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Note

Biochemical characterization of family 43 glycosyltransferases in the *Populus* xylem: challenges and prospects

Anders Winzell[1], Gea Guerriero[1], Henrik Aspeborg[1], Yiqiang Wang[1], Alex S. Rajangam[2], Tuula T. Teeri[1], Inés Ezcurra[1,*]

[1]Royal Institute of Technology, Department of Biotechnology, AlbaNova University Center, SE-10691 Stockholm, Sweden; [2]Warwick HRI, University of Warwick, Wellesbourne, CV35 9EF Warwickshire, United Kingdom
* E-mail: ines@biotech.kth.se Tel: +46-85537-8388 Fax: +46-85537-8468

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Abstract Wood formation is a biological process of great economical importance. Genes active during the secondary cell wall formation of wood fibers from *Populus tremula*×*tremuloides* were previously identified by expression profiling through microarray analyses. A number of these genes encode glycosyltransferases (GTs) with unknown substrate specificities. Here we report heterologous expression of one of these enzymes, PttGT43A, a putative IRREGULAR XYLEM9 (IRX9) homologue. Expression trials in *Pichia pastoris* and insect cells revealed very low levels of accumulation of immunoreactive PttGT43A, whereas transient expression in *Nicotiana benthamiana* leaves by *Agrobacterium* infiltration (agroinfiltration) using a viral vector produced substantial amounts of protein that mostly precipitated in the crude pellet. Agroinfiltration induced weak endogenous xylosyltransferase activity in microsomal extracts, and transient PttGT43A expression further increased this activity, albeit only to low levels. PttGT43A may be inactive as an individual subunit, requiring complex formation with unknown partners to display enzymatic activity. Our results suggest that transient co-expression in leaves of candidate subunit GTs may provide a viable approach for formation of an active xylan xylosyltransferase enzymatic complex.

Key words: GT43 glycosyltransferase, IRX9, *populus* xylem, xylan, xylosyltransferase.

During the past decade *Populus* has emerged as a convenient model organism to study wood biosynthesis (xylogenesis) (Mellerowicz et al. 2001). A large gene expression database was assembled by expressed sequence tag (EST) sequencing and microarray analysis of different *Populus* tissues (Sterky et al. 2004; Sterky et al. 1998). Genes that are active during xylogenesis were identified by expression profiling of cDNAs isolated from cryosections corresponding to narrow zones of the developing xylem of *Populus tremula*×*tremuloides* (hybrid aspen) (Hertzberg et al. 2001). As a result, over 200 genes were identified that are up-regulated during early secondary cell wall synthesis and among these, 14 genes were annotated as putative glycosyltransferases (GTs) that are not involved in cellulose biosynthesis (Aspeborg et al. 2005).

The secondary cell walls of plants consist of two types of carbohydrate polymers, cellulose and hemicelluloses, which—together with a phenolic polymer, lignin—contribute to the strength and durability of wood. Hemicelluloses are branched carbohydrate polymers that are, like cellulose, synthesized by GTs. The major hemicellulose in hardwoods is glucuronoxylan (Ebringerova 2006), a polymer with a linear backbone consisting of β-(1,4)-linked D-xylosyl and with sidechains of either glucuronic acid or 4-*O*-methyl-glucuronic acid.

Mutant analysis in *Arabidopsis thaliana* showed that the IRREGULAR XYLEM (IRX) and FRAGILE FIBER (FRA) genes FRA8/IRX7, IRX8, IRX9, IRX10, IRX14, as well as PARVUS, which are members of the glycosyltransferase families 47, 8, 43, 47, 43 and 8, respectively, are essential for normal xylan biosynthesis (Brown et al. 2005, 2007, 2009; Lee et al. 2007a, 2007b; Peña et al. 2007; Persson et al. 2007; Wu et al. 2009; Zhong et al. 2005). Current data suggests that IRX8, PARVUS and FRA8/IRX7 are involved in the synthesis of a starting primer, the reducing end oligosaccharide, whereas IRX9, IRX10 and IRX14 may be required to synthesize the xylan backbone (Brown et al. 2007, 2009; Wu et al. 2009). In hybrid aspen, two highly conserved family 43-GTs, PttGT43A and PttGT43B, were identified as highly expressed during the secondary growth stage of xylogenesis (Aspeborg et al. 2005). Here

Abbreviations: CPM, counts per minute; DXD, aspartic acid-any amino acid-aspartic acid; EST, expressed sequence tags;; FRA, FRAGILE FIBER; GTs, glycosyltransferases; IRX, IRREGULAR XYLEM; kDA, kiloDalton; PttGT43A, *Populus tremula*×*tremuloides* glycosyltransferase 43 A; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

This article can be found at http://www.jspcmb.jp/

we report data on sequence and expression analysis of PttGT43A and PttGT43B as well as heterologous expression of PttGT43A, and attempts towards enzymatic characterization of the obtained protein.

We identified the gene models *estExt_fgenesh1_pg_v1.C_280131* and *estExt_Genewise1_v1.C_LG_XVI2679* in the genome of *Populus trichocarpa* (version 1.1, http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Tuskan et al. 2006), as the likely orthologs of *PttGT43A* and *PttGT43B* (GenBank accession numbers AY935504 and AY935505), respectively, thus confirming that *PttGT43A* and *PttGT43B* are paralogous genes and not copies of the same gene from the two species in the hybrid. However, the annotation of both genes in the *Populus trichocarpa* genome version 1.1 was too short, and did not include the entire protein-coding region. After careful re-annotation the full-length genes were found to exhibit 97% sequence identity between *P. trichocarpa* and the hybrid aspen, while the identity between the two genes was below 90% in both species. From a strictly phylogenetic perspective PttGT43B is closer to IRX9, as their percentage identity is higher both in the overall protein sequence and also in the catalytic domain. Consistent with this, the *Populus alba×tremula* GT43B, PoGT43B, complements the Arabidopsis *irx9* mutant, suggesting that it has xylan xylosyltransferase activity (Zhou et al. 2007). Although *Populus* GT43A and GT43B are highly similar (Figure 2), it still remains to be established whether GT43A is functionally redundant with IRX9/GT43B.

Although PttGT43A and PttGT43B are xylem-specific and coregulated in the developing cambium (Aspeborg et al. 2005; Figure 1A), the distribution of EST sequences in different tissue-specific cDNA libraries reveals a slight bias with ESTs corresponding to *PttGT43A* dominating in the tension wood library while *PttGT43B* has most ESTs in the cambial library (Figure 1B). The bias in PttGT43A expression towards tension wood is interesting since tension wood generally contains less hemicelluloses than normal wood. However, tension wood has a higher content of galactan compared to normal wood (Timell 1969). Tension wood galactan contains a highly branched arabinogalactan with a terminal glucuronic acid on some of the sidechains (Meier 1962; Timell 1967). Although mutant studies show that *irx9* is deficient in xylosyltransferase activity, the only biochemically confirmed activity in family 43-GTs is the transfer of glucuronic acid to glycosaminoglycan sidechains of proteoglycans (EC 2.4.1.135) (Gulberti et al. 2005). It is thus possible that while PttGT43B is a xylosyltransferase in the developing cambium, PttGT43A could transfer glucuronic acid to tension wood-specific polysaccharides.

An alignment of the translated protein sequences of *Populus* GT43 sequences and their predicted closest

