrophotometrically (Hitachi U2000 spectrophotometer) under 260 and 280 nm wavelengths. RNA concentration of all samples was adjusted to 300 ng µL⁻¹. Physical integrity of the RNA was assessed in 1% agarose gel (Wilson and Walker 2000). Afterwards, 2 µg of total RNA from each organ were treated with DNase I (Promega, USA) according to the manufacture's recommendations. The cDNA synthesis was carried out with Superscript II (Invitrogen, USA). Control experiments consisted of omitting Superscript II in two samples from each tissue. The RT-PCR was conducted from the cDNA library and the controls with primers for the *EgMUR3* gene and for two housekeeping genes: histone H2B and ribosomal protein

L23A. *EgMUR3* primers for qPCR were based on the *EgMUR3* sequence. The following *EgMUR3* primers were used: qEgMUR3_for 5' GACAGCGAAGAACCAGCACTC 3' and qEgMUR3_rev 5' GAGTTTCCAAATGGCTCT CAGTC 3'. Primers for constitutive genes were designed according to EST database information for *Eucalyptus* (<http://www.lge.ibi.unicamp.br/eucalyptus/>). Expression of the histone H2B and the ribosomal protein L23A were monitored in each qPCR experiment. Primers for histone H2B were: H2B_for 5' GAGCGTGGAGACGTACAAGA 3' and H2B_rev 5' GGCGAGTTTCTCGAAGATGT 3'. Primers for the ribosomal protein L23A were: RPtL23A_for 5' AAGGACCCTGAAGAAGGACA 3' and RPtL23A_rev 5' CCTCAATCTTCTTCATCGCA 3'. All primers were used at a final concentration of 0.12 pmol in each primer-specific reaction. The 20-µL final volume reaction contained 1× *High Fidelity PCR Buffer*; 3.0 mM MgCl₂, 25 µM of each dNTPs; 0.04 µL of ROX; 0.08 µL BSA; 2.0 µL *SYBR Green* (1:10,000 dilution), and 0.25 U Taq Platinum (all reagents from Invitrogen, USA). Three technical and two biological replicates for each sample were performed. The real-time PCR was conducted in a Step-One Plus equipment (Applied Biosystems, USA).

Primers validation based on the relative standard curve was performed with three technical replicates of leaf samples from the biological pool 1 as four geometric dilutions (1:5) of the cDNA reaction (1, 5, 25, and 125 times diluted). Afterwards, 0.28 µL of the cDNA sample was used per reaction. The PCR program consisted of 90°C for 5 min, 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s during the amplification steps. The dissociation curve was determined starting from 58°C increasing 0.3°C each 20 s until 95°C was reached. Primer pairs assessed exhibited determination coefficients above 95%, variance coefficient in two repetitions less than 10%, amplification efficiencies of 95% to 100% and slope values near to -3.0 (Applied Biosystems, USA).

The relative quantitation was performed by the comparative C_T (ΔΔC_T) method using the Step-One Plus C_T (ΔΔC_T) program (Applied Biosystems, USA). The cycling conditions were the same as for the relative standard curve. The experimental design consisted of two biological pools with three technical replicates for each sample: root, stem, or leaves.

Data analysis was carried out with the Step-One Plus software v. 2.0 (Applied Biosystems, USA). Threshold cycle (C_t) values for the technical replicates were calculated out and set by the relative quantitation method of copy-level amplification (Ali-Benali et al. 2005). In order to determine the relative expression of the target genes in the respective tissues, homogeneity was assessed by the Bartlett test ($p=0.05$) for the three technical replicates of each biological sample. For comparative test,

root sample was used. Relative *EgMUR3* expression level in the analyzed tissues was assessed by comparison with the relative expression level of the housekeeping genes (Nicot et al. 2005). The expression level (R) was then determined: $R = 2^{-\Delta\Delta C_t}$, where $-\Delta\Delta C_t = (\Delta C_T \text{ of } EgMUR3) - (\Delta C_T \text{ of the housekeeping genes})$. Comparative analysis was performed for both constitutive genes. The variance components comprised of the results obtained from gene expression analysis in both biological replicates of each organ in comparison with both constitutive genes. The relative *EgMUR3* expression in each organ was evaluated by ANOVA and the average of each treatment compared by Scott–Knot test ($p=0.05$).

