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RESEARCH PAPER

The synthesis of the rhamnogalacturonan II component 3-deoxy-D-*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-D-*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, *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 (*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 it accounts for 75% of the mRNAs to be translated into Kdo-8-P synthase. Furthermore, this predominant expression of *AtKDSA2* over *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 *AtkdsA1-AtkdsA2-* genotype to form an elongated pollen tube properly and perform fertilization.

Key words: *Arabidopsis thaliana*, 3-deoxy-D-manno-oct-2-ulosonate-8-phosphate, 3-deoxy-D-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 *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 *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 *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

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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 (KDSA; EC 4.1.2.16) which catalyses the condensation of phospho*enol*pyruvate 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*^{ts} 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* (At1g79500) and *AtKDSA2* (At1g16340), 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

At*kdsA2-V* mutant plants of *Arabidopsis thaliana* are in the Wassilewskija (WS) ecotype background. At*kdsA1*-S, At*kdsA2*-S, and *qrt1-2* 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′-GCGTCACTACTGTATGATCAG-3′ and FST3′, 5′-TGACTCGACTCATGAACGTC-3′. These *AtkdsA2* gene-specific primers were used in combination with primers located in the T-DNA sequence: TAG31, 5′-CCGAAAGAACATT-GCGCGAAAGGGTGG-3′, and TAG51, 5′-GCGTAGACAACC-CTCAACTGGAAACGG-3′. The insertion site within *AtkdsA2*-V genomic DNA was mapped by sequencing PCR products spanning the borders of T-DNA.

To characterize the At*kdsA1*-S mutant, primers KdsA15′, 5′-CTCTTTTTATGCCTTGGGATG-3′ and KdsA13′, 5′-ACTGTA-TCCGAACATGGTTCCT-3′) were used in combination with primer LBa1, 5′-TGGTTCACGTAGTGGGCCATCG-3′, located within the LB sequence of the T-DNA. PCR reactions were performed with primers KdsA25′, 5′-GGACAATTTTGTGGTCA-CTCTG-3′, and KdsA23′, 5′-CTACGGCGGTTCTTGCTATG-3′, in combination with LBa1 in order to amplify the T-DNA insertion site within the At*kdsA2*-S genomic DNA.

RNA extraction and cDNA synthesis

Wild-type (WS and Col0) or At*kdsA1*-S, At*kdsA2*-S, and At*kdsA2*-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)$_{16}$ as a primer, SuperScript™ II RNase H$^-$ reverse transcriptase (Invitrogen) in a total volume of 20 µl. The cDNA was then diluted 10 times, and 1 µl of the diluted cDNA was used as a template for semi-quantitative PCR analysis.

RT-PCR analysis

RT-PCR analysis was performed as described by Joubès *et al*. (1999). Specific amplification for At*kdsA1* and At*kdsA2* cDNAs was obtained using as a 5′ primer: AtkdsA1Ex2-5′, 5′-CTGCTGA-ACCATTCTTCTTGTTGG-3′ and AtkdsA2Ex2-5′, 5′-TGCTGA-GCCGTTTTTCTTATTG-3′, respectively, in combination with the respective 3′ primers: AtkdsA1Ex3-3′, 5′-CTTCAGACCCTCGG-CCATGC-3′ and AtkdsA2Ex3-3′, 5′-GAGCCCCTCAGCATGC-CAGGA-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′-GGATCTGTACGGTAA-CATTGTGCTC-3′ as a 5′ primer, and AtActin23′, 5′-CTTTGAT-CCCATTCATAAAACCCCAGC-3′ as a 3′ primer. After an initial

denaturation step of 5 min at 95 °C, the reaction programme was as follows: 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C for 33 cycles for At*kdsA1* and At*kdsA2* or 25 cycles for *AtACTIN2*, and a final step of 5 min at 72 °C. The amount of first-strand cDNA and the number of cycles allowed the reaction to be in the linear range of PCR amplification. As a control for DNA contamination, a PCR reaction was performed using the *AtACTIN2*-specific primers in the absence of any added DNA.

