DR AI-MIN WU (Orcid ID : 0000-0001-8322-8833)

Article type : Original Article

UDP-Api/UDP-Xyl synthases affect plant development by controlling the content of UDP-Api to regulate the RG-II-borate complex

Xianhai Zhao^{1,2,†}, Berit Ebert³, Baocai Zhang⁴, Huabin Liu⁵, Yutao Zhang⁶, Wei Zeng^{7,8}, Carsten Rautengarten³, Huiling Li^{1,2}, Xiaoyang Chen^{1,2}, Antony Bacic^{7,8,9}, Guodong Wang⁶, Shuzhen Men⁵, Yihua Zhou⁴, Joshua L. Heazlewood³ and Ai-Min Wu^{1,2,*}

¹State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, South China Agricultural University, Guangzhou 510642, China

²Guangdong Key Laboratory for Innovative Development and Utilization of Forest Plant Germplasm, College of Forestry and Landscape Architecture, South China Agricultural University, Guangzhou 510642, China

³School of BioSciences, The University of Melbourne, Parkville, VIC 3010, Australia

⁴State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

⁵Department of Plant Biology and Ecology, College of Life Sciences, Nankai University, Tianjin 300071, China

⁶National Engