

Molecular characterization of two *Arabidopsis thaliana* glycosyltransferase mutants, *rra1* and *rra2*, which have a reduced residual arabinose content in a polymer tightly associated with the cellulosic wall residue

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Abstract Two putative glycosyltransferases in *Arabidopsis thaliana*, designated reduced residual arabinose-1 and -2 (*RRA1* and *RRA2*), are characterized at the molecular level. Both genes are classified in CAZy GT-family-77 and are phylogenetically related to putative glycosyltransferases of *Chlamydomonas reinhardtii*. The expression pattern of the two genes was analyzed by semi-quantitative RT-PCR using mRNA extracted from various organs of bolting *Arabidopsis thaliana* plants. In addition, *promoter::gusA* analysis of transgenic *Arabidopsis thaliana* containing a fusion between either the *RRA-1* or -2 promoter fragment and the *gusA* reporter gene showed that whereas the *RRA1* promoter was primarily active in the apical meristem, the expression pattern of the *RRA2* promoter was more diverse but also highly active in the meristematic region. In addition, T-DNA mutant insertion lines of both *RRA-1* and -2, were identified and characterized at the molecular and biochemical level. Monosaccharide compositional analyses

of cell wall material isolated from the meristematic region showed a ca. 20% reduction in the arabinose content in the insoluble/undigested cell wall residue after enzymatic removal of xyloglucan and pectic polysaccharides. These data indicate that both *RRA-1* and -2 play a role in the arabinosylation of cell wall component(s).

Keywords Plant cell wall · Glycosyltransferase · CAZy database · T-DNA insertional mutants.

Abbreviations

a.a.	Amino acid
AG	Arabinogalactan
AG II	Arabinogalactan type II
AGP	Arabinogalactan-proteins
CAZy	Carbohydrate-Active enZYmes
CW	Cell wall
EST	Expressed sequence tag

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GT	Glycosyltransferase
HG	Homogalacturonan
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
XG	Xylogalacturonan
XEG	Xyloglucanase
GUS	β -glucuronidase

Introduction

The cell wall (CW) of higher plants represents a unique type of extra-cellular matrix with numerous structural, protective and growth-regulating functions. Plant CWs need to be strong enough to prevent the protoplast from bursting under turgor and at the same time possess sufficient yielding properties to permit controlled cell expansion (Carpita and Gibeaut 1993).

The chemical composition of plant CW is well characterized, whereas little is known about the biosynthesis of wall polysaccharides and the principles of CW assembly. The earliest detailed CW model was based on studies of *Platanus occidentalis* suspension culture cells and proposed a CW of covalently interconnected biopolymer networks (Keegstra et al. 1973). Later models emphasized instead the separability of the different classes of CW polysaccharides observed during wall extraction, and proposed a wall structure that relied heavily on hydrogen bonding and other non-covalent forces (Talbot and Ray 1992; Carpita and Gibeaut 1993; Cosgrove 2001; Somerville et al. 2004). Covalent cross-linking was then mostly viewed as wall maturation processes that followed the completion of cell expansion. Recent observations by Popper and Fry (2005) have re-introduced emphasis on covalent cross-links as crucial to the integrity of the expanding primary wall.

Both types of models maintain that some polymers, notably the tethering heteroxylans and xyloglucans, associate strongly with microfibrillar cellulose, and researchers invariably find galactose- (Gal) and arabinose- (Ara) containing remnants bound to cellulose, even after exhaustive chemical extraction.

The major Ara- and Gal-containing CW polysaccharides are arabinans and type I (arabino)galactans associated with the pectic polysaccharides and the arabinoxylans (Bacic et al. 1988). The CW polysaccharides are, however, not the only carbohydrate-containing components that may bind strongly to cellulose or resist sequential extraction. Some of the less readily extractable polymers may be glycoproteins. The apoplastic space contains a substantial number of glycoproteins, of which a major group are the hydroxyproline-rich glycoproteins (HRGPs). These include the

readily soluble arabinogalactan-proteins (AGPs), a class of highly abundant proteoglycans, which contain up to 98% carbohydrate composed mainly of D-Gal and L-Ara (Showalter 1993; Johnson et al. 2003). Two types of glycomodules occur in the AGPs, the complex AGP-glycomodules and the extensin-type glycomodule associated with the Ser-Hyp₃ or Ser-Hyp₄ motif. Extensins are characterized by the Tyr-X-Tyr or Val-Tyr-Lys crosslinking motif in addition to the extensin glycomodule motifs (for reviews see Showalter 1993; Johnson et al. 2003). The extensin glycomodule consists of short arabinan chains and single galactosyl residues attached directly to the protein. Extensins are deposited in the wall as monomers, and remain extractable for a very short time before they are rendered resistant to extraction (Lampert 1963) probably by peroxidase catalyzed cross-linking (e.g., Popper and Fry, 2005).

Initially, CAZy GT-family-77 (Coutinho and Henrissat 1999; Coutinho et al. 2003; <http://afmb.cnrs-mrs.fr/CAZY/index.html>) was formed using two retaining *Arabidopsis thaliana* xylosyltransferases (XylTs), At4g01770 and At4g01750 (Egelund et al. 2004). The two XylTs have been shown to transfer xylose on to fucose forming an α -(1,3)-linkage and have been shown to be involved in the synthesis of pectic rhamnogalacturonan-II (Egelund et al. 2006). Together with the UDP-Gal:fucoside (1,3)- α -D-galactosyltransferase (GalT) from *Dictyostelium discoideum*, these are presently the only known activities in CAZy GT-family-77.

In the present work, two homologous *Arabidopsis thaliana* genes, At1g75120 and At1g75110, belonging to CAZy GT-family-77, are characterized by *promoter::gusA* mediated expression analysis and by analysis of the corresponding T-DNA insertion lines. CWs isolated from the meristematic tissues from T-DNA insertion lines in both At1g75120 and At1g75110, displayed a 20% reduction in the Ara content of the cellulosic residue following enzyme mediated extraction of the wall and were thus designated, *reduced residual arabinose 1* and *-2* (*rra1*; At1g75120 and *rra2*; At1g75110), respectively. Possible functions in the synthesis of less readily extractable CW polymers and glycoproteins are discussed.

Materials and methods

Sequence retrieval and phylogenetic analysis

The identification of the six putative GTs (At4g01220, At1g56550, At4g01770, At4g01750, At1g75120 and At1g75110) has recently been published (Egelund et al. 2004). All other accessions are available through the CAZy database (<http://www.afmb.cnrs-mrs.fr/CAZY/index.html>).

Alignments and phylogenetic trees were generated using Muscle (Edgar 2004) and modified using TreeView version 1.6.6 (Page 1996).

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0; WT) and T-DNA insertional mutants, were grown in soil in a controlled-environment growth chamber (Percival AR-60 I, Boone, IA, USA) at a photosynthetic flux of 100–120 mol photons m⁻² s⁻¹ at 8 h light/16 h dark cycle, 20°C and 70% relative humidity.

T-DNA insertional mutants in At1g75120 (Garlic_76_G04 and SAIL_590_G09), and in At1g75110 (Garlic_244_A03 and SAIL_70_D08), were obtained through Syngenta (Garlic lines, http://www.tmri.org/pages/collaborations/garlic_files/GarlicAnalysis.html) and the Salk Institute (SAIL lines, Alonso et al. 2003), respectively.

Transcripts detected by RT-PCR

RNA was extracted from root, rosette leaves, cauline leaves, stem, siliques and flower from 4-week old bolting *Arabidopsis thaliana* plants. The RNA was extracted using the TRIzol reagent (Invitrogen A/S, Taastrup, Denmark) according to the manufacturer's instructions. RT-PCR using 3 µg of RNA from each tissue type, was performed according to Mikkelsen et al. (2003), with the exception of the cycle parameters in the PCR which were as follows: 3 min at 94°C, (25 cycles for 18S and 45 cycles for At1g75120 and At1g75110) of: 94°C for 30 s, 50°C for 30 s and 68°C for 30 s followed by 12 min at 72°C. The following primer sets were used to amplify each transcript specifically: At1g75120_forward, 5'-tgagaaggatgaggaaatga-3'; At1g75120_reverse, 5'-atggtccaatttctctca-3'; At1g75110_forward, 5'-gaaagaatcaagaactgaaga-3'; At1g75110_reverse, 5'-aggataactgattataacaaca-3'; 18S_forward, 5'-taaggattgacagactgagagct-3'; 18S_reverse, 5'-aatacatcagtgtagcgcgct-3'. To ensure reproducibility the experiment was replicated; both experiments produced identical results.

Screening for homozygous T-DNA insertional mutants

Homozygous plants were identified by PCR, using a pair of gene-specific primers designed to anneal outside of the T-DNA insertion, which in case of homozygosity does not produce a band of the predicted size (negative selection): forward primer (5'-atggcggttcgtaagag-3' for At1g75120, 5'-atggcgggtcgcagagac-3' for At1g75110) and reverse primer (5'-ctatgaaccatcacggaac-3' for At1g75120, 5'-tcaatct-

gaaccatcggg-3' for At1g75120). As a control for the negative selection, a set of At4g01750 specific primers (5'-atggcgagaacaacag-3' forward and 5'-ttactgcaatttcctaag-3'), were used. Genomic DNA was isolated using the Nucleon PhytoPure kit (Amersham Biosciences, UK).

Strategy for cloning of the *RRA-1* and -2 promoters

The promoter regions 2.0 kb upstream from the start codon of *RRA-1* (At1g75120) and -2 (At1g75110) were amplified from genomic DNA using PCR as described in the "Polymerase chain reaction" section and sub-cloned into pCR2.1-TOPO (as described by the manufacturer, Invitrogen Life Technologies) and cloned into pCambia 1301 (NcoI and KpnI).

The final pCambia 1301 vectors containing either the At1g75120 or At1g75110 promoter fragments were sequenced in both orientations, using the services provided by MWG (MWG-Biotech AG, Germany, <http://www.mwg-biotech.com/html/all/index.php>).

The pCambia 1301 vector (Clunies Ross Street at Dickson Rd, Black Mountain ACT 2601, Australia) contains the 35S::*gusA* cassette. The catalase intron (GIS) is inserted within the coding sequence of *gusA*, which ensures that the gene is not expressed in bacteria but only upon transfer to plants.

Arabidopsis thaliana transformation

The binary plasmid pCambia 1301, containing the promoter::*gusA* fusion construct, was mobilized into *Agrobacterium tumefaciens* strain pGV3850 by electroporation. Overnight cultures in LB medium supplemented with rifampicin (100 mg/l) and kanamycin (50 µg/ml) were grown to OD₆₀₀ = 2, centrifuged and the cells re-dissolved in infiltration medium (0.22% Murashige and Skoog medium (Sigma-Aldrich, Denmark), 0.32% g Gamborgs B5 medium (Sigma), 5% w/v sucrose, 0.03% v/v Silwet (Lehle Seed)) to give a OD₆₀₀ = 0.8. *Arabidopsis thaliana* plants were placed upside-down in the solution for 15 min and placed in the greenhouse. Seeds were harvested after ca. 8 weeks. The T₁ transgenic plants were selected on MS plates supplemented with hygromycin (50 µg/ml). Before plating on MSO plates, seeds were sterilized by incubating 2 × 10 min in 5% NaOCl; 0.02% Triton X-100, followed by washes in sterile water, and then germinated on MS agar containing 50 µg/ml hygromycin until positive transformants could be identified and transferred to soil. The transformed plants were subjected to PCR to confirm the T-DNA inserts, and 10 independent T₂ lines of the *RRA1* and *RRA2 promoter>::gusA* transformed *Arabidopsis thaliana* plants were analyzed for β-glucuronidase (GUS) activity.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to clone promoter regions from gDNA, and to check transformed *Arabidopsis thaliana* plants to ensure the presence of the T-DNA construct harboring the *promoter::gusA* construct in the genome. Taq polymerase (Invitrogen Life Technologies) was used for analytical purposes and PFU-polymerase (Stratagene) was used to generate the various constructs. Reaction mixture, mixed in 0.2 ml MicroAmp[®] Reaction Tube With Cap (Perkin Elmer): 3.0 µl Gibco BRL[®] 10 × PCR buffer (Invitrogen Life Technologies), 1 µl 50 mM MgCl₂, 1.0 µl 5 mM dNTP's, 2.0 µl 10 pM 5' primer, 2.0 µl 10 pM 3' primer, MQ water up to 30 µl, approximately 1–5 ng template DNA, 0.1 µl Gibco BRL[®] Taq polymerase (5 U/µl) (Invitrogen Life Technologies) or 0.5 µl Stratagene[®] cloned PFU polymerase (2 U/µl) (Stratagene). MgCl₂ was not added when using PFU. The PCR reaction was carried out on a Progene thermal cycler (Techne) using the following standard program: 97°C, 3 min, then 30 cycles at: 94°C, 30 s, followed by 50°C 30 s (annealing) and elongation for 1 min per kilobase (kb) fragment at 72°C, followed by 10 min at 72°C and ∞ at 4°C. Three microliter of the PCR reaction was tested on 1% agarose gels. The following primers were used (all listed from 5' to 3'): At1g75120_promoter_forward, atattgatccgggtaccaataccaagattggctgcta; At1g75120_promoter_reverse, atatgtcgaccatggtccaattgaagtatccgaa; At1g75110_promoter_forward, atatggatccgggtacctatatgctgttaa gcaagata; At1g75110_promoter_reverse, atatgtcgaccatggtgccgaaatgacgcagaagccaa.

Analysis of β-glucuronidase expression

Transformed *Arabidopsis thaliana* plants carrying the *promoter::gusA* reporter gene constructs were tested for GUS activity, using the protocol from Jefferson et al. (1987), with a few modifications. Plant material was incubated in GUS staining buffer (50 mM sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM EDTA, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 0.1% X-gluc (w/v)), and incubated at 37°C O/N. Samples were then cleared in 80% ethanol. All experiments were performed in triplicate with 10 independent transgenic lines from each construct. For each construct, all individual lines had a similar GUS staining pattern, with slight differences in intensity.

All pictures were taken using a digital camera (Nikon Coolpix 990) and were later modified using the Adobe Photoshop Version 7.0. © Adobe Systems Incorporated software. Small details were viewed using a microscope (Olympus BH-2, Olympus Instruments, UK) equipped with a normal light source.

Crude cell wall extraction: alcohol insoluble residues (AIR)

Alcohol insoluble residues were isolated as described by Abdulrazzak et al. (2005), with few modifications. Briefly, plant material was harvested and frozen in liquid nitrogen. The tissue was then homogenized using a Retschmill machine (model MM200, Retsch, Haan, Germany) at 25 Hz for 1 min. The ground tissue was then suspended in 70% ethanol, vortexed, and pelleted by centrifugation at 10,000 g for 10 min. The ethanol was decanted, and this procedure was then repeated using chloroform:methanol (1:1,v/v), until all chlorophyll was removed. The pellet was then washed twice in acetone. The remaining pellet was dried under vacuum for 5 min and either processed directly or stored until further use.

Fractionation of the cell wall polymers

Isolation and characterization of xyloglucan (XG) was performed as follows. The isolated CW pellet (100 µg AIR) was incubated with 3 U of xyloglucanase (XEG was provided by Novozymes (Bagsvaerd, Denmark), and purified according to Pauly et al. 1999), in 1 ml of 50 mM ammonium formate buffer, for 36 h at 37°C (shaking). After 24 h of incubation a further 3 U of XEG was added to maximize the removal of XG from the CW pellet (1 U of XEG releases 1 µmol of reducing XG oligosaccharide per hour). Samples were then briefly centrifuged to pellet the insoluble CW components, and the supernatant containing the solubilized XG collected for analysis using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) as described by Lerouxel et al. (2002). Xylan was extracted in a similar manner by digestion of CW material using a 50 mM ammonium formate buffer (pH = 4.5) with 3 U of xylanase M1 (Megazyme, Bray Ireland). After incubation for 24 h at 37°C (shaking) the supernatant was collected by centrifugation (10 min at 20,000g) and stored for analysis.

The remaining CW pellet was washed twice in 50 mM ammonium formate buffer, and used for extraction of the pectic polysaccharides as described by Sørensen et al. (2000), with the following modifications. The CW pellet was incubated with 3 U of pectin methylesterase (PME) and 20 U of endo-polygalacturonanase (EPG), in 1 ml of 50 mM ammonium formate buffer, for 36 h at 37°C (shaking). After 24 h of incubation a further 3 U of PME and 20 U of EPG were added to maximize the removal of the pectic polysaccharides from the CW pellet. Samples were then briefly centrifuged to pellet the large insoluble CW components, and the supernatant containing the solubilized pectic polysaccharides collected and further separated into rhamnogalacturonan I (RG I),

rhamnogalacturonan II (RG II) and homogalacturonan (HG), using size exclusion chromatography on a Superose 12 column (1 × 30 cm, Amersham Pharmacia), as described by Sørensen et al. (2000). The remaining CW pellet containing insoluble and undigested CW material was washed twice in 50 mM ammonium formate buffer, and twice in acetone before further processing. Extraction of water-soluble polysaccharides and proteoglycans was done as described by Schultz et al. (2000). Precipitation and collection of AGPs from the water soluble extract were done by using the β -glucosyl Yariv reagent (β -GlcY) as described by Gane et al. (1995). All CW extracted fractions were freeze-dried and stored for further analysis. The following CW fractions were obtained: total CWs (AIR), RG I, RG II, HG, XG, xylan and the remaining insoluble/undigested CW pellet.

Monosaccharide composition analysis

For neutral sugar analysis the freeze-dried CW fractions were hydrolysed with TFA, reduced and acetylated to form their corresponding alditol acetates and then analysed by gas chromatography mass-spectrometry (GC-MS) as previously described (Albersheim et al. 1967; Sims and Bacic 1995). Measurement of uronic acid was done using the protocol described by Blumenkrantz and Asboe-Hansen (1973).

Results

Organization of CAZy GT-family-77

The CAZy GT-family-77 presently comprises 36 accessions, 18 from *Arabidopsis thaliana*, 15 from *Oryza sativa*, one from *Linum usitatissimum* and the *Dictyostelium discoideum* (1,3)- α -D-GalT, AX3, (Fig. 1A). At5g40900 appears to be incomplete and as the flanking sequences do not immediately suggest how to reconstruct the full gene. We hypothesize that it is a pseudogene and have thus omitted it from the phylogenetic analysis. The *Dictyostelium discoideum* (1,3)- α -D-GalT has neither a signal peptide nor a membrane anchor and has been shown to galactosylate Skp1, a cytoplasmic signalling molecule (Ketcham et al. 2004). It is noteworthy that two of the plant sequences are also predicted to be soluble glycosyltransferases (GTs) (At1g28695 and At1g70630) whereas the remainder are predicted to be classical type II transmembrane proteins. All of the sequences are homologous, albeit quite divergent, and the *Dictyostelium discoideum* (1,3)- α -D-GalT clearly forms an outgroup relative to the higher plant accessions. The phylogenetic tree shown in Fig. 1A cannot be assumed to reliably represent evolutionary relationships

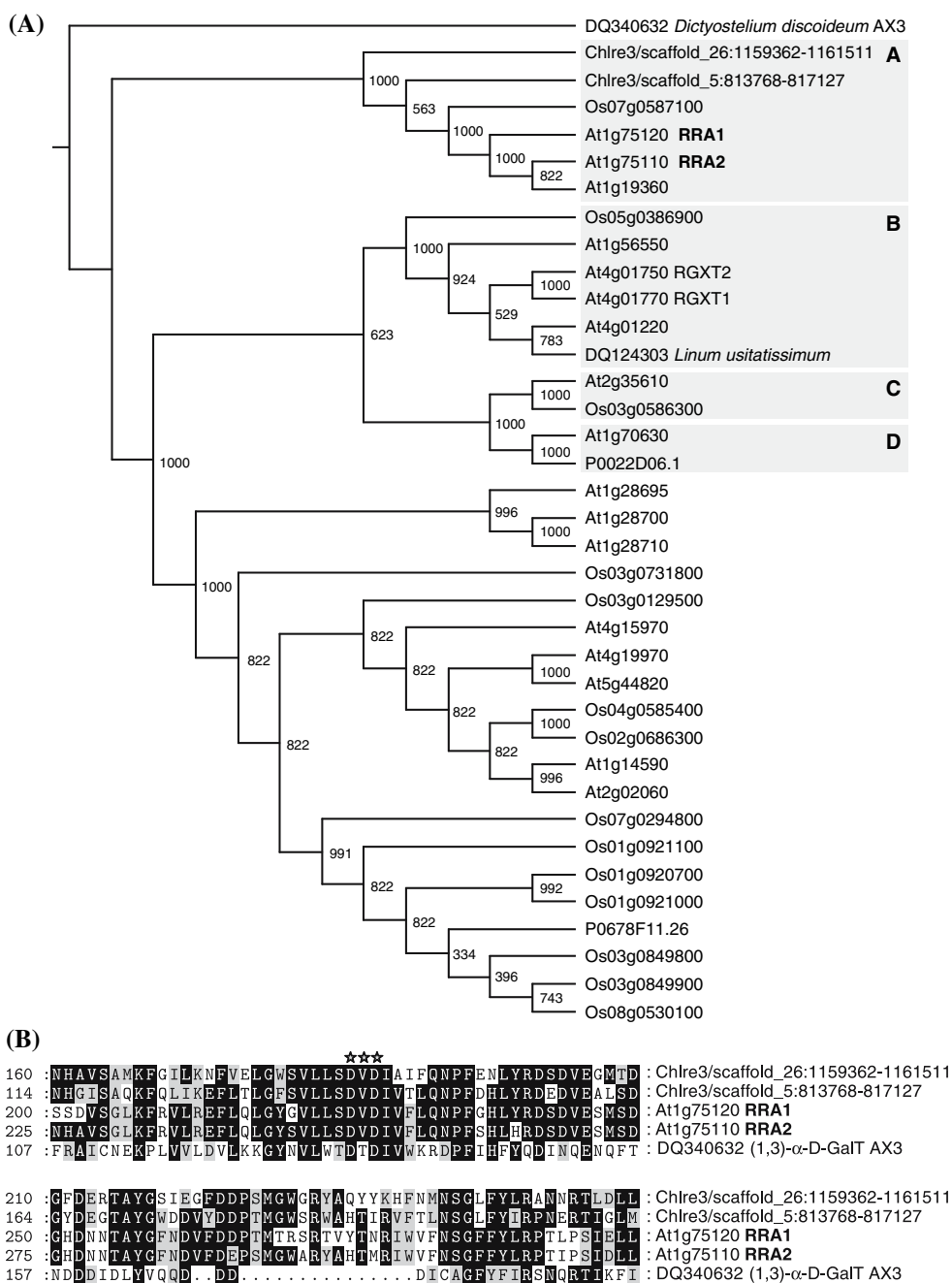
due to the low level of identity as well as large differences in length of the coding sequences (Supplemental Figure 1). However, the four clades labeled A to D appear to be robust to different choices of alignment algorithms (e.g., ClustalX v1.81, data not shown) and clade members are joined by 33% or higher identity.

RRA1 (At1g75120), *RRA2* (At1g75110) and a third *Arabidopsis thaliana* gene, At1g19360 belong to the A-clade, which, we propose, also comprises two *Chlamydomonas reinhardtii* accessions Chlre3/scaffold_5:813768–817127 (acegs_kg.scaffold_5000061) and Chlre3/scaffold_26:1159362–1161511 (estExt_gwp_1W.C_260040) accessible via the *Chlamydomonas reinhardtii* database v3.0 (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>). The algal sequences are 33–36% identical to the *Arabidopsis thaliana* genes and 35–37% identical to the rice sequence. Alignment of the sequences surrounding the catalytic domain (100 a.a. containing the D×D motif, Fig. 1B) between the two *Chlamydomonas reinhardtii* accessions and the *RRA* genes display up to 86% identity, indicating a strong evolutionary relationship. The significance of the *Chlamydomonas reinhardtii* accessions in the A-clade will be elaborated upon in the “Discussion” section. Both *RRA1* and *RRA2* are located on chromosome 1, separated by only 1.2 kb. In the *Arabidopsis thaliana* genome, many highly homologous genes are situated in clusters created by recent, local duplication (Coutinho et al. 2003) and it is thus likely that the two genes are paralogs.

The B-clade comprises the two highly identical (1,3)- α -D-XylTs (89% identity), two additional very similar *Arabidopsis thaliana* genes with unknown function, and the *Linum usitatissimum* accession. Each of the clades includes one *Oryza sativa* sequence and clades C and D may represent orthologous pairs of *Oryza sativa* and *Arabidopsis thaliana* genes.

RRA1 and *RRA2* encode polypeptides of 402 and 428 a.a., respectively. They are predicted to be type II membrane proteins and are 17–23% identical to the accessions in the B-clade. Similarities are in all cases most pronounced in the vicinity of the D×D motif, e.g., as seen in the focused alignment of the out-group accession *Dictyostelium discoideum* (1,3)- α -D-GalT (AX3), the *RRA* genes and the two *Chlamydomonas reinhardtii* sequences (Fig. 1B). Corresponding expressed sequence tags (ESTs) have been detected in various monocot and dicot plants, e.g., as seen in Table 1. ESTs representing the *RRA* genes have also been found, but the two genes are so similar that individual EST-sequences usually cannot be assigned to either gene. ESTs thus provide very little information about *RRA1* and *RRA2* expression profiles. Homologous sequences from organisms outside the plant kingdom other than the *Dictyostelium discoideum* (1,3)- α -D-GalT, have not been found.

Fig. 1 Phylogenetic analysis of the CAZy GT-family-77. (A) A rooted cladogram comprising 35 accessions of CAZy GT-family-77 (17 from *Arabidopsis thaliana*, 16 from *Oryza sativa*, one from *Linum usitatissimum*, the *Dictyostelium discoideum* (1,3)- α -D-GalT (AX3) and the two additional *Chlamydomonas reinhardtii* accessions) was generated as described in “Material and methods” section, resulting in four clades (A to D), Clade A contains the two genes *RRA1* (At1g75120) and *RRA2* (At1g75110), selected for analysis in the present study. Accessions starting with a ‘P’ are from *Oryza sativa*. Initially CAZy GT-family-77 was seeded using the two *Arabidopsis thaliana* (1,3)- α -D-XylTs, RGXT1 and RGXT2 (Egelund et al., 2006), which are found in clade B. Bootstrap values were generated from 1.000 re-samplings. (B) *RRA-1* and -2 and the two *Chlamydomonas reinhardtii* sequences were aligned over a range of 100 a.a. comprising the DxD-motif (indicated by ‘stars’). The *Dictyostelium discoideum* (1,3)- α -D-GalT (AX3) was added to this alignment using the profile option in muscle



Expression analysis

The level of *RRA-1* and -2 transcripts in various tissues was investigated using RT-PCR (Fig. 2). Whereas a strong 18S rRNA derived band was obtained (18S in Fig. 2), neither *RRA-1* nor -2-derived transcripts were detectable after 25 cycles of PCR (data not shown). After 45 cycles of PCR amplification, *RRA2* mRNA was detectable (Fig. 2), with strongest expression in roots, flowers and siliques, medium expression in rosette leaves and cauline leaves and low expression in stems. In comparison, *RRA1* mRNAs only produced a very faint band in siliques (Fig. 2).

In addition, the expression pattern of *RRA1* and *RRA2* was analyzed by *promoter::gusA* fusions in *Arabidopsis thaliana*. Expression of the *RRA1* promoter was restricted to meristematic tissue and only seen from the stage in which development of the first true leaves had begun and subsequently throughout the lifespan of the plant (Fig. 3A–D). Tissues where vascular connections are established with the developing leaves also stain intensely (Fig. 3B). In addition, expression was occasionally observed in points of cauline leaf attachments on the primary stem (Fig. 3E). The absence of *RRA1* promoter mediated GUS staining, where *RRA1* transcripts were detected by RT-PCR, might

Table 1 Expressed sequence tags (ESTs) found for *RRA1* (At1g75120) and *RRA2* (At1g75110)

Organism	Acc. No.	Tissue
<i>Medicago truncatula</i>	BM814575	Roots from 3-weeks old plants
<i>Gossypium hirsutum</i>	AI729290	Immature cotton fibers
<i>Hordeumvulgare</i> subsp. vulgare	BQ766243	Roots from 3-weeks old drought stressed, barley
<i>Ipomoea nil</i>	BJ567442	Mixture of flower and flower bud from Japanese morning glory
<i>Oryza sativa</i>	CA997950	Rice seedlings from cold-stressed germination
<i>Casipum annuum</i>	BM066150	Flower buds from red pepper 10 weeks after germination
<i>Populus tremula</i> × <i>Populus tremuloides</i>	BU814066	Bark from the Populus tree
<i>Trotoci aestivum</i>	AL820371	Embryo after 2 days post-germination
<i>Sorghum bicolor</i>	AW922738	Five-days old dark grown seedlings
<i>Arabidopsis thaliana</i>	AI999697	Rosettes from 4- to 7-weeks old plants
<i>Zea mays</i>	AW042361	Root from 3 to 4-days old plants
<i>Prunus persica</i>	BU041548	Developing peach
<i>Citrus sinensis</i>	BQ624172	Seedlings from sweet oranges
<i>Chlamydomonas reinhardtii</i>	BQ824326	15–60 min after being deflagellated by pH shock

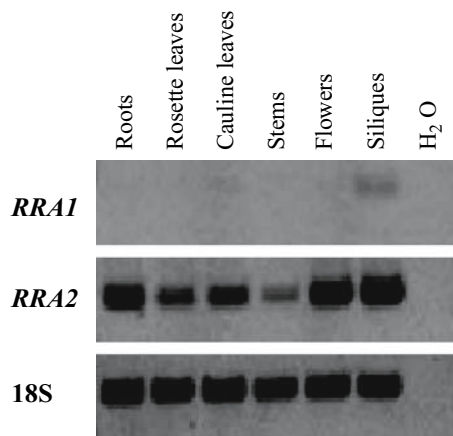


Fig. 2 Detection of *RRA-1* and *-2* transcripts in bolting *Arabidopsis thaliana* plants, using RT-PCR. RT-PCR was performed with specific primer sets for *RRA1*, *RRA2* and for ribosomal 18S (control) as described in the “Material and methods” section. The RNA used was isolated from tissues of 5-weeks old bolting WT plants. H₂O was included as control. Each RT-PCR reaction was performed twice to ensure reproducibility

result from the absence of potential enhancer elements present in, e.g., intron- or the 3'UTR regions of the *RRA1* gene. Similar observations have, e.g., been reported from RT-PCR versus GUS analysis of the UDP-D-glucuronate-4-epimerase in *Arabidopsis thaliana* (Usadel et al. 2004).

Strong GUS activity was found throughout germinating seedlings of *RRA2 promoter::gusA* transformed *Arabidopsis thaliana* (Fig. 4A), and throughout seedlings, in which the development of the first true leaves had begun (Fig. 4B). GUS staining activity was rapidly decreasing, though still found throughout all tissues in plants in which the development of fourth pair of leaves had begun (Fig. 4C), and in mature plants GUS activity was restricted

to rosette leaf hydathodes and trichome support cells in the adaxial epidermis (Fig. 4D). In bolting *Arabidopsis thaliana* plants GUS activity was found in cauline leaves (Fig. 4E), in petals (Fig. 4E, G–I), and in both the proximal and distal ends of siliques (Fig. 4F–G). A more detailed analysis revealed that high GUS activity was found in leaves of 2–3-week old plants (Fig. 5A), which rapidly weakened later in development (Fig. 5B–C). The GUS activity was weak in leaves from mature plants and found in rosette leaf hydathodes and trichome support cells in the adaxial epidermis (Fig. 5D–F). GUS activity was found in rosette leaf hydathodes (Fig. 5G), and in trichome support cells (Fig. 5G–H). Strong GUS activity was also found in the apical meristem, in the junction of newly developing leaves (Fig. 5I). GUS activity was found in the vascular tissue in both root tip (Fig. 5J), and in older non-elongating roots (Fig. 5K). These data are consistent with the RT-PCR analysis where *RRA1* transcripts could not be detected in roots, rosette leaves, cauline leaves, stems and flowers from 4 week bolting WT plants whereas *RRA2* transcripts were detected in all tissues tested (Fig. 2).

T-DNA insertional mutants for At1g75120 and At1g75110: *rra1* and *rra2*

Two T-DNA insertional mutants for *RRA1*, Garlic_76_G04 & SAIL_590_G09, with insertions in the first intron and in the last exon, respectively (Fig. 6A) and two T-DNA insertional mutants for *RRA2*, Garlic_244_A03 & SAIL_70_D08, with insertion in the last exon (Fig. 6B), as determined by sequencing from the T-DNA left border (Alonso et al. 2003) were selected for this investigation. Homozygous plants for all four T-DNA insertional lines were identified using PCR-based screening (Fig. 6C–D).

Fig. 3 Expression of *RRA1 promoter::gusA* in *Arabidopsis thaliana*. (A) GUS activity was found in meristematic tissue in seedlings, at the stage in which development of the first true leaves had begun. (B) Close up view of GUS staining of the meristematic region in seedlings, in which the development of the third pair of leaves had begun. (C) GUS staining of the meristematic region in seedlings, in which the development of the third pair of leaves had begun. (D) GUS staining in the meristematic region of adult non-bolting plants. (E) GUS staining observed on the primary stem, adjacent to the point of cauline leaf attachment

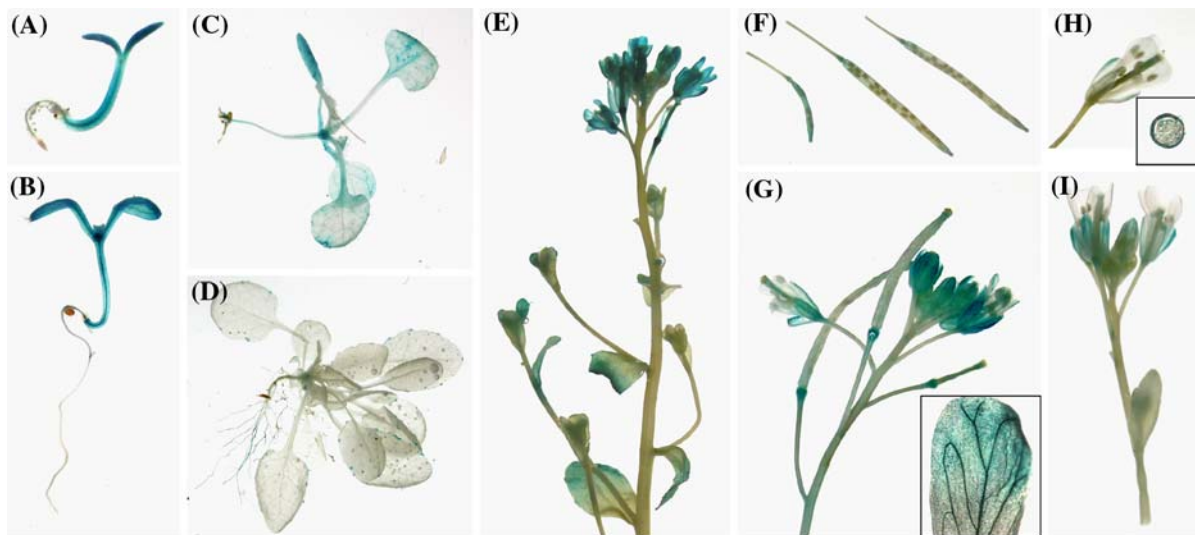
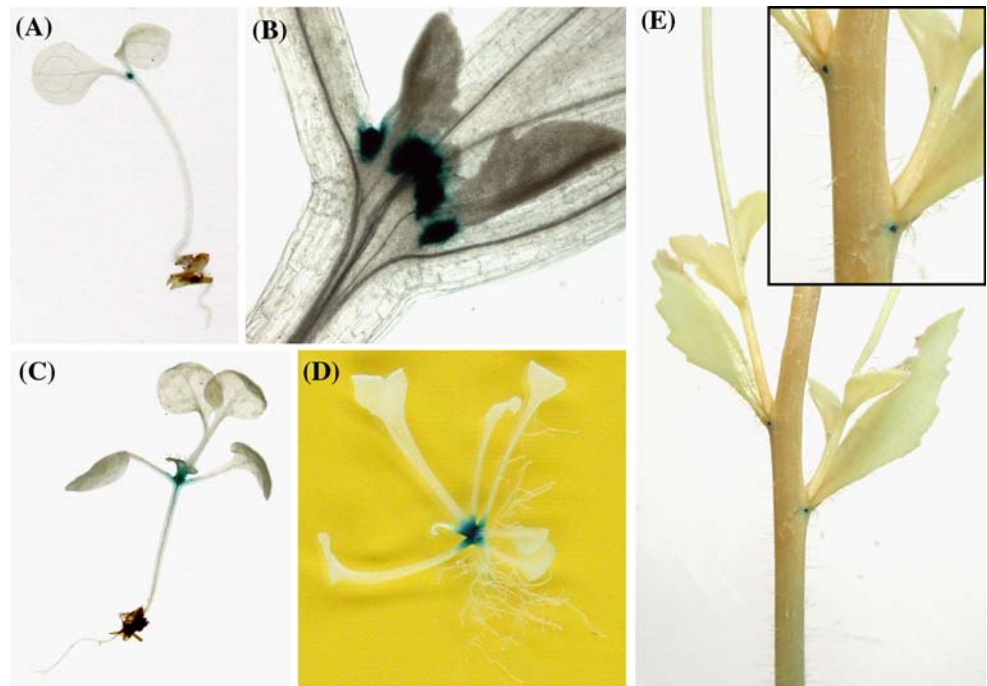