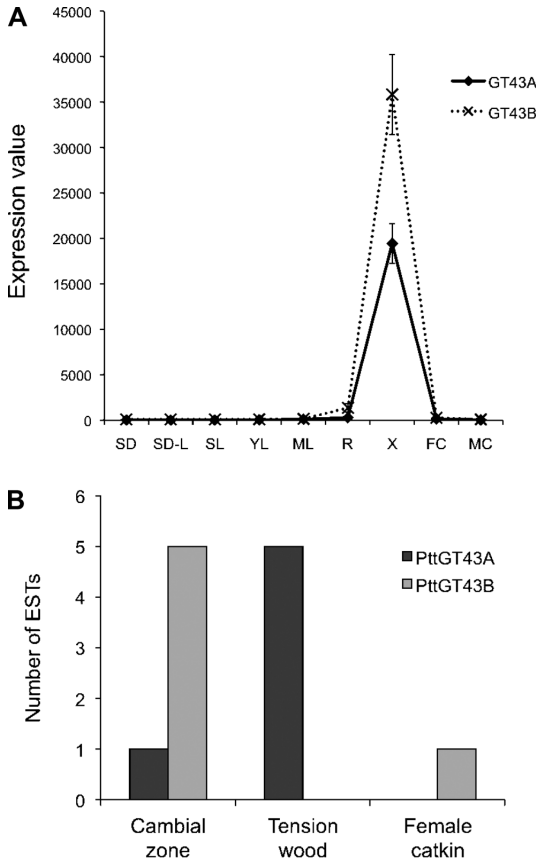

Figure 1. Expression of *Populus* GT43A and GT43B. (A) Expression levels of GT43A and GT43B in tissues of *Populus trichocarpa*. Microarray data was extracted from Poplar eFP Browser (http://bar.utoronto.ca; Wilkins et al. 2009). Expression levels are the average of three or two experiments. Developmental stages are: SD, dark-grown seedlings, etiolated; SD-L, dark-grown seedling, etiolated, exposed to light for 3 hr; SL, continuous light- grown seedling; YL, young leaf; ML, mature leaf, R, root; X, xylem; FC, female catkins; MC, male catkins. Error bars indicate standard deviation. (B) Distribution and number of ESTs in different tissues of hybrid aspen. Data were extracted from the *Populus* EST database at http://poppel.fysbot.umu.se/ (Sterky et al. 2004).

homologues are shown in Figure 2. Predicted transmembrane helices are highlighted. As expected of glycosyltransferases involved in biosynthesis of carbohydrate polymers in the Golgi lumen (Keegstra and Raikhel 2001), all the aligned protein sequences appear to be type-II transmembrane proteins with an amino-terminal cytosolic tail, a hydrophobic transmembrane region, a putative stem region and a carboxy-terminal catalytic domain. A DXD signature motif has been identified in the catalytic domains of family 2, 6, 7, 8, 13, 15, 27, 43, 64 and 78 glycosyltransferases, which have known crystal structures and GT-A fold (Breton et al. 2006). This motif has been shown to be involved in the coordination of the nucleotide donor and a divalent cation required for catalysis in these structures, and was also observed in a fiber-specific cotton GT43 (Wu and Liu 2005). However, the present sequence alignment

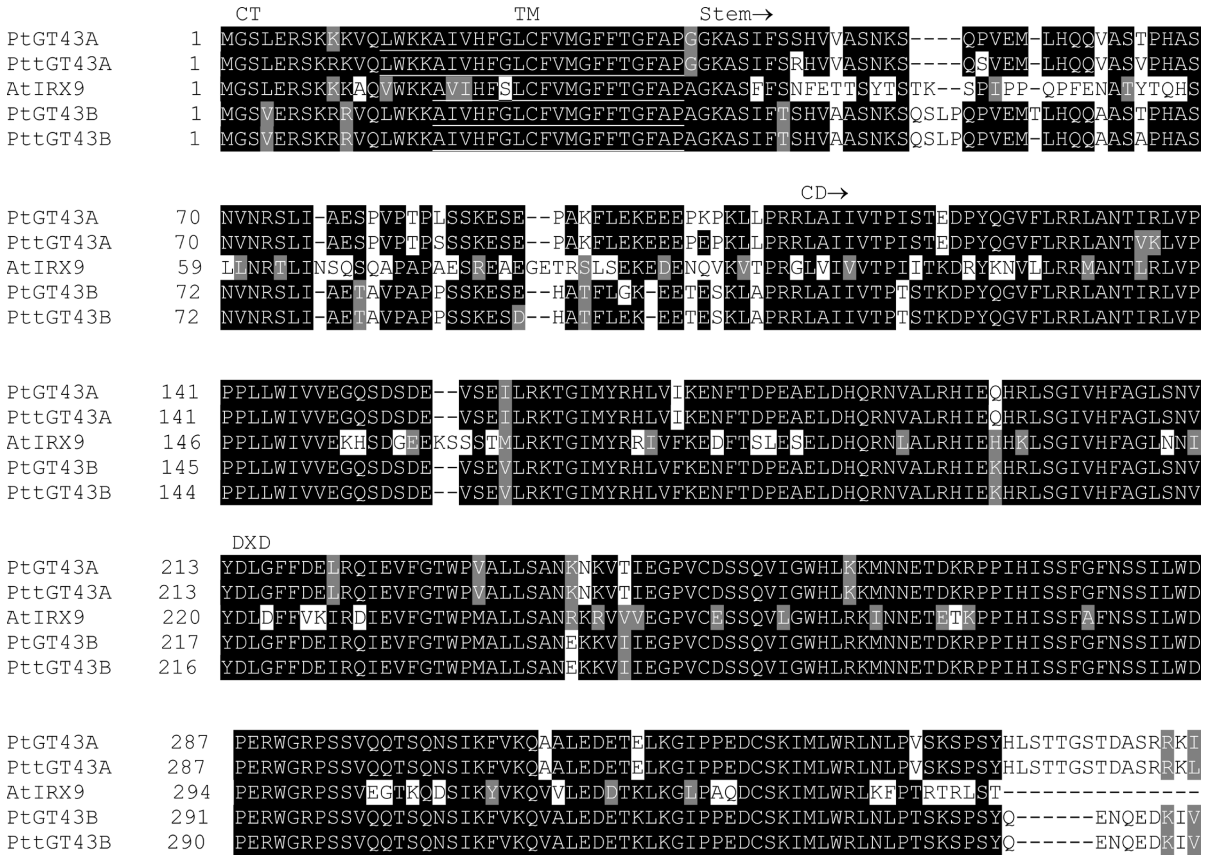

Figure 2. Alignment of *Populus* GT43A and GT43B with closest homologue AtIRX9 (AT2G37090), performed using Kalign (http://msa.cgb.ki.se/cgi-bin/msa.cgi; Lassmann and Sonnhammer 2005) and the Boxshade server version 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Transmembrane helices, predicted using CBS TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/; Krogh et al. 2001) are underlined. CT, cytosolic tail; TM, transmembrane helices; CD, catalytic domain; DXD, DXD motif.

reveals that the DXD motif is only present in IRX9, with the remaining poplar sequences exhibiting a DXXFFD sequence at this location. In the absence of crystal structures and enzymatic data for these proteins it is impossible to predict if this sequence could fulfill the same or a similar function as the DXD motif. Figure 2 highlights two major differences between PttGT43A and PttGT43B, namely a small insert in the stem region of PttGT43B (possibly a small loop), and dissimilar C-termini. However, the high similarity over their catalytic domains suggests that PttGT43A and PttGT43B could be functionally redundant paralogs with partially overlapping expression, in homology to IRX10-L and F8H, which are completely or partially redundant to IRX10 and FRA8, respectively (Brown et al. 2009; Lee et al. 2009; Wu et al. 2009). Thus, woody species like *Populus* could have evolved two redundant paralogs with IRX9 function for more efficient wood formation.

In order to get further insight into the function and role of the various glycosyl transferases in plant cell wall biosynthesis, biochemical characterization will be necessary. As truncated forms of some plant glycosyltransferases and other cell wall-associated enzymes have been successfully expressed in yeast by us and others (Edwards et al. 1999), we initially chose *P. pastoris* as host for expressing PttGT43A. However, only small amounts of immunoreactive protein were detected by Western blotting using antibodies against recombinant PttGT43A, and the broad distribution and high molecular weight of the signal suggested hyper-glycosylation. Purification of the over-glycosylated protein was not successful, and all attempts to deglycosylate the protein enzymatically or by glycan engineering did not lead to higher yield of specific protein.

Baculovirus-infected insect cells were also tried for the expression of PttGT43A, but, again, the level of protein production was too low for the detection of the corresponding bands on Coomassie-stained SDS-PAGE. Due to the persistently low protein yield, all attempts to obtain even small amounts of pure recombinant protein using ion exchange chromatography failed for both expression systems (data not shown).

In spite of vivid interest in characterizing plant glycosyltransferases, reports of successful expression of these proteins are scarce. An emerging trend in papers appears to be expression of the full length proteins

including the transmembrane domain, and using microsomal fractions as an enriched source of the enzyme (Wagner et al. 2006). It is thus possible that these enzymes fail to fold properly in the absence of the N-terminal domains, or that their folding is facilitated by the membrane environment, and that the lack of the membrane spanning domain is reflected in the extensive hyper-glycosylation of the proteins in yeast. It is also possible that the enzymes involved in hemicellulose biosynthesis work in close association with other glycosyltransferases in so far unidentified protein complexes in the Golgi. In the case of xylan-active GTs, it has been suggested that IRX9, IRX10 and IRX14 might form a functional complex that synthesizes the glucuronoxylan backbone (Brown et al. 2007; Wu et al. 2009), or alternatively, that IRX10 adds the first xylose to the reducing end oligosaccharide, and that IRX9 and IRX14 act together to extend the backbone chain (Brown et al. 2009).

In an attempt to overcome problems in heterologous expression we focused on developing transient expression systems in plants. Using *Agrobacterium* infiltration (agroinfiltration) in leaves of *N. benthamiana* combined with improved viral vectors for high-level expression (Marillonnet et al. 2005), we reproducibly obtained expression of a protein of expected size (40.3 kDa) that was immunoreactive against anti-PttGT43 antibodies and detectable by Coomassie Blue-stained SDS-PAGE (Figure 3). Unexpectedly, the expressed protein was mainly detected in the pellet after initial centrifugation of the crude leaf extract, suggesting that strong protein overexpression induces protein secretion to the cell wall by exocytosis, or, alternatively, aggregation and precipitation. The high similarity between PttGT43A and IRX9 prompted us to address whether it has xylosyltransferase activity, by measuring transfer of radiolabeled xylose from UDP-[C^{14}]xylose to exogenously added xylan. Initial attempts using total protein extracts failed to detect enzyme activity in all samples, including expected positive controls from untransfected xylem tissues, suggesting that measurement of xylosyl transferase activity requires purification of microsomal membranes. By using microsomal fractions we detected substantial xylosyltransferase activity in xylem controls both from *Populus tremula* and *N. benthamiana*, as well as low, albeit statistically significant ($P=0.011$), activity in leaves transiently expressing PttGT43A (Figure 4).

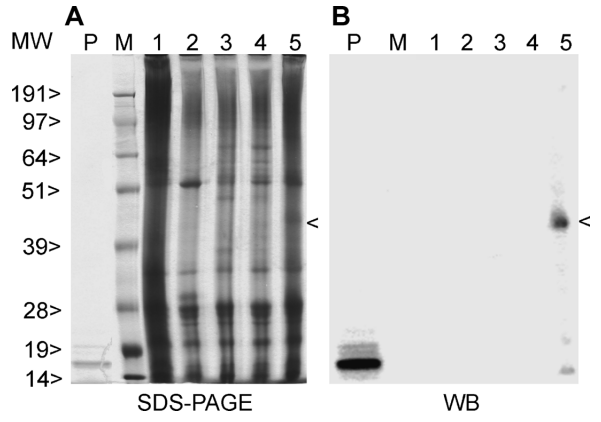

Figure 3. Transient expression of full-length PttGT43A in leaves of *N. benthamiana*. Agroinfiltration was performed as described (Giritch et al. 2006; Marillonnet et al. 2005) with vectors from Icon Genetics (Haale, Germany). PttGT43A-OE contains the full-length coding region of PttGT43A cloned into the 5′ viral promodule pICH11599. *Agrobacterium tumefaciens* GV3101 strains containing PttGT43A-OE, the 3′ promodule pICH17388 and the integrase module pICH14011, were mixed and infiltrated into leaves of *N. benthamiana.* After 8 days leaves were harvested, mechanically disrupted, and cell walls and debris were pelleted at 10,000 g 10 min. (A) SDS-PAGE analysis from crude cell wall extracts. P, PttGT43A recombinant fragment, 15 kDa; M, size marker; 1, untransfected leaves; 2–4, unrelated constructs and 5, PttGT43A-OE. (B) Western blot using PttGT43A-specific antibodies, obtained using the purified recombinant soluble domain of PttGT43A (aa 41-359) to immunize rabbits (Agrisera AB, Vännäs, Sweden) combined with the ECL Western Blotting analysis system (GE healthcare, UK). Samples are as in A.