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 at s 72 °C) from genomic DNA of *Arabidopsis* (WS) plantlets using the following primers: 5′-CAATCTGGTGCTCTGGG-3′ introducing a *Bam*HI site and 5′-CTATAGTAGAGCTTAAGATC-3′ introducing a *Nco*I site for subsequent cloning. The 831 bp product was digested by *Bam*HI and *Nco*I and ligated into the plasmid pCAMBIA 1381 (http://www.cambia.org.au) between the corresponding sites to yield pCKDSA2. A transcriptional fusion of the CaMV 35S constitutive promoter with the *GUS* gene (pC35S plasmid) was obtained by cloning a *Bam*HI–*Nco*I 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 pC35S and pCKDSA2 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 plates containing hygromycin (25 μg ml^{-1}). T1 transformants were selected and grown in a growth chamber until mature seeds were obtained. T2 plants were analysed for GUS activity by histochemical assays.

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 NH_4OH before being dried again in order to be derivatizated 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

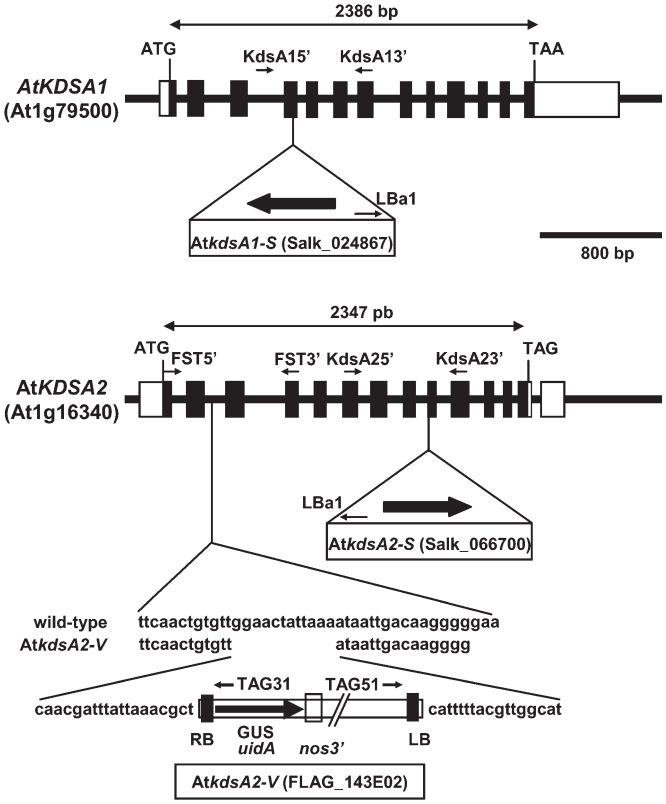

Fig. 1. Schematic representation of the T-DNA insertion *AtkdsA1-S*, *AtkdsA2-S*, and *AtkdsA2-V* null mutants. The transcribed region of the *AtKDSA1* and *AtKDSA2* genes is boxed. *AtKDSA1* and *AtKDSA2* consist of 13 and 14 exons, respectively, represented as black boxes. White boxes represent the untranslated 5′ and 3′ region of the corresponding mRNA. LB and RB indicate the orientation of the left and right borders of the T-DNA, respectively. The location of the PCR primers used for the molecular characterization of the mutants is indicated as black arrows above the genes.

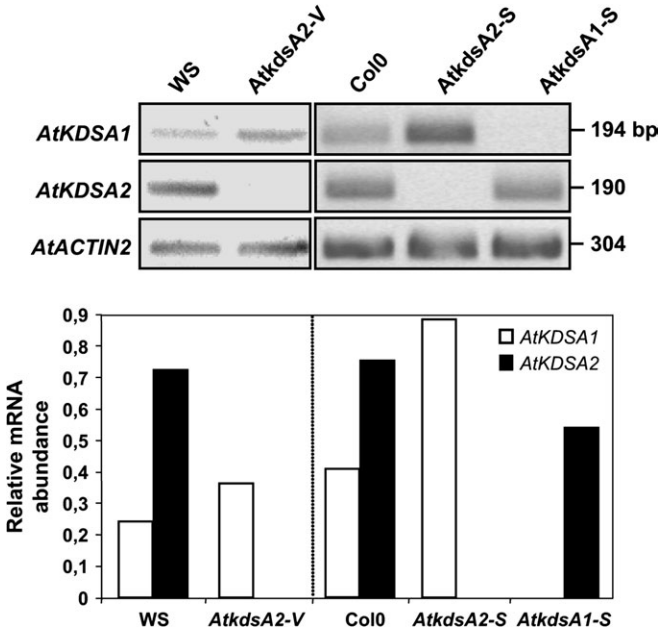

Fig. 2. *AtKDSA1* and *AtKDSA2* gene expression analysis in the T-DNA insertion *AtkdsA1-S*, *AtkdsA2-S*, and *AtkdsA2-V* null mutants. The *AtKDSA1* and *AtKDSA2* mRNA expressions in seedlings of the mutant lines and corresponding WT ecotypes were analysed by semi-quantitative RT-PCR and the *AtKDSA1* and *AtKDSA2* mRNA relative abundance was quantified by image scanning of the autoradiogram using the Quantity One software from BioRad Laboratories (Hercules, CA, USA), normalized to *AtACTIN2* used as a positive control of PCR, and expressed as a ratio of arbitrary units for pixel intensities. Relative quantification data were the mean value of two repeats.

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

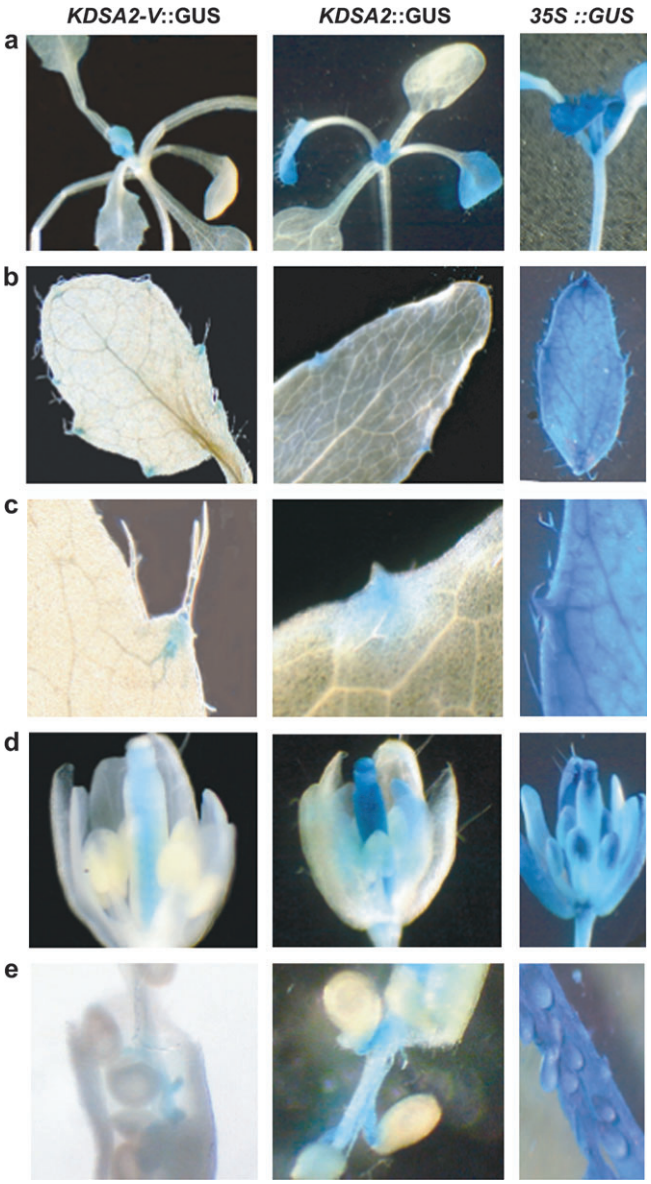

Fig. 3. Spatial and developmental analysis of reporter gene expression *KDSA2::GUS* in *Arabidopsis thaliana* plants. GUS staining was performed using the *AtkdsA2-V* mutant plants (*KDSA2-V::GUS*), transformants with a T-DNA harbouring the cloned *KDSA2* promoter upstream of the *uidA* coding region (*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.

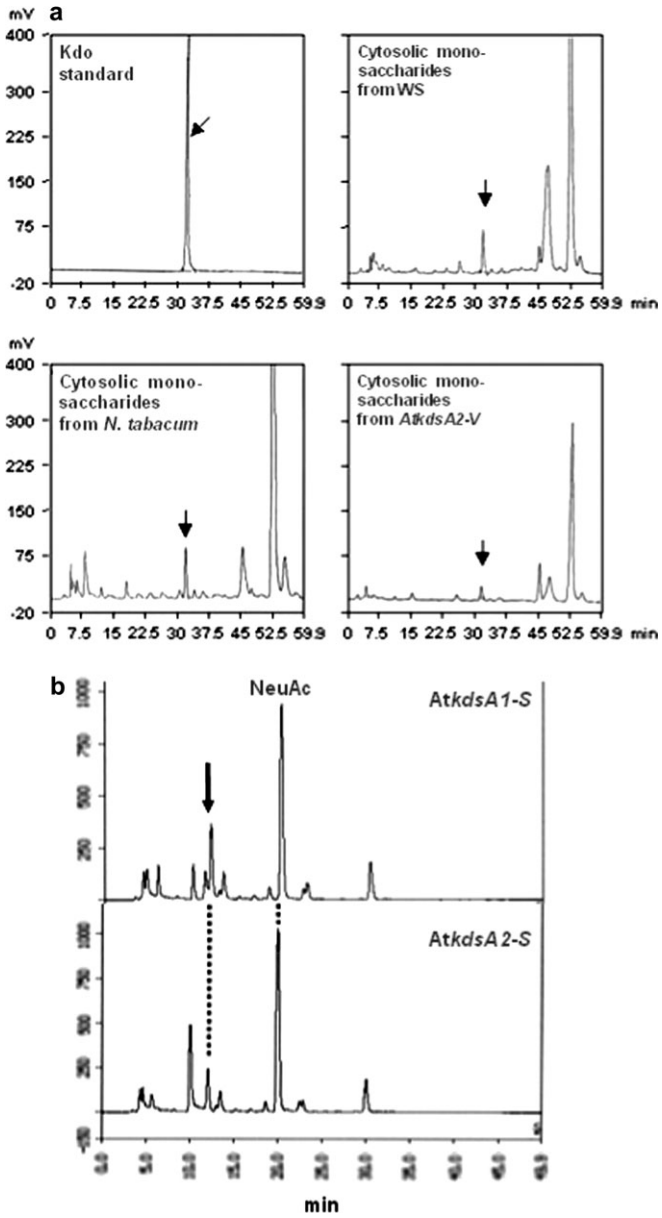

Fig. 4. Kdo-8-P synthesis in the T-DNA insertion *AtkdsA1-S*, *AtkdsA2-S*, and *AtkdsA2-V* null mutants. (a) Comparison of HPLC profiles obtained with a standard of Kdo derivatized with DMB, DMB-derivatized cytosolic monosaccharides extracted from wild-type WS *A. thaliana* leaves, wild-type *Nicotiana 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.

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 siliquo′ analysis of the progeny from one silique *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 silique. 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 silique, 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 methylesterase 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