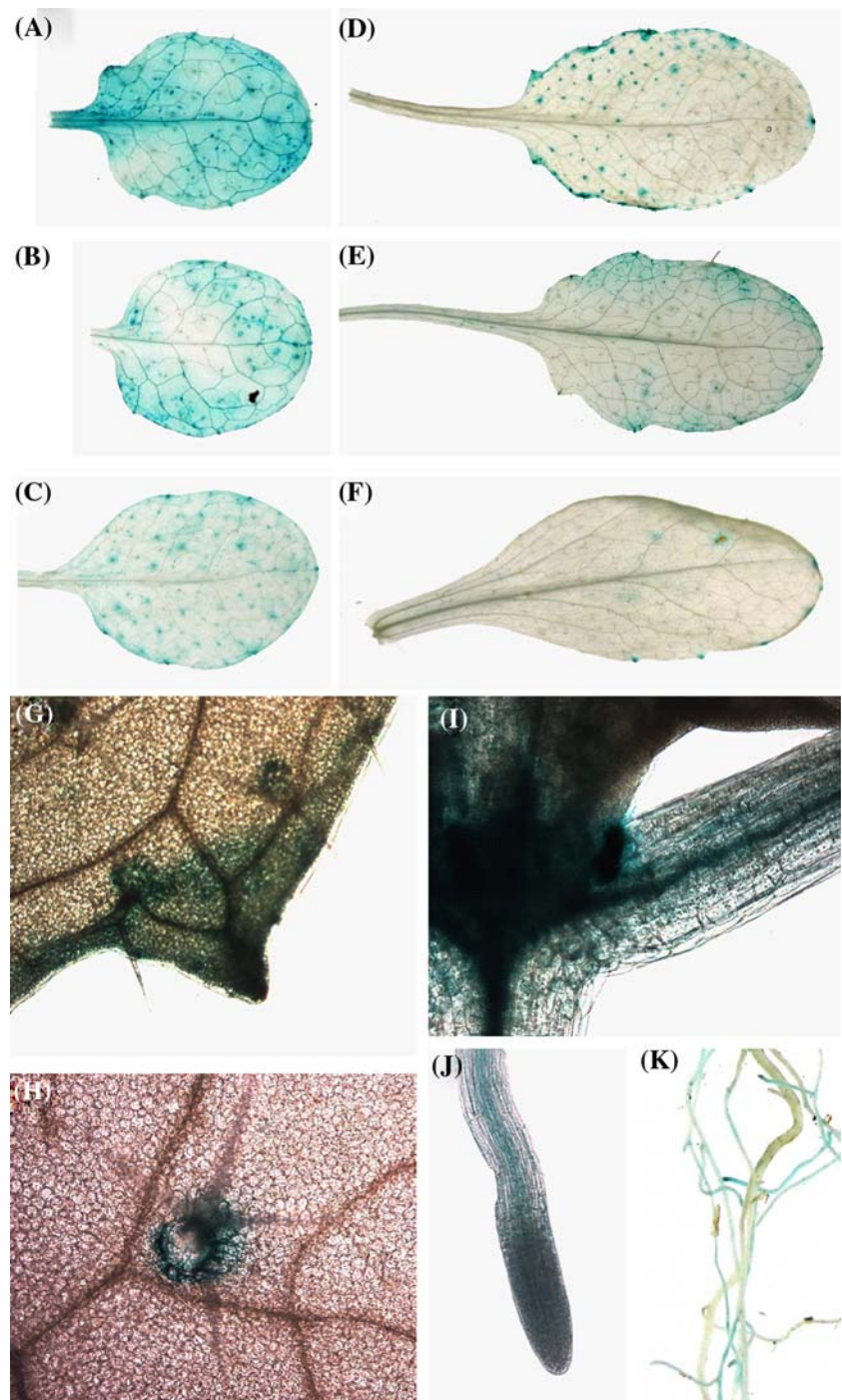
Fig. 4 Expression of *RRA2 promoter::gusA* in *Arabidopsis thaliana*. (A–B) Strong GUS activity was found throughout newly developing seedlings (A), and throughout seedlings, in which the development of the first true leaves had begun (B). (C–D) GUS activity was rapidly decreasing, though still found throughout all tissues, in plants in which the development of fourth pair of leaves had begun (C) and in

mature plants GUS activity was restricted to rosette leaf hydathodes and trichome support cells in the adaxial epidermis (D). (E–I) In bolting *Arabidopsis thaliana* plants GUS activity was found in cauline leaves (E), in petals (E, G, H and I), and in both proximal and distal ends of siliques (F and G)

No obvious morphological differences between the WT and the four mutant lines were observed. Histological analysis of root and leaf tissue of 1-week old seedlings, and of 5-week old bolting plants did not reveal any differences between WT and mutant plants. In addition, 1-week old seedlings were analyzed using a panel of antibodies raised against CW specific epitopes both by applying the “whole-mount” immunolabelling technique to the primary roots

(Willats et al. 2001) and by ELISA of fractionated CW-material from whole seedlings. The panel of antibodies comprised: LM6 (RG I, (1,5)- α -L-arabinan), LM5 (RG I, (1,4)- β -D-galactan), MAC 207 (plasma membrane AGPs), LM2 (AGPs, β -D-glucuronosyl), JIM5 (low esterified HG) and IGII (RG I, unesterified GalUA). No significant differences between the T-DNA mutants and WT were observed (our unpublished results).

Fig. 5 Detailed expression analysis of *RRA2 promoter::gusA* in *Arabidopsis thaliana* (A–C) High GUS expression was found in leaves from 2- to 3-weeks old plants (A), and then the GUS activity rapidly weakened in more mature leaves (B and C). (D–F) The GUS activity was weak in leaves from mature plants and only found throughout cell hydathodes and trichome support cells in the ad axial epidermis (D, E and F). (G–H) GUS activity was found in rosette leaf hydathodes (G), and in trichome support cells (G and H). (I) Strong GUS activity was found in the apical meristem, in the junction of newly developing leaves. (J–K) GUS activity was found in root tips (J), and in older non-growing roots (K)



Monosaccharide composition of alcohol insoluble residues, from cell walls of the T-DNA insertional mutants *rra-1* and *-2*

Alcohol insoluble residues (AIR) were prepared from CW material isolated from the following tissues: roots and leaves isolated from seedlings, root, leaves, stems and flowers of 5-week old bolting plants, and the apical meristem of 3-week old plants. AIR of all the tissues were

sequentially extracted to yield RG I, RG II, HG and xyloglucan. In addition, water-soluble extracts and AGPs were isolated from rosette leaves and meristematic tissues of 3-week old plants. The fractions were then analyzed by either oligosaccharide mass profiling (xyloglucan) or by monosaccharide composition analysis. No significant differences were observed in these analyses (data not shown). However, a significant reduction of Ara was observed in the CW pellet after enzymatic removal of the pectic

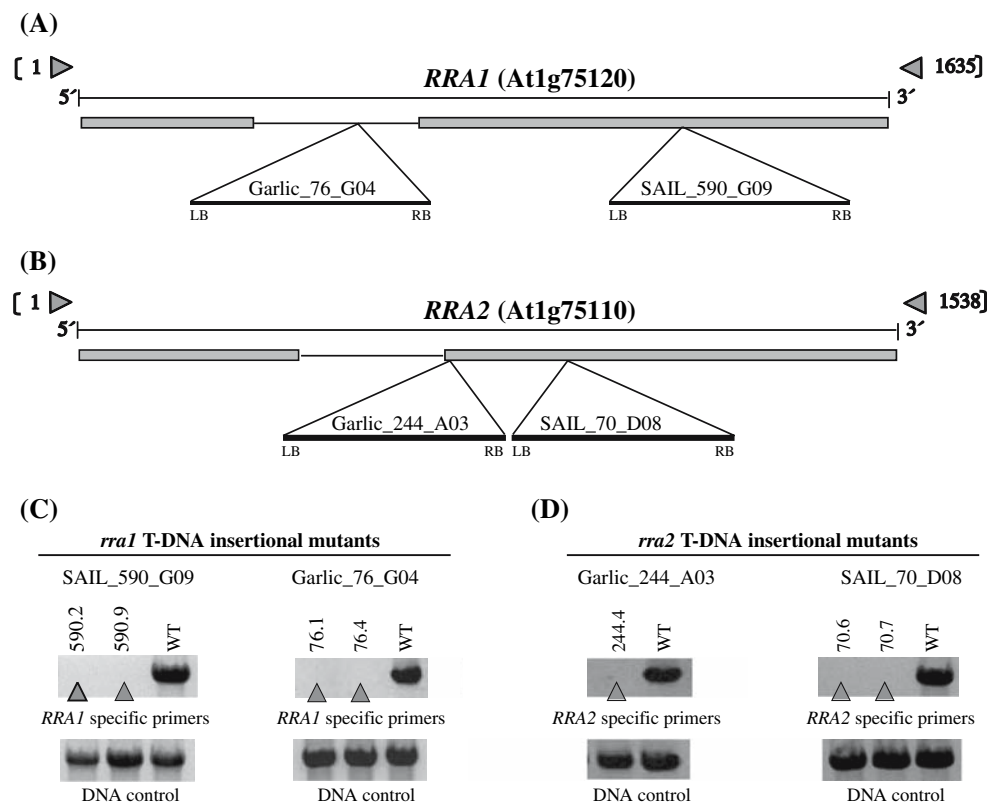


Fig. 6 T-DNA insertion mutants in *RRA-1* and *-2*. Positions of the two allelic T-DNA insertion mutants in both of the *RRA1* and *-2* gene. Boxes indicate exons and lines indicate introns. (A) The T-DNA is inserted in the first intron (Garlic_76_G04) and in the last exon (SAIL_590_G09). (B) Both T-DNAs are inserted into the second exon (Garlic_244_A03 and SAIL_70_D08). (C) Homozygous lines identified in SAIL_590_G09 and Garlic_76_G04, respectively.

(D) Homozygous lines identified in Garlic_244_A03 and SAIL_70_D08, respectively. The PCR analysis was carried out using a set of gene specific primers outside of the T-DNA insertions. 'DNA control' refers to the amplification of At4g01750. Triangles indicate homozygous lines. The results were confirmed using three independent analyses, and were also confirmed in the T_2 generation (data not shown)

polysaccharides and xyloglucan and this reduction was only observed in CWs isolated from meristematic tissue (Table 2). The reduction was observed in both *rra1* mutants (Garlic_76_G04: $p \leq 0.00002$ & SAIL_590_G09: $p \leq 0.0002$), and in both *rra2* mutants (Garlic_244_A03: $p \leq 0.0010$ & SAIL_70_D08: $p \leq 0.010$).

The Garlic_244_A03 line (*rra2* mutant) contains elevated levels of Glc (Table 2). No clear trend was seen in the relative levels of other monosaccharides compensating for the Ara reduction. The two alleles appear not to respond similarly, but it is important to appreciate that these differences are not statistically significant. However, the reduction of Ara was seen in both alleles of *RRA2*.

The method used for determining the monosaccharide compositions of the neutral sugars, does not allow for the simultaneous quantification of acidic sugars such as e.g., GalA, and these were therefore analyzed separately (no differences were observed, data not shown).

The monosaccharide profile of the CW remaining pellet suggests the presence of tightly bound non-cellulosic polysaccharides, notably xyloglucans and (arabino)xylans.

While xyloglucan in solanaceous plants contains Ara, xyloglucan in *Arabidopsis thaliana* does not (Zablackis et al. 1995), so changes in xyloglucan composition cannot account for the reduced Ara content of the CW-pellet. Glucuronoarabinoxylans do contain Ara (Zablackis et al. 1995; Bacic et al. 1988), and the xylans were therefore digested with a xylanase and the solubilized degradation products analyzed for arabinose content. No differences between WT and mutants were observed (data not shown) suggesting that the Ara is not derived from heteroxylans.