Cell wall analysis

For the characterization of XyG in WT, *mur3* and the generated transgenic plants oligosaccharide mass profiling was performed using a purified xyloglucan-specific endo- β -1,4-glucanase (Pauly et al. 1999b) in combination with MALDI-TOF mass spectrometry (Lerouxel et al. 2002). The XyG oligosaccharides released by the digestion were assessed in a Voyager DE-Pro MALDI-TOF (Applied Biosystems, Germany) equipment using an acceleration voltage of 20,000 V and a delay time of 350 ns. Mass spectra were obtained in the positive reflection mode.

Results

Cloning and sequence analysis of *EgMUR3* gene

EgMUR3 sequence (GenBank accession no. GU013763) consisted of an intronless sequence of 1,854 bp coding for a predicted protein with 617 amino acid residues with a deduced molecular mass of 70.8 kDa and an isoelectric point of 8.19. Alignment analysis revealed a striking similarity of 82% and identity of 73% over 617 amino acids between *EgMUR3* and the *MUR3* protein from *A. thaliana* (Fig. 1). Searches for catalytic domains in *EgMUR3* protein were performed by comparing its amino acid sequence with others, available through the Conserved Domains Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). This analysis revealed that *EgMUR3* contains a pfam03016 motif from the exostosin superfamily, ubiquitous to GT47 glycosyltransferases from many organisms including microorganisms, animal, fungi, and plant. Local alignment of their predicted catalytic domains (GT47 signature motif for exostosins) revealed similarity and identity of 96% and 90%, respectively. Searches of Genolyptus database (<http://www.lge.ibi.unicamp.br/eucalyptus>), which contains a large collection of *Eucalyptus* ESTs from different organs

and species, did not return any nucleotide or amino acid sequence showing significant homology to *EgMUR3*.

TMHMM v. 2.0, used to predict transmembrane helices in proteins, identified an N-terminal cytoplasmic tail followed by a stretch of hydrophobic residues in *EgMUR3*, which is consistent with a putative transmembrane domain (TMD) typical of type II glycosyltransferases (Fig. 1). In addition, TopPred analysis showed that the charge distribution along *EgMUR3* is in agreement with typical type II GTs, and the most negative values for hydrophobicity observed after the TMD could correspond to the enzyme stem (not shown). Hydrophobic cluster analysis (HCA) of *EgMUR3* revealed four putative DxD motifs (Fig. 1). This analysis takes into consideration the hydrophobicity of the amino acids and the folding possibilities in aqueous environment to give rise to catalytic pockets in the enzyme. These sites could potentially accept a UDP sugar, the putative donor substrate to this type of GT.

Phylogenetic analysis

Alignment of *EgMUR3* with relevant GTs from the GT47 family was performed, and a phylogenetic tree with the evolutionary relationship between them is shown in Fig. 2. Family GT47 GTs are abundant in plants and exhibit a diversity of enzyme specificities, including β (1,2)-galactosyltransferase, β (1,3)-xylosyltransferase, β (1,4)-xylosyltransferase and possibly also α (1,5)-arabinoxyltransferase and β (1,4)-glucuronyltransferase activities. The GT47 family might comprise genes encoding GTs involved in cell wall architecture and storage carbohydrates. Despite the abundance of GT47 family members to date, only the function of a few of those have been determined, and representative members for some subfamilies are still not functionally characterized, hampering the assignment of putative enzyme activities for related entries in the same subfamily branch. The branches comprising FRA8 and IRX10 are better characterized and contain GTs involved in xylan or pectin biosynthesis. NpGUT1 is a pectin glucuronyltransferase involved in the transfer of glucuronic acid to the rhamnogalacturonan II domain in pectin (Iwai et al. 2002), whereas FRA8 and PoGT47C are involved in xylan biosynthesis (Zhong et al. 2005). According to expression profile analysis (Aspeborg et al. 2005), poplar PttGT47A has been suggested to transfer glucuronyl side chains to rhamnogalacturonan II in ray cells or alternatively, to 4-*O*-methylglucuronoxylan during secondary wall thickening of poplar stem. Nevertheless, poplar PttGT47A was closely related to IRX10 and IRX10-L (Fig. 2), and in two recent studies, IRX10 and IRX10-L were shown to participate in the xylan backbone biosynthesis (Brown et al. 2009 and Wu et al. 2009). The authors showed that the double mutant *irx10 irx10-l* exhibits strong reduction of xylan content, polymerization, and β (1,4)-xylosyltransferase

Fig. 1 Sequence alignment of EgMUR3 and MUR3 amino acid sequences and prediction of transmembrane domain and DxD motifs in EgMUR3. Exostosin motif is underlined. The putative transmembrane domain (TMD) is boxed and encompasses amino acids 26 to 48 as determined by TMHMM 2.0. The enzyme stem rich in charged

residues was localized by TopPred after TMD and before the exostosin motif. Hydrophobic cluster analysis (HCA) identified four candidate DxD motifs (the four numbered boxes along the EgMUR3 amino acid sequence relates to the encircled residues in the HCA map)

activity. Furthermore, Wu et al. 2009 demonstrated that the N-terminal portion of IRX10/IRX10-L contains a signal peptide determinant for xylan biosynthesis and that NpGUT1 lacks this N-terminal portion. Interestingly, IRX10 N-terminal portion fused to NpGUT1 conferred the ability of NpGUT1 to successfully rescue the *irx10* phenotypes. Recently, F8H, another gene involved in xylan biosynthesis, has been characterized. F8H shares the same biochemical function with FRA8, as shown by complementation studies of *fra8* mutant (Lee et al. 2009). A gene encoding for an alpha-1,5-arabinosyltransferase (ARAD1) of *A. thaliana* (Harholt et al. 2006) was also included in our analysis. EgMUR3 and ARAD1 share 20% aminoacidic identity (8e-07) and were distantly related in our phylogenetic tree. In addition to MUR3, a poplar entry (gi, 224065537) was found to be highly conserved with EgMUR3 and could potentially be a poplar xyloglucan galactosyltransferase.

EgMUR3 expression analysis in *E. urograndis*

Endogenous expression of *EgMUR3* was investigated in roots, expanding leaves and growing stems of 90-day-old *E. urograndis* plants by qPCR. *EgMUR3* transcript abundance differs in the investigated organs. *EgMUR3* expression is almost tenfold higher in growing stem in comparison to roots and about fivefold higher in young expanding leaves (Fig. 3).

Generation and identification of transformant plants

The 4.6-kb contig obtained from the sequenced subclones of the EUGR_SG_006 library showing sequence overlap to the *A. thaliana* MUR3 ortholog contained the complete coding sequence of the *EgMUR3* gene. PCR of BAC 87E04 DNA as template and *EgMUR3* specific primers with *KpnI* and

Fig. 2 Evolutionary relationship of *EgMUR3* with other GT47 glycosyltransferases. Only GT47 members showing relevant homology with *EgMUR3* or functionally characterized GTs were selected for the analysis. Common name was used according to the authors that described the respective gene/enzyme and gi numbers were used for the not yet characterized GT47 members. Po and At after each GT mean Poplar or *A. thaliana*, respectively. Common names represent: MUR3/KAM1—xyloglucan galactosyltransferase from *A. thaliana*; EgMUR3—xyloglucan galactosyltransferase from *E. grandis*; NpGUT1—pectin glucuronyltransferase from *Nicotiana plumbaginifolia*; PoGT47C—glycosyltransferase involved in the biosynthesis of glucuronoxylan in *Populus alba*×*tremula*; FRA8—