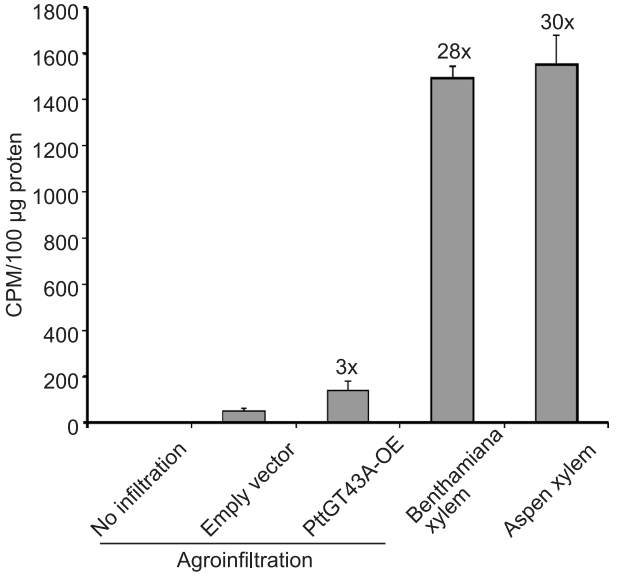

Figure 4. Measurement of xylosyltransferase activity in microsomal membrane preparations from agroinfiltrated leaves of *N. benthamiana* and from xylem of *N. benthamiana* and *P. tremula*. Microsomal membranes were isolated as described (Brown et al. 2007; Porchia and Scheller 2000) freshly from leaves and stem of *N. benthamiana* as well as from *P. tremula* vascular cambium, the latter obtained as described (Gray-Mitsumume et al. 2004). Xylosyltransferase activity was monitored in 40 μl reactions containing 1.5 kBq UDP-[^{14}C]-D-Xyl donor (Perkin Elmer; http://perkinelmer.com) and 50 μg 4-*O*-methyl-glucuronoxylan acceptor (Institute of chemistry, Slovak academy of science, Bratislava, Slovakia) for 1 h and terminated by precipitation with >2 volumes ethanol. The precipitate was added onto glass microfiber filters (Whatman; http://www.whatman.com) in a vacuum manifold and washed using water and ethanol. Incorporation of radiolabeled xylose was quantified by liquid scintillation counting of dried filters. Columns represent the average of three independent measurements. Values above columns indicate fold activation over empty vector control. Error bars indicate standard error of the mean.

Similar results were obtained when the experiment was repeated, and xylanase treatment of xylem control reactions reduced levels of labeled product by 70%, indicating that the product is xylan (not shown). Although much of the expressed protein is removed upon initial centrifugation of crude extracts, low levels of the 40.3 kDa protein were detected in microsomal extracts by western analysis (not shown), suggesting that residual PttGT43A in Golgi membranes causes the obtained low-level xylosyltransferase activity. We show that infiltration with *Agrobacterium* harboring an empty vector induces weak xylosyltransferase activity in leaves (Figure 4), in line with recent data showing that *Agrobacterium* infiltration induces pathogen-related and developmental responses in leaves of *Nicotiana tabaccum* (Pruss et al. 2008). It is thus possible that the expressed PttGT43A interacts with weakly induced endogenous xylan-active GTs, and that the low abundance of these is the limiting factor causing only weak levels of enzyme activity. In summary, the obtained low levels of activity may be caused by the low levels of protein retained in the Golgi, by limiting amounts of required partners, or by a combination of both reasons. In addition, it cannot be excluded that PttGT43A displays only weak xylosyltransferase activity, and that its primary enzymatic activity is something other than transferring xylose to the xylan backbone. Finally, our results highlight challenges in heterologous expression of plant proteins (also discussed by Farrohki et al. 2009 and Kaewthai et al. this issue), and suggest that transient co-expression in leaves of candidate subunit GTs may be a viable approach for formation of an active xylan xylosyltransferase enzymatic complex.

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