RESEARCH PAPER

The synthesis of the rhamnogalacturonan II component 3-deoxy-\textit{d}-\textit{manno}-2-octulosonic acid (Kdo) is required for pollen tube growth and elongation

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Abstract

Despite a very complex structure, the sugar composition of the rhamnogalacturonan II (RG-II) pectic fraction is extremely conserved. Among its constituting monosaccharides is the seldom-observed eight-carbon sugar 3-deoxy-\textit{d}-\textit{manno}-octulosonic acid (Kdo), whose phosphorylated precursor is synthesized by Kdo-8-P synthase. As an attempt to alter specifically the RG-II structure in its sugar composition and assess the consequences on the function of RG-II in cell wall and its relationship with growth, \textit{Arabidopsis} null mutants were sought in the genes encoding Kdo-8-P synthase. Here, the isolation and characterization of one null mutant for the isoform 1 (\textit{AtkdsA1-S}) and two distinct null mutants for the isoform 2 of \textit{Arabidopsis} Kdo-8-P synthase (\textit{AtkdsA2-V} and \textit{AtkdsA2-S}) are described. Evidence is provided that \textit{AtkdsA2} gene expression is preferentially associated with plantlet organs displaying a meristematic activity, and that it accounts for 75% of the mRNAs to be translated into Kdo-8-P synthase. Furthermore, this predominant expression of \textit{AtKDSA2} over \textit{AtKDSA1} was confirmed by quantification of the cytosolic Kdo content in the mutants, in a variety of ecotypes. The inability to identify a double knockout mutant originated from pollen abortions, due to the inability of haploid pollen of the \textit{AtkdsA1-AtkdsA2} genotype to form an elongated pollen tube properly and perform fertilization.

Key words: \textit{Arabidopsis thaliana}, 3-deoxy-\textit{d}-\textit{manno}-2-octulosonic acid-8-phosphate, 3-deoxy-\textit{d}-\textit{manno}-oct-2-ulosonate-8-phosphate synthase, pollen tube growth, rhamnogalacturonan II.

Introduction

Cell wall pectin consists of three structurally well-characterized polysaccharides: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (RG-II) (Willats \textit{et al.}, 2001).

Despite a complex structure, RG-II is evolutionarily conserved in the plant kingdom as it is present in the primary cell wall of all higher plants predominantly in the form of a dimer that is cross-linked by a borate di-ester (dRG-II-B) between two apiosyl residues (O’Neill \textit{et al.}, 2004). RG-II is the only known borate-binding polysaccharide in the primary cell wall, sequestering up to 80% of the cellular boron (Matoh \textit{et al.}, 1996). Boron deficiency results in altered plant growth and changes in cell wall architecture, which indicates the primordial function of boron-mediated cross-linking of RG-II to generate...
a covalently cross-linked pectic network that is involved in the regulation of cell wall properties and plant growth (Fleischer et al., 1998, 1999; Ishii et al., 1999, 2001). Nevertheless the precise function of RG-II remains unclear.

As a first step to decipher the role of RG-II within plant cells, a great deal of effort was put in establishing the composition and structure of RG-II. RG-II has an α-1,4-linked homogalacturonan backbone that is substituted with four structurally different oligosaccharide side chains (O’Neill et al., 2004). At least 12 different glycosyl residues are present in RG-II. Among these residues are the seldom observed sugars: aceric acid, apiose, 3-deoxy-D-lyxo-heptulosonic acid (Dha), and 3-deoxy-D-manno-octulosonic acid (Kdo) (O’Neill et al., 1996; Pérez et al., 2000).

Historically it was believed that only Gram-negative bacteria synthesized Kdo as a component of the outer membrane lipopolysaccharides (Rick, 1987) until Kdo was identified as a component of the primary cell walls of higher plants and of cell wall polysaccharides of some green algae (York et al., 1985; Becker et al., 1995). Kdo is synthesized through the activity of Kdo-8-P synthase (KDS; EC 4.1.2.16) which catalyses the condensation of phosphoenolpyruvate with D-arabinose-5-phosphate to yield Kdo-8-P and inorganic phosphate. Up to now, cDNAs for Kdo-8-P synthase in plants have been isolated from Pisum sativum (Brabetz et al., 2000), Solanum lycopersicum (Delmas et al., 2003), and Arabidopsis thaliana (Matsuura et al., 2003). Functional complementation of a kdsA**null** mutant of Salmonella enterica was obtained with the pea and tomato enzyme, and all plant Kdo-8-P synthases studied so far displayed the efficient catalytic activity to produce Kdo-8-P. While Kdo-8-P synthase is encoded by a single-copy gene in tomato, two different isozymes are present in pea and Arabidopsis.

The two genes in Arabidopsis, designated as AtKDSA1 (At1g16340) and AtKDSA2 (At1g16330), display a differential expression in plantlets, the former one being predominantly expressed in shoots and the latter one in roots (Matsuura et al., 2003).

Here, the isolation and characterization of one null mutant for the isoform 1 (AtkdsA1-S) and two distinct null mutants for the isoform 2 of Arabidopsis Kdo-8-P synthase (AtkdsA2-V and AtkdsA2-S) are described. Evidence is provided that AtkdsA2 gene expression is preferentially associated with plantlet organs displaying a meristematic activity, and that the loss of AtkdsA2 gene expression results in a decrease of up to 90% of the cytosolic Kdo content in the AtkdsA2-V mutant when compared with the wild type. The inability to obtain a double knockout mutant is due to the inability of haploid pollen grains of the AtkdsA1–AtkdsA2– genotype to form an elongated pollen tube properly. Consequently this results in an impairment to the fertilization of the ovule.

### Materials and methods

#### Plant materials

AtkdsA2-V mutant plants of Arabidopsis thaliana are in the Wassilewskija (WS) ecotype background. AtkdsA1-S, AtkdsA2-S, and gtrl-2 mutant plants of Arabidopsis thaliana are in the ecotype Columbia (Col0). For germination, seeds were surface sterilized for 15 min in 12.5% (v/v) sodium hypochlorite and 0.02% (v/v) Triton X-100, rinsed at least five times, and plated in Petri plates containing MS growth medium (Murashige and Skoog, 1962). After cold treatment at 4 °C for 2 d in the dark, the plates were incubated in a growth chamber at 22 °C in a cycle of 16 h light/8 h darkness. After 10 d of growth the plantlets were transferred to soil in a growth chamber in the same conditions. The pollen germination experiment was performed as described by Hicks et al. (2004).

#### Characterization of T-DNA insertion sites

PCR reactions were performed to characterize the T-DNA insertion site within the homozygous AtkdsA2-V null mutant genomic DNA. Primers located within the AtkdsA2 gene sequence were designed as follows: FST5’, 5’-GGGTCAATCTGATGATCAG-3’ and FST3’, 5’-TGACTCAGCTCAGAAAAGCT-3’. These AtkdsA2 gene-specific primers were used in combination with primers located in the T-DNA sequence: TAG31, 5’-CGGAAGAACATT-GGGCAGAAAGGTG-3’, and TAG51, 5’-GGTGAGACAAACC-CTCAACTGGGAAAAGC-3’. The insertion site within AtkdsA2-V genomic DNA was mapped by sequencing PCR products spanning the borders of T-DNA.

To characterize the AtkdsA1-S mutant, primers KdsA15’, 5’-CCTCTTTTATGCTGGATG-3’ and KdsA13’, 5’-ACTGTTACAGACATGGTTCTC-3’ were used in combination with primer LBA1, 5’-TGTTTCAGTTGAGGGCCATCG-3’, located within the LB sequence of the T-DNA. PCR reactions were performed with primers KdsA25’, 5’-GGACAATTGGTGTTAC-CTCAGT-3’, and KdsA23’, 5’-CTAGCGGGTTCTGCTTATG-3’, in combination with LBA1 in order to amplify the T-DNA insertion site within the AtkdsA2-S genomic DNA.

#### RNA extraction and cDNA synthesis

Wild-type (WS and Col0) or AtkdsA1-S, AtkdsA2-S, and AtkdsA2-V seedlings grown under light conditions in the growth medium for 10 d were used for RNA analysis. Total RNA were isolated using the TRIzol® Reagent (Invitrogen) and were treated with DNase RQI (Promega) according to the manufacturer’s protocol. Two micrograms of total RNA from plantlets were reverse transcribed into cDNA using oligo(dT)12, as a control of RT-PCR expression, a 304 bp cDNA fragment for the Actin2 gene (AtACTIN2) was amplified using the following set of primers: AtActin25’, 5’-GGATCTGTAACGCTTACT-3’, respectively, in combination with the respective 3’ primers: AtkdsA1Ex1-3’, 5’-CTTCAGACCCCTCGGGCCATGC-3’, and AtkdsA2Ex1-3’, 5’-GAGCCCTCTAGCAGGAGCA-3’. As a control of RT-PCR expression, a 304 bp cDNA fragment for the Actin2 gene (AtACTIN2) was amplified using the following set of primers: AtActin25’, 5’-GGATCTGTAACGCTTACT-3’, respectively, in combination with the respective 3’ primers: AtkdsA1Ex1-3’, 5’-CTTCAGACCCCTCGGGCCATGC-3’, and AtkdsA2Ex1-3’, 5’-GAGCCCTCTAGCAGGAGCA-3’ as a 3’ primer. After an initial
Plasmid constructions

The AtkdsA2 promoter sequence was amplified by PCR (35 cycles: 30 s at 94 °C, 30 s at 53 °C, 1 min 30 s at 72 °C) from genomic DNA of Arabidopsis (WS) plantlets using the following primers: 5'-CAATCTGGTGTCTGGG-3' introducing a BamHI site and 5'-CTATAGTAGGCTTAAGATC-3' introducing a NcoI site for subsequent cloning. The 831 bp product was digested by BamHI and NcoI and ligated into the plasmid pCAMBIA 1381 (http://www.cambia.org.au) between the corresponding sites to yield pCKDS2A. A transcriptional fusion of the CaMV 35S constitutive promoter with the GUS gene (pC3SS plasmid) was obtained by cloning a BamHI–NcoI fragment harbouring the duplicated 35S promoter from pCAMBIA 1301 (http://www.cambia.org.au) into the corresponding sites of pCAMBIA 1381.

Plant transformation

GUS fusion vectors pC3SS and pCKDS2A were electroporated into disarmed Agrobacterium strain GV3101 cells. Plants were transformed by a dipping procedure (Clough and Bent, 1998) using transformed Agrobacterium GV3101. After transformation, seeds were harvested from T0 plants, pooled, and sown on MS medium containing hygromycin (25 mg l⁻¹). Twenty T1 plantlets were vacuum infiltrated for 10 min in a reaction buffer (50 mM sodium phosphate, pH 7, and 0.5% (v/v) Triton X-100, and then incubated at 37 °C for 12 h. Samples were then washed and cleared in 70% ethanol for several hours.

Analysis of β-glucuronidase (GUS) activity

For histochemical assays of GUS activity, fragments of Arabidopsis T2 plantlets were vacuum infiltrated for 10 min in a reaction buffer containing 20 mg ml⁻¹ 5-bromo 4-chloro 3-indolyl glucuronide (X-gluc), 50 mM sodium phosphate, pH 7, and 0.5% (v/v) Triton X-100, and then incubated at 37 °C for 12 h. Samples were then washed and cleared in 70% ethanol for several hours.

Quantification of cytosolic Kdo

One-month-old leaves were taken from the Arabidopsis plants. After weighing, leaves were suspended in 70% (v/v) ethanol and heated at 70 °C for 15 min to inactivate enzymes. Leaves were then ground in a Potter homogenizer, and the homogenate was washed twice with hot 70% (v/v) ethanol at 70 °C for 15 min. After centrifugation (5000 g for 5 min), the three ethanol fractions were combined, lyophilized, and then dissolved in 2 ml of water. This solution was purified by elution in water through a C18 Bond Elut cartridge (Varian, Sugarland, TX, USA), lyophilized, and then treated with 200 μl of 2 M acetic acid at 80 °C for 5 h to hydrolyse the Kdo-8-P and CMP-Kdo into free Kdo.

The solution was neutralized with 200 μl of 1 M NH₄OH before being dried again in order to be derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB). A 14 mM solution of DMB (Sigma) was prepared by dissolving DMB in an aqueous solution of 80 mM β-mercaptoethanol, 40 mM sodium hydro-sulphite, and 2.8 M acetic acid. The sample containing cytosolic monosaccharides was solubilized in 90 μl of deionized water and 90 μl of the DMB solution. The mixture was heated at 50 °C for 2.5 h in the dark. Twenty microlitres of the resulting solution were injected in a liquid chromatograph (Kontron, Milan) equipped with a reverse-phase C18 column (300×4.5 mm) and an SFM-25 fluorescence spectrophotometer (Kontron). Elution of the DMB derivatives was performed at a flow rate of 0.9 ml min⁻¹ at room temperature using solvent A (acetonitrile:methanol:water, 4:6:90, v/v/v) and solvent B (acetonitrile:methanol:water, 11:7:82, v/v/v), with an A to B linear gradient from 50:50 to 0:100 (v/v) over 40 min. The DMB derivatives were detected by fluorescence using excitation and emission wavelengths of 373 nm and 448 nm, respectively. DMB-derivatized Kdo was used as a standard. N-Acetyl neuraminic acid (NeuAc) (400 ng) was added to the cytosolic extracts prior to DMB derivation, and used as an internal standard for quantification of Kdo.

Results

Characterization of null mutants for KDSA genes in Arabidopsis

Two T-DNA insertion lines targeting the AtKDSA1 gene (SALK_024867) and the AtKDSA2 gene (SALK_066700) were obtained from the Arabidopsis Biological Resource Center and a third one targeting the AtKDSA2 gene (FLAG_143E2) was obtained from the large collection of T-DNA insertion transformants of Arabidopsis thaliana plants from the Institut National de la Recherche Agronomique (INRA), Versailles, France. These T-DNA insertion mutants were referred to as AtkdsA1-S and AtkdsA2-S (S for SALK) and AtkdsA2-V (V for Versailles). The T-DNA insertion sites in the three mutants are illustrated in Fig. 1. In AtkdsA1-S, the AtKDSA1 gene was disrupted within exon 4. In AtkdsA2-S and AtkdsA2-V, the AtKDSA2 gene was disrupted between exon 8 and exon 9 and between exon 2 and exon 3, respectively. This was confirmed by PCR analyses using combinations of primers as indicated in Fig. 1 and genomic DNA compared with control wild-type Columbia (for AtkdsA1-S and AtkdsA2-S) and Wassilevskija (Ws) (for AtkdsA2-V) plants (data not shown).

The general development and growth phenotype of AtkdsA1-S, AtkdsA2-S, and AtkdsA2-V plants appeared to be quite similar to those of the respective wild-type Col0 and WS plants in standard growth conditions. At the mutational level, this can be explained by the functional redundancy of the two KDSA isogenes. This was indeed demonstrated by the transcriptional characterization of AtKDSA1 and AtKDSA2 gene expression in the different mutants (Fig. 2). In whole wild-type WS and Col0 plantlets, both genes are expressed but to different levels: AtKDSA2 and AtKDSA1 mRNAs represented, respectively, 75% and 25% of the transcripts coding for Kdo-8-P synthase in Arabidopsis wild-type plants no matter what ecotype, thus confirming previous observations (Matsuura et al., 2003). AtKDSA2 is predominantly expressed since the quantification of the relative mRNA abundance for AtKDSA2 indicated a 3-fold higher level than that of
AtKDSA1 (Fig. 2). In the AtkdsA1-S mutant, the sole expression of the AtKDSA2 gene could be detected, and similarly in the AtkdsA2-S and AtkdsA2-V mutants, only AtKDSA1 transcripts were detected, thus confirming that AtkdsA1-S and AtkdsA2-S and AtkdsA2-V are, respectively, mRNA null mutants for the AtKDSA1 and AtKDSA2 genes (Fig. 2).

In planta analysis of the AtkdsA2 spatial expression
Since the AtKDSA2 gene appears to be predominantly expressed in wild-type Arabidopsis plantlets (Fig. 2), an in planta analysis of the AtKDSA2 spatial expression was performed using the AtkdsA2-V mutant. Indeed, the inserted T-DNA originating from the pGKB5 vector (Bouchez et al., 1993) harbours the uidA gene which allows putative promoter trapping using the GUS expression as a reporter system. In the AtkdsA2-V mutant, the T-DNA is inserted in the 5’ part of the AtKDSA2 gene as such that the uidA gene expression may be driven by the endogenous KDSA2 promoter (Fig. 1). Therefore, the ability of the endogenous KDSA2 promoter (KDSA2-V) to drive the expression of the uidA gene was investigated in the AtkdsA2-V plants by measuring the GUS activity as revealed by GUS staining (Fig. 3). In seedlings at the four-leaf stage (Fig. 3a), the apical meristem and the emerging leaves were heavily stained. In young leaves, the GUS staining was restricted to hydathodes (Fig. 3b, c). In mature flowers, only the style displayed the blue staining (Fig. 3d). In mature siliques, only the funicule was stained, and the mature seeds were devoid of any staining (Fig. 3e). To demonstrate the specificity of this expression driven by the endogenous AtKDSA2 promoter in the null mutant plants, the putative promoter of the AtKDSA2 gene (KDSA2) was cloned into the plant transformation vector pCAMBIA-1381 so as to govern the expression of the uidA reporter gene. The resulting construct (KDSA2::GUS) was introduced into Arabidopsis plants of the WS ecotype, and GUS activity was revealed by GUS staining in almost identical regions to those in the AtkdsA2-V mutant plants. It is noteworthy that a clear GUS activity was detected in both the style and the anthers. The uidA gene expression appeared to be higher than that observed in the AtkdsA2-V mutant. However, the overall data were strikingly reproducible, indicating that AtKDSA2 gene expression as reported by the GUS activity in the mutant was specifically detected.
Biochemical characterization of AtkdsA1-S, AtkdsA2-S, and AtkdsA2-V null mutants

Since no phenotypical differences could be observed between wild-type plants and the three null mutant plants, it was interested to investigate the consequence of the respective kdsA null mutations on the accumulation of cytosolic Kdo-8-P at a quantitative level. Cytosolic extracts of wild-type Arabidopsis and tobacco leaf cell extracts were treated with mild acid to remove the phosphate groups. The extracts as well as a standard of purified Kdo were then treated with DMB, a reagent able to specifically transform α-ketoacids, such as Kdo or sialic acids, into fluorescent derivatives (Hara et al., 1989). The accumulation of cytosolic Kdo-8-P was then measured by HPLC (Fig. 4). The analysis of wild-type WS Arabidopsis and

Fig. 3. Spatial and developmental analysis of reporter gene expression KDSA2::GUS in Arabidopsis thaliana plants. GUS staining was performed using the AtkdsA2-V mutant plants (KDSA2-V::GUS), transformants with a T-DNA harbouring the cloned KDSA2 promoter upstream of the uidA coding region (KDSA2::GUS) to confirm the specificity expression and transformants harbouring 35S::GUS as a control of GUS staining. (a) Four-leaf plantlets; (b) young leaves; (c) hydathodes of young leaves; (d) fully developed flowers; (e) dissected siliques.

Fig. 4. Kdo-8-P synthesis in the T-DNA insertion AtkdsA1-S, AtkdsA2-S, and AtkdsA2-V null mutants. (a) Comparison of HPLC profiles obtained with a standard of Kdo derivatized with DMB, DMB-derivatized cytosolic monosaccharides extracted from wild-type WS A. thaliana leaves, wild-type Nicotiana tabacum leaves, and AtkdsA2-V mutant leaves. The arrow points out the specific peak of DMB-derivatized Kdo. (b) HPLC analysis of DMB-derivatized cytosolic monosaccharides extracted from AtkdsA1-S and AtkdsA2-S mutant leaves. The arrow points out the specific peak of DMB-derivatized Kdo, and the DMB-derivatized N-acetyl neuraminic acid (NeuAc), used as an internal standard for quantification of Kdo, is indicated.
together, the tobacco leaf extracts used as a control revealed the presence of a typical peak, co-eluting with the DMB-derived Kdo standard (Fig. 4a). In the AtkdsA2-V mutant, the peak corresponding to Kdo was significantly lowered. The quantification of the Kdo peak surface from the AtkdsA2-V extracts compared with that of wild-type Arabidopsis WS extracts revealed that only about 10% of the amount of Kdo present in the wild-type remained in AtkdsA2-V mutant plants (Fig. 4a). In the AtkdsA1-S and AtkdsA2-S mutants, the amount of cytosolic Kdo was estimated to be of 110 ng and 62 ng per 30 mg fresh weight, respectively (Fig. 4b). These quantities would indicate that the AtKDSA2 enzyme accounts for merely 64% of the total cytosolic Kdo to be produced in Arabidopsis cells, and conversely the AtKDSA1 enzyme represents 36% of synthesized Kdo.

Kdo synthesis is essential for proper pollen tube elongation

Since the single mutation in each KDSA gene gave no clear phenotypes compared with the wild type, an attempt was made to generate a double knockout mutant for the AtkdsA1 and AtkdsA2 genes by pollinating flowers of the AtkdsA1-S mutant plants with pollen from AtkdsA2-S mutant plants, and vice versa. The analysis of 75 individual plants resulting from these crosses revealed that homozygous AtkdsA1−/− AtkdsA2−/− plants were not obtained as determined by molecular characterization of the progeny. It was then decided to perform an ‘in silico’ analysis of the progeny from one siliques containing two AtkdsA1−/− AtkdsA2+/− and one AtkdsA1+/− AtkdsA2−/−. No embryonic defects were detected in mature siliques of these genotypes, so the seeds from those siliques were carefully taken out and organized on an MS plate in order to get the exact position of the seeds from the top to the bottom part of the siliques. After growth and molecular characterization of the seedlings of two siliques from each genotype, no homozygous AtkdsA1−/− AtkdsA2−/− plants were found, but 50% of the seedlings was from the parental genotype (AtkdsA1−/− AtkdsA2+/− or AtkdsA1+/− AtkdsA2−/−) and 50% was from the wild-type genotype (AtkdsA1−/− AtkdsA2+/- or AtkdsA1+/- AtkdsA2−/−). Moreover, from this positional genotyping it was seen that the different genotypes were randomly organized through the siliques, meaning that the haploid pollen grains AtkdsA1− AtkdsA2+ or AtkdsA1+ AtkdsA2− developed and germinated properly. This suggested the defect was in the haploid pollen grains AtkdsA1− AtkdsA2−.

To test whether this could originate from a gametophytic or a sporophytic defect, a tetrad analysis was performed taking advantage of the quartet1 mutant (McCormick, 2004). The QUARTET1 gene encodes a pectin methyl-esterase that is essential for pectin cleavage in the pollen mother cell primary wall (Francis et al., 2006). As a result, the four products of microsporogenesis remain fused and pollen grains are released as tetrads in the quartet1 mutant (Preuss et al., 1994), but each pollen grain can germinate normally. Flowers from quartet1 (qrt1-2) mutant plants were pollinated with pollen from AtkdsA1−/− AtkdsA2+/- mutant plants. After two generations, plants were screened for the quartet phenotype and AtkdsA1−/− AtkdsA2−/- genotype. The analysis of the pollen grains revealed intact tetrads with no obvious defects (Fig. 5). Dry tetrads obtained from the cross qrt1-2×AtkdsA1−/− AtkdsA2+/- displayed the same morphology as those from the qrt1-2 mutant (Fig. 5a). Furthermore, hydration of the pollen

![Fig. 5. Characterization of pollen grains from a qrt1-2×AtkdsA1−/− AtkdsA2+/- tetrad. (a) Comparison of the morphology of dry tetrads from qrt1-2 and from the cross qrt1-2×AtkdsA1−/− AtkdsA2+/-; (b) aniline blue staining of hydrated tetrads from qrt1-2 and from the cross qrt1-2×AtkdsA1−/− AtkdsA2+/-; (c) comparison of the germination of tetrads from qrt1-2 and from the cross qrt1-2×AtkdsA1−/− AtkdsA2+/-; (d) higher-scale view showing the four elongated pollen tubes from qrt1-2 tetrad and only two elongated pollen tubes from qrt1-2×AtkdsA1−/− AtkdsA2+/- tetrads. Scale bars = 0.1 mm.](image-url)
grains and visualization with an aniline blue staining revealing the callose of the cell wall structures (Smith and McCully, 1978) showed no differences between the two genotypes (Fig. 5b). However the pollen germination assay showed a defect of elongation in the pollen tetrad qrt1-2×AtkdsA1−/+− AtkdsA2+− compared with the qrt1-2 background (Fig. 5c, d). The pollen grains did germinate but failed to develop pollen tubes. The statistical analysis of the germinated pollen grains showed that a maximum of two out of four pollen grains of a qrt1-2×AtkdsA1−/+− AtkdsA2+− tetrad form a proper pollen tube (Table 1), thus indicating the homozygous AtkdsA1−/+− AtkdsA2−/+− double mutant results in a gametophytic phenotype (McCormick, 2004).

Discussion

Altering specifically the RG-II structure in its sugar composition is of interest for understanding the function of RG-II in the cell wall and its relationship with growth. Hence the isolation of plant mutants is useful to cell wall investigations.

The first known mutant to be altered in the glycosyl residue composition of RG-II was mur1 in Arabidopsis (Reiter et al., 1993; O’Neill et al., 2001). mur1 plants are defective in an isomerase of GDP-α-mannose-4,6-dehydratase which is involved in the synthesis of GDP-L-fucose. In mur1 plants, the L-fucose residues present in side chains A and B of RG-II are replaced by L-galactose residues, leading to a reduced content in dRG-II-B and development of defects such as dwarfism and altered growth of rosette leaves (O’Neill et al., 2001; Reuhs et al., 2004). In tobacco, Iwai et al. (2002) identified the mutant line nolac-H18 which is affected in the NpGUT1 gene encoding a glycosyltransferase thought to be involved in the borate cross-linking of pectin RG-II. nolac-H18 displays defective intercellular attachments resulting in crumbled callus, due to the inability to transfer a glucuronic acid (GlcA) residue to the side chain A of RG-II which contains the apiosyl residue involved in the borate cross-linking of monomeric RG-II. Recently, Iwai et al. (2006) showed that NpGUT1 is required for male and female reproductive tissues and fertilization. Thus, altering the composition of the main side chain A in RG-II in the vicinity of the apiosyl residue provokes important developmental and reproductive disorders.

In this work, the aim was to investigate the functional consequences of the absence of the seldom-observed sugar Kdo in the RG-II structure. Kdo is α-linked to the RG-II GalA backbone and participates in the formation of the side chain C composed of the α-D-Kdo-α-L-Rhap disaccharide (O’Neill et al., 2004). Hence, it would be expected from the impairment of Kdo synthesis that the whole side chain C would be missing in RG-II.

In plants, the biosynthetic pathway leading to Kdo is almost fully conserved. Most of the genes have been identified in Arabidopsis (Wu et al., 2004). A single gene (At3g54690) encodes a putative homologue of α-arabinose-5-P isomerase (Escherichia coli YrbH; Meredith and Woodward, 2003) which converts α-ribulose-5-P into α-arabinose-5-P. Two genes code for Kdo-8-P synthase (AtKDSA1, At1g79500; AtKDSA2, At1g16340; Matsuura et al., 2003). No candidate gene for a putative Kdo-8-P phosphatase has been identified so far. Prior to its incorporation into the pectin RG-II, the dephosphorylated Kdo is activated by coupling to CMP in a reaction catalysed by CMP-Kdo synthetase which is encoded by a single gene: AtKDSB (At1g53000), the homologue of the previously characterized gene in maize (Royo et al., 2000). Finally Wu et al. (2004) mentioned the occurrence of a putative Kdo transferase in Arabidopsis (AtKDTA encoded by At5g03770).

Therefore knockout mutants targeting the two genes encoding Kdo-8-P synthase which synthesizes the phosphorylated precursor of Kdo were characterized. Three T-DNA insertion lines called AtkdsA1-S, AtkdsA2-V, and AtkdsA2-S, respectively, extinguishing AtKDSA1 and AtKDSA2 activity, were isolated (Fig. 1). Single knockout mutations had no phenotypical consequences on plants, most probably due to genetic redundancy. This was also observed for the rgx1 and rgx2 single mutants which are affected in the (1,3)-α-D-xylosyltransferases, responsible for the synthesis of the unique type of glycosidic α-(1,3) linkage between a 2-O-Me-α-D-Xylp residue and a α-L-Fucp residue found in the RG-II side chain (Egelund et al., 2006).

Although their expression data were not quantified, Matsuura et al. (2003) clearly showed that AtKDSA2 is predominantly expressed in wild-type Arabidopsis plantlets both in shoots and roots. By contrast, the expression of AtKDSA1 seems to be restricted to the aerial parts of the plantlets. Here, these results have been confirmed and this difference in expression quantified. AtKDSA2 and AtKDSA1 mRNAs represent, respectively, 75% and 25% of the transcripts coding for Kdo-8-P synthase in Arabidopsis (Fig. 2). At the level of the end product accumulation, the present results suggest that the AtKDSA2 gene accounts for 64–90% of the total cytosolic

Table 1. Analysis of pollen tetrads obtained from the cross qrt1-2×AtkdsA1−/+− AtkdsA2+−

| Genotype                  | % of developing pollen tubes per tetrad |
|---------------------------|----------------------------------------|
|                           | 0 | 1  | 2  | 3  | 4  |
| qrt1-2 × AtkdsA1−/+−      | 2.6 | 19 | 30.1 | 38.8 | 9.5 |
| qrt1-2 × AtkdsA1−/+−      | 28.4 | 45.6 | 26 | 0 | 0 |

a Total number of tetrads analysed=116.

b Total number of tetrads analysed=169.
Kdo synthesized in whole Arabidopsis plants (Fig. 4). However, altering the total cytosolic Kdo content by as much as 90% has no obvious effect on the growth and development of Arabidopsis plants. The synthesis of Kdo within Arabidopsis cells thus appears to be in large excess and as low as 10% of remaining Kdo seems enough to allow a correct incorporation into the RGII fraction and a correct development of the plant. The present quantification data for the relative contribution of AtKDSA1 and AtKDSA2 in the cytosolic production of Kdo are in remarkably good agreement with those obtained at the level of KDSA gene transcription.

As reported by GUS reporter gene expression, the AtKDSA2 gene promoter is preferentially induced in young tissues: cotyledons, primary leaves of the shoot, hydathodes, style and anthers of mature flowers, and funicule in the siliques (Fig. 3). It was not possible to obtain any clear induction of the GUS reporter gene in roots, and especially in the meristematic part (data not shown). Therefore it was not possible to confirm the results from Matsuura et al. (2003) who attributed a preferential expression in the roots for AtKDSA2 and in the shoots for AtKDSA1. However, these authors did still observe a very high expression of AtKDSA2 in the shoots. Together these data are in accordance with previous work describing the preferential expression of the LeKDSA gene in tomato dividing tissues and organs (Delmas et al., 2003), and also indicate that AtKDSA2 is expressed in reproductive tissues similar to NpGUT1 (Iwai et al., 2006).

It was not possible to isolate a double knockout AtkdsA1−/− AtkdsA2−/− mutant. When AtkdsA1+/− AtkdsA2−/− or AtkdsA1−/− AtkdsA2+/− plants were self-crossed, no embryo defect was observed in the developing siliques, indicating that the ovules of the AtkdsA1− AtkdsA2− genotype were viable. The lack of a double homozygous mutant was due to the inability of haploid pollen grains of the AtkdsA1− AtkdsA2− genotype to form a pollen tube properly (Fig. 5), and consequently to fertilize the egg cell. It has been reported that the walls of growing tips of pollen tubes are composed predominantly of pectic polysaccharides including RG-II and that the normal growth of pollen tubes requires borate- and calcium-dependent cross-linking of pectin (O’Neill et al., 2004). Interestingly, it was recently demonstrated that the extinction of NpGUT1 in germinating pollen inhibited pollen tube elongation, together with the absence of pectin RG-II and boron in the pollen tube tip (Iwai et al., 2006). The present observations using the qrt1−2×AtkdsA1−/− AtkdsA2+/− cross are strikingly similar to the effects induced by NpGUT1 misexpression. This study shows a new example of pollen tube defect related to the RGII within the plant cell wall. Moreover, pollen tube elongation appears to be a useful assay for understanding the role of cell wall mutants.

With respect to the so-called RG-II mutants, mur1 and NpGUT1 which both affect the composition and structure of the side chain A, it has been demonstrated that the absence of Kdo biosynthesis in Arabidopsis affects pollen tube elongation. The present study suggests that conservation of the RG-II composition is critical for plant development, and provides new insights towards the elucidation of the major physiological role of such a quantitatively minor component in the cell wall.

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