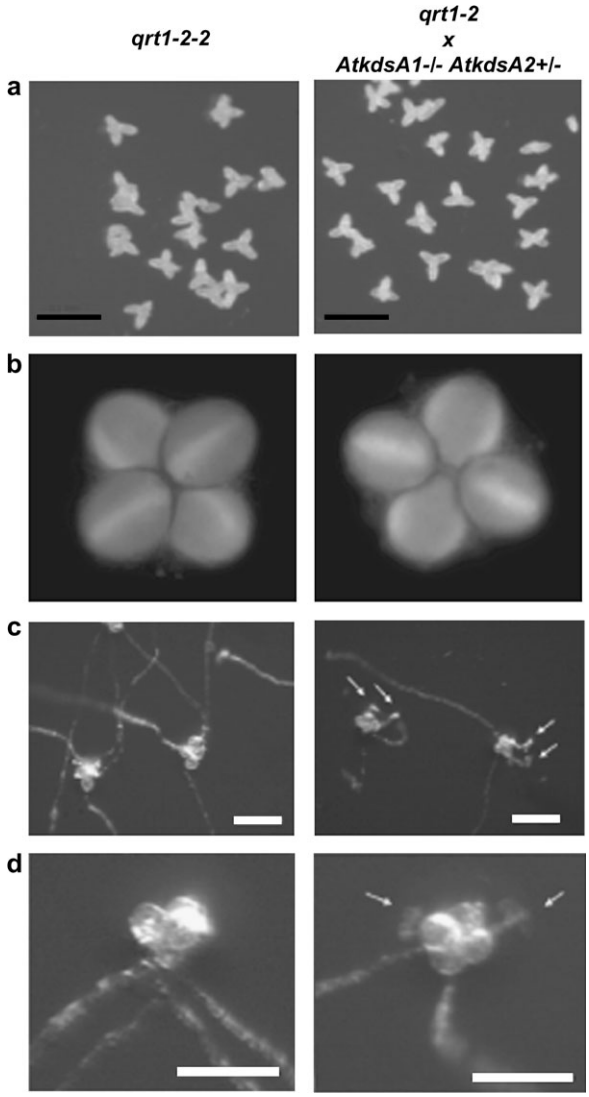

Fig. 5. Characterization of pollen grains from a *qrt1-2*×*AtkdsA1*−/− *AtkdsA2*+/− tetrad. (a) Comparison of the morphology of dry tetrads from *qrt1-2* and from the cross *qrt1-2*×*AtkdsA1*−/− *AtkdsA2*+/−; (b) aniline blue staining of hydrated tetrads from *qrt1-2* and from the cross *qrt1-2*×*AtkdsA1*−/− *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* tetrads and only two elongated pollen tubes from *qrt1-2*×*AtkdsA1*−/− *AtkdsA2*+/− tetrads. Scale bars = 0.1 mm.

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 isozyme of GDP-D-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 developmental 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.

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
quartet (*qrt1-2*)[a]	2.6	19	30.1	38.8	9.5
qrt1-2×*AtkdsA1*−/− *AtkdsA2*+/−[b]	28.4	45.6	26	0	0

[a] Total number of tetrads analysed=116.
[b] Total number of tetrads analysed=169.

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-Rha*p* 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 D-arabinose-5-P isomerase (*Escherichia coli YrbH*; Meredith and Woodward, 2003) which converts D-ribulose-5-P into D-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 *rgxt1* and *rgxt2* 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-Xyl*p* residue and a α-L-Fuc*p* 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

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.

References

Brabetz W, Wolter FP, Brade H. 2000. A cDNA encoding 3-deoxy-D-*manno*-oct-2-ulosate-8-phosphate synthase of *Pisum sativum* L. (pea) functionally complements a *kdsA* mutant of the Gram-negative bacterium *Salmonella enterica*. *Planta* **212,** 136–143.

Becker B, Lommerse JPM, Melkonian M, Kamerling JP, Vliegenthart JFG. 1995. The structure of an acidic trisaccharide component from a cell wall polysaccharide preparation of the green alga *Tetraselmis striata* Butcher. *Carbohydrate Research* **267,** 313–321.

Bouchez D, Camilleri C, Caboche M. 1993. A binary vector based on Basta resistance *in planta* transformation of *Arabidopsis thaliana*. *Comptes Rendus de l'Académie des Sciences Serie III Sciences de la Vie* **316,** 1188–1193.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Delmas F, Petit J, Joubès J, Seveno M, Paccalet T, Hernould M, Lerouge P, Mouras M, Chevalier C. 2003. The gene expression and enzyme activity of plant 3-deoxy-D-*manno*-2-octulosonic acid-8-phosphate synthase are preferentially associated with cell division in a cell cycle-dependent manner. *Plant Physiology* **133,** 348–360.

Egelund J, Petersen BL, Motawia MS, Damager I, Faik A, Olsen CE, Ishii T, Clausen H, Ulvskov P, Geshi N. 2006. *Arabidopsis thaliana RGXT1* and *RGXT2* encode golgi-localized (1,3)-α-D-xylosyltransferases involved in the synthesis of pectic rhamnogalacturonn-II. *The Plant Cell* **18,** 2593–2607.

Fleisher A, O'Neill MA, Ehwald R. 1999. The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturnan II.. *Plant Physiology* **121,** 829–838.

Fleisher A, Titel C, Ehwald R. 1998. The boron requirement and cell wall properties of growing and stationary suspension-cultured *Chenopodium album* L. cells. *Plant Physiology* **117,** 1401–1410.

Francis KE, Lam SY, Copenhaver GP. 2006. Separation of Arabidopsis pollen tetrads is regulated by QUARTET1, a pectin methylesterase gene. *Plant Physiology* **142,** 1004–1013.

Hara S, Yamaguchi M, Takemori Y, Furuhata K, Ogura H, Nakamura M. 1989. Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. *Analytical Biochemistry* **179,** 162–166.

Hicks GR, Rojo E, Hong S, Carter DG, Raikhel NV. 2004. Geminating pollen has tubular vacuoles, displays highly dynamic vacuole biogenesis, and requires VACUOLESS1 for proper function. *Plant Physiology* **134,** 1227–1239.