Discussion

CAZy GT-family-77: retaining glycosyltransferases that form (1,3)-linkages

A phylogenetic analysis of CAZy GT-family-77 lead us to propose a sub-division of some members into four clades labeled A to D, and a large remainder of accessions that could not be subdivided. If clades A and B reflect not only

Table 2 Monosaccharide composition of the remaining cell wall pellet after enzymatic removal of pectin and xyloglucan

Genotype	<i>rra1</i>			<i>rra2</i>	
	WT	76_G04	590_G09	244_A03	70_D08
Glc	14.9±2.4	13.8±1.4	13.3±0.3	19.4±0.6	12.3±0.2
Gal	10.5±1.4	13.7±0.9	13.9±0.4	12.3±0.5	12.6±1.0
Man	4.9±0.7	6.8±0.3	6.6±0.1	5.7±0.1	6.3±0.2
Xyl	28.9±2.1	33.4±0.7	31.3±1.2	28.5±0.5	31.7±0.9
Ara	31.0±2.2	24.1±0.5	25.3±0.4	26.2±0.8	27.4±0.7
Fuc	3.6±0.3	3.0±0.2	3.3±0.2	3.0±0.1	3.2±0.0
Rha	6.1±1.0	5.2±0.3	6.2±0.4	4.9±0.3	6.7±0.4

The cell walls (AIR) was isolated from the shoot apical meristem of wild type and the four T-DNA insertional mutants, Garlic_76_G04 & SAIL_590_G09 (*rra1*) and Garlic_244_A03 & SAIL_70_D08 (*rra2*) (Fig. 6)

Molar percentages (mol%) of each glycosyl residue analyzed by GC and GC/EI-MS. The values are shown as the average of five independent experiments ± SE

evolutionary relatedness, but also correlate with catalytic function, it would appear that *Arabidopsis thaliana* devotes several GT-genes where *Oryza sativa* employs one. It is, however, premature to conclude that the *Arabidopsis thaliana* genes of clade A and B, respectively, are redundant; currently only RGXT1 and RGXT2 of clade B have been functionally characterized, the annotation as a GalT of the *Linum* sequence, which is also assigned to clade B, is premature (C. Morvan personal communication). The accessions not yet assigned to clades seem to split according to species rather than being organized as sets of orthologous *Oryza sativa* and *Arabidopsis thaliana* genes (see bottom of the phylogenetic tree in Fig. 1A). This may hint at functions in the synthesis of carbohydrates that are typical of *Gramineae* monocots and other angiosperms, there are several polysaccharides and proteoglycans that differ between Type-I and Type-II CWs, *sensu* Carpita and Gibeaut (1993). We recommend that definition of a clade of e.g., *Poaceae*-specific genes should await the functional characterization of some of the *Oryza sativa* accessions.

The (1,3)- α -D-GalT (AX3) from the slime mold *Dictyostelium discoideum* has also been classified to CAZy GT-family-77. It is fairly distantly related to the plant sequences (below 10%) and is not included in any of the plant clades. The *Dictyostelium discoideum* (1,3)- α -D-GalT is a soluble protein, which takes part in the glycosylation of Skp1, a cytoplasmic and nuclear protein involved in cell cycle regulation (Ketcham et al. 2004). The Skp1 pentasaccharide (to which the (1,3)- α -D-GalT transfers the penultimate galactosyl residue) is attached to a hydroxyproline residue and is assembled in the cytoplasm. This cytoplasmic O-glycosylation pathway is known from lower eukaryotes, possibly including *Chlamydomonas reinhardtii* (West et al. 2004), but has yet to be demonstrated in vascular plants, like *Arabidopsis thaliana*. The *Chlamydomonas reinhardtii* accessions, which clearly belong in clade A, might suggest the

existence of a similar pathway in higher plants (the CAZY database does not list sequences from genome projects. Bernard Henrissat personal communication). Another possibility is that the *Chlamydomonas reinhardtii* genes encode GTs involved in CW biosynthesis. Interestingly RG II is not present in the CWs of algae (O'Neill et al. 2004) and the CW of *Chlamydomonas reinhardtii* is unusual in that it is almost entirely composed of HRGPs, which are related to the extensins of higher plants (Waffenschmidt et al. 1993). It may thus be speculated that the *Chlamydomonas reinhardtii* genes could encode either GalTs or AraTs involved in glycosylation of extensin-like cell wall proteins.

Organ specific and developmentally determined expression of *RRA-1* and *-2*

Both *RRA-1* and *-2* were shown by *promoter::gusA* studies to be active in the meristematic region. Pectic arabinan, but not galactan, has been shown to be enriched in apical meristems of potato stolons (Bush et al. 2001) and has also been shown to be essential to meristematic function in potato (Borkhardt et al. 2005). The observation that arabinans are associated with meristematic regions in carrot (Willats et al. 1999) may be relevant to the observed GUS expression in root tips of the *RRA2 promoter::gusA* transformants.

The expression pattern in mature plants in specialised tissues and cell types: hydathodes, trichome support cells in the adaxial epidermis, cauline leaves, petals, mature roots and in both the proximal and distal ends of siliques, does not allow us to make inferences with regard to the function(s) of *RRA-1* and *-2*.

Characterization of the *rra1* and *rra2* mutants

T-DNA insertional lines, *rra-1* and *-2*, for At1g75120 and At1g75110, respectively, are similar to WT-plants with regard

to morphology and growth rate. A significantly altered CW monosaccharide profile was only observed in the residual CW pellet after enzymatic removal of the pectic and xyloglucan polysaccharides, and only observed in tissues isolated from meristematic tissue. A significant reduction in Ara was observed in both mutants for *RRA-1* and *-2*. These results suggest that both genes may encode arabinosyltransferases (AraTs), although other more complex explanations such as indirect effects on arabinosylation cannot be ruled out.

Inferring the catalytic function of *RRA-1* and *-2*

A mutant phenotype reduced in Ara in a tightly bound wall fraction would be most easily explained if *RRA-1* and *-2* encode AraTs. A reduction in Ara can, however, also be the consequence of a knocked out GalT. Extensins contain α -linked galactosyl residues as single substituents directly on serine residues of the protein backbone, and the arabinosylation of adjacent Hyp residues could, for example, depend on correct galactosylation of the Ser-Hyp₄ repeat.

Ara is a major component in various plant polysaccharides e.g., the pectic polysaccharides RG I and RG II, in the non-cellulosic arabinoxylans, in extensins as well as in type II AGs/AGPs (reviewed by Ishii et al. 2005a). Ara is predominately found in the arabinofuranose (Araf) form in plants (Carpita and Gibeaut 1993). Arabinopyranose (Arap) has been found as a terminal sugar in galactans from soybean (Huisman et al. 2001), in AGPs from maize (Bacic et al. 1986), in the pectic polysaccharide RG II (O’Niell et al. 2004), and in arabinans isolated from various plant species (reviewed by Ishii et al. 2005a). The biochemistry of Araf incorporation into pectic polysaccharides from UDP-Arap has received much attention recently (Numan and Scheller 2003; Ishii et al. 2005b; Konishi et al. 2006; Harholt et al. 2006), but is unresolved at present. As long as this is the case, it cannot be predicted with certainty whether a GT-family-77 AraT would form α - or β -glycosidic linkages, although we consider the latter more likely, however, α -linked Araf is much more prevalent in the CW than β -linked Ara. The terminal Arap of soybean RG I has been suggested to be β -(1,4)-linked (Huisman et al. 2001). β -(1,3)-Linked Arap has so far only been found in the gymnosperm *Larix dahurica* AG II (Odonmaig et al. 1994). The internal Ara residues of the extensin oligo-arabinoside side chains are Araf- β -(1,2)-linkages (Akiyama and Kato 1977). Extensins become tightly bound to the CW soon after their deposition, so a change in CW composition of the mutants should be observed in the pellet rather than in any of the CW extracts. It should be noted, however, that Zykwinska et al. (2005) recently showed that pectic arabinan and galactan side chains are able to bind tightly to specific sites on cellulose microfibrils in vitro.

The most persuasive approach to resolving whether the *RRA-1* and *-2* play roles (and not necessarily the same role) in either arabinosylation of xylan, RG I side chain or extensin, would be to heterologously express the *RRA-1* and *-2* gene products and demonstrate transfer of Ara to acceptors originating from the three candidate biopolymers.

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