glucuronoxylan glucuronyltransferase from *A. thaliana*; ARAD1—alpha-1,5-arabinosyltransferase from *A. thaliana*; ARAD2—ARAD1 homolog from *A. thaliana*; PttGT47A—glycosyltransferase possibly involved in the transference of glucuronyl side chains to 4-*O*-methylglucuronoxylan of secondary wall or to rhamnogalacturonan II in the ray cells; PttGT47B and PttGT47C—up-regulated during secondary wall thickening of poplar cambial region; XGD1—xylogalacturonan xylosyltransferase 1 from *A. thaliana*; F8H—glycosyltransferase that complements *fra8* mutation in *A. thaliana*; IRX10-L and IRX10—involved in glucuronoxylan biosynthesis in *A. thaliana*

Sall adaptamers for cloning into pBinAr MCS resulted a PCR product of approximately 1.8 kb that facilitate the construction of pBinAr-EgMUR3 plant transformation vector. This vector was successfully transformed into *A. thaliana mur3* plants using floral dip method, and transformants were identified by growth on kanamycin media.

Phenotypic alterations were not observed in transgenic plants constitutively expressing *EgMUR3*. After 5 weeks, leaf material was collected for transgenic and cell wall analysis. Transgenic status was confirmed by RT-PCR for most of the plants selected on kanamycin media (Fig. 4a). Since *MUR3* transcript is still present in *mur3* plants (The *mur3* mutant allele has a miss-sense mutation) and *EgMUR3* shares high homology of sequence with *MUR3*, primers for characterization of the transformants were designed to a region of minimal alignment between *EgMUR3* and *MUR3*. To confirm the specificity of the primers, a PCR test was performed with Col-0 cDNA preparation from young leaves as template and primers for

the endogenous *MUR3* or *EgMUR3*. *MUR3* message was detected in the cDNA preparation from Col-0 leaf material only with the *MUR3* specific primers, but not with *EgMUR3* primers, confirming that *EgMUR3* primers were specific for the detection of transgenic plants (Fig. 4b). Plants with strong and weak expression of *EgMUR3* were probably identified due to positional effects of the inserted transgene (Fig. 4a).

Cell wall analysis

Cell wall material was prepared from young leaves of Col-0, *mur3*, empty vector control plants, EgM4 (weak expressor), EgM9 and EgM10 (strong expressors). Cell wall material was digested with XEG, and the released XyG oligosaccharides (XyGOs) were analyzed by MALDI-TOF MS. *MUR3* has previously been shown to add a galactosyl unit solely to the third xylosyl residue from the non-reducing end in the XXXG xyloglucan oligosaccharide

Fig. 3 Relative expression of *EgMUR3* in roots, leaves, and stem of *E. urograndis*. Relative expression level of *EgMUR3* normalized to root (fold-change=1) is shown in the columns. Letters indicate statistic differences of transcript level. Statistics consisted of ANOVA and Scott–Knot test at 5% probability

core (Madson et al. 2003). Since fucosylation at this position depends on previous galactosylation, all galactosylation (XXLG) and fucosylation (XXFG and XLFG) ions are lacking in *mur3* mutant (Fig. 5). In the EgM9 and EgM10 transformant plants, the peaks corresponding to *m/z* 1,393.2 (XXFG) and 1,555.2 (XLFG) were restored, confirming that *EgMUR3* possesses a xyloglucan galactosyltransferase activity. Also, *EgMUR3*-expressing plants exhibited an increase in fucosylation, galactosylation, and *O*-acetylation (XXLG/XLXG, XXFG and XLFG) in comparison to Col-0 (Fig. 5, lower panel), suggesting that the abundance of the galactosyltransferase gene product might be limiting or that the *E. grandis* *EgMUR3* might have different enzymatic properties. Despite the EgM4 line

exhibiting kanamycin resistance and *EgMUR3* expression, the XyG structure was not restored. Thus, this line cannot be considered to contain a functional MUR3.

Discussion

Cell wall genes from woody species have successfully complemented *A. thaliana*, proving to be a suitable and fast approach for investigation of gene function in tree species (Dornelas et al. 2004; Park et al. 2003; Zhou et al. 2006). In this study, transformation of *Arabidopsis* with a newly identified and cloned *EgMUR3* gene from *E. grandis* permitted the successful identification of this gene as a xyloglucan galactosyltransferase.

In the *A. thaliana* genome, *MUR3* is represented as a single-copy gene, and its non-functional redundancy is confirmed by the impact of *mur3* mutation on xyloglucan structure (Reiter et al. 1997; Tamura et al. 2005). Although the subcellular localization of *EgMUR3* needs to be confirmed, in silico analysis determined that it is a type II GT that carries a TMD. *EgMUR3* is expected to be anchored in the Golgi, with its C-terminal portion probably facing the Golgi lumen, as is the case with other type II GTs (Fleischer 1981). *EgMUR3* has a stretch of charged amino acids after the TMD that could be the “stem” segment, which is responsible for the proper positioning of the catalytic domain of anchored GTs away from the lipid bilayer in the lumen face (Breton et al. 2001). *EgMUR3* belongs to the GT47 CAZy family represented by several exostosin-like glycosyltransferases. Analysis within the exostosin domain of *EgMUR3* (pfam03016) revealed important putative DxD motifs that might be directly involved in the galactosyltransferase activity. Several GTs that utilize nucleotide sugars as donor substrate and sugar moieties as acceptor contain conserved DxD motifs [aspartic acid (D), any (X), aspartic acid (D)] (Breton and Imberty 1999). Variants of these

Fig. 4 Transcript analyses by RT-PCR of *Arabidopsis* lines. **a** EgM1 to EgM12—*EgMUR3* transformant plants, BAC—DNA of BAC 85E07 was used as template (positive control), Col-0 and *mur3*—DNA from Col-0 and *mur3* plants were used as templates (negative controls). **b** RT-PCR with Col-0 cDNA preparation to test the specificity of the primers used in the analysis of transgenic plants.

APT+—amplification with primers for the adenine phosphoribosyltransferase 1 constitutive gene, *EgMUR3*—amplification using *EgMUR3* primers, *MUR3*—amplification using *A. thaliana* *MUR3* primers, *APT-*—Test for genomic DNA contamination using *APT* primers in a sample which reverse transcriptase was not added during cDNA synthesis

Fig. 5 XyG MALDI-TOF MS spectra derived from *Arabidopsis* leaf cell wall material of the various lines. The lower panel shows the relative abundance of XyGO ions as displayed above. Ions representing known XyGOs are labeled using the one-letter code according to

Fry et al. 1993. *O*-acetylated residues in XyGOs in the MALDI-TOF MS spectra are underlined. Error bars represent the standard deviation from two technical and two biological replicates

motifs are EDD, EED, or [DE]×[DE], where (E) represents glutamic acid. These residues are negatively charged in the cellular environment and therefore might contact divalent cations, which are believed to coordinate the binding between substrates and enzyme, functioning as nucleotide binding domain.

Functional analysis of EgMUR3

Analysis of *EgMUR3* expression in *E. urograndis* by qPCR revealed that *EgMUR3* is mainly expressed in elongating stems and expanding leaves of young plants. In roots, transcripts were detected, but in much lower levels.

In order to investigate the ability of *EgMUR3* to rescue the *mur3* phenotype, an oligosaccharide mass profiling (Lerouxel et al. 2002; Obel et al. 2006) of *EgMUR3*-transformed lines was performed. The results obtained from MALDI-TOF MS analysis clearly confirmed that *EgMUR3* gene encodes for a galactosyltransferase that recognizes acceptor substrates for the xyloglucan biosynthesis in *A. thaliana* and decorates the third xylosyl residue within the XXXG core structure just like *MUR3*. This was confirmed by the restoration of the fucosylated side chains leading to the detection of XXFG and XLFG peaks in the transgenic plants in contrast to *mur3* plants. A compensation for the lack of galactosylation/fucosylation at the third xylosyl seemed to take place, as detected by the remarkable increase of XLXG abundance when one compares *mur3* and Col-0 spectra. On the other hand, EgM9 and EgM10 transformants exhibited an increase of fucosylated XyGOs (XXFG and XLFG) and a decrease of XXXG in comparison to Col-0 levels. These results support the idea that XXXG is a core structure for further decorations of XyG, since the lack of galactosylation/fucosylation in *mur3* leads to increased levels of XXXG and XXLXG/XLXG, whereas the constitutive expression of *EgMUR3* in *Arabidopsis mur3* plants decreases XXXG core below to the WT levels. Additionally, *EgMUR3* overexpression remarkably increases fucosylation level concomitantly with an increase of *O*-acetyl substitutions. This suggests that the expression level of endogenous *MUR3* in *Col-0* plants might be limiting with regards to XyG galactosylation and further substitutions associated with it. Alternatively, *MUR3* from *E. grandis* might have different enzymatic properties than that of *A. thaliana* resulting in overgalactosylation of XyG.

Overexpression of the fucosyltransferase AtFUT1 in *A. thaliana* also led to a high xyloglucan *O*-acetylation (Perrin et al. 2003). In contrast, mutants with reduced or lack of fucose in their XyG structure (*mur1* and *mur2*) exhibited a significant reduction of *O*-acetylation, suggesting that fucosyl residues on the XyG structure are the primary determinant for galactosyl *O*-acetylation. However, the biological role of *O*-acetyl-substituents on XyG or any polysaccharide remains unknown. Structurally, *O*-acetylation has been suggested to block enzymatic degradation of XyG (Pauly and Scheller 2000).

In *A. thaliana*, different *MUR3* mutant alleles exert different effects on plant development (Madson et al. 2003; Reiter et al. 1997; Tamura et al. 2005; Tedman-Jones et al. 2008). Madson et al. (2003) reported that the galactosylation and consequently, fucosylation at the third xylosyl residue (XXXG) is completely abolished while the galactosylation at the second xylosyl (XLXG) is enhanced in *mur3* mutants (*mur3-1* and *mur3-2*) carrying amino acid substitutions in different positions within the exostosin domain, without major consequences for normal plant development. In contrast, Tamura et al. (2005) reported

drastic effects due to the expression of a truncated form of KATAMARI1/MUR3 protein in the plant. These authors reported the loss of physical integrity of cytoskeleton elements that could be somehow connecting to the KATAMARI1/MUR3 protein. More recently, Tedman-Jones et al. (2008) isolated *mur3/ciel*, an *Arabidopsis* mutant showing constitutive defense responses in the petiole. *ciel* carries two amino acids substitutions in the *MUR3* gene: one at the putative exostosin domain and another at the C-terminal region.

One issue that remains to be solved in XyG biosynthesis is the precise mechanism by which sugar moieties are added to XyG. In *E. grandis*, for instance, the basic XyG structure is constituted of XXXG and XXXXG, but also many galactosylated XyGOs forms can be found, such as XXLXG/XLXG, XLXG, XLJG, and XLXXG (Dr. Marcos S. Buckeridge, personal communication). It has yet to be determined how many glycosyltransferases are required to produce all those XyGOs or even if a single or a few xyloglucan galactosyltransferase could have their transferase activity modulated by other factors at the cell level in the context of the XyG biosynthesis.

The functional characterization of novel eucalypts genes showing temporal or tissue-specific expression offers new opportunities for studies on modification of plant metabolism. For instance, since *EgMUR3* relates to primary cell wall metabolism in young expanding stem and leaves, functional studies using *EgMUR3* promoter to target gene expression of candidate genes controlling plant development and other aspects of cell wall metabolism could be investigated. Biological processes such as the early patterns of lignin biosynthesis and deposition, carbon relations through alteration of the sink strength, resistance responses to plant pathogens which preferentially start invading young tissues, and metabolism of many plant hormones which control cell wall constitution and the overall plant architecture are just few examples of potential studies using the *EgMUR3* promoter.

As functional genomic reports for this important forestry species become available, the understanding of how particular genes may affect plant development and biomass properties will pave the way for designing trees more suitable for pulping and bioenergy.

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