Ishii T, Matsunaga T, Hayashi N. 2001. Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. *Plant Physiology* **126,** 1698–1705.

Ishii T, Matsunaga T, Pellerin P, O'Neill MA, Darvill AG, Albersheim P. 1999. The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. *Journal of Biological Chemistry* **274,** 13098–13104.

Iwai H, Hokura M, Oishi M, Chida H, Ishii T, Sakai S, Satoh S. 2006. The gene responsible for borate cross-linking of pectin rhamnogalacturonan-II is required for plant reproductive tissue development and fertilization. *Proceedings of the National Academy of Sciences, USA* **103,** 16592–16597.

Iwai H, Masaoka N, Ishii T, Satoh S. 2002. A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem. *Proceedings of the National Academy of Sciences, USA* **99,** 16319–16324.

Joubès J, Phan TH, Just D, Rothan C, Bergounioux C, Raymond P, Chevalier C. 1999. Molecular and biochemical characterization of the involvement of cyclin-dependent kinase A during the early development of tomato fruit. *Plant Physiology* **121,** 857–869.

Matoh T, Kawaguchi S, Kobayashi M. 1996. Ubiquity of a borate-rhamnogalacturonan II complex in the cell walls higher plants. *Plant Cell Physiology* **37,** 636–640.

Matsuura K, Miyagawa I, Kobayashi M, Ohta D, Matoh T. 2003. *Arabidopsis* 3-deoxy-D-*manno*-oct-2-ulosonate-8-phosphate synthase: cDNA cloning and expression analyses. *Journal of Experimental Botany* **54,** 1785–1787.

McCormick S. 2004. Control of male gametophyte development. *The Plant Cell* **16,** S142–S153.

Meredith TC, Woodard RW. 2003. *Escherichia coli* YrbH is a D-Arabinose-5-P isomerase. *Journal of Biological Chemistry* **278,** 32771–32777.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiologia Plantarum* **15,** 473–497.

O'Neill MA, Eberhard S, Albersheim P, Darvill AG. 2001. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science* **294,** 846–849.

O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review in Plant Biology* **55,** 109–139.

O'Neill MA, Warrenfeltz D, Kates K, Pellerin P, Doco T, Darvill AG, Albersheim P. 1996. Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. *Journal of Biological Chemistry* **271,** 22923–22930.

Pérez S, Mazeau K, Hervé du Penhoat C. 2000. The three-dimensional structures of the pectic polysaccharides. *Plant Physiology and Biochemistry* **38,** 37–55.

Preuss D, Rhee RY, Davis RW. 1994. Tetrad analysis possible in Arabidopsis with mutation of the QUARTET (QRT) genes. *Science* **264,** 1458–1460.

Reiter WD, Chapple CCS, Sommerville CR. 1993. Altered growth and cell walls in a fucose-deficient mutant of Arabidopsis. *Science* **261,** 1032–1035.

Reuhs BL, Glenn J, Stephens SB, Kim JS, Christie DB, Glushka JG, Zablackis E, Albersheim P, Darvill AG, O'Neill MA. 2004. L-Galactose replaces L-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose deficient *mur1* Arabidopsis mutant. *Planta* **219,** 147–157.

Rick P. 1987. Lipopolysaccharide biosynthesis. In: Neidhardt FC, ed. Escherichia coli and Salmonella typhimurium: *cellular and molecular biology*. Washington, DC: American Society for Microbiology.

Royo J, Gomez E, Hueros G. 2000. A maize homologue of the bacterial CMP-Kdo synthetases: similar pathways operate in plants and bacteria for the activation of Kdo prior to its incorporation into cellular envelopes. *Journal of Biological Chemistry* **275,** 24993–24999.

Smith MM, McCully ME. 1978. Enhancing aniline blue fluorescent staining of cell wall structures. *Stain Technology* **53,** 79–85.

Willats WGT, McCartney L, Mackie W, Knox JP. 2001. Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology* **47,** 9–27.

Wu J, Patel MA, Sundaram AK, Woodard RW. 2004. Functional and biochemical characterization of a recombinant *Arabidopsis thaliana* 3-deoxy-D-*manno*-2-octulosonate-8-phosphate synthase. *Biochemistry Journal* **381,** 185–193.

York WS, Darvill AG, McNeil M, Albersheim P. 1985. 3-Desoxy-D-manno-2-octulosonic acid (KDO) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell walls of plants. *Carbohydrate Research* **138,** 109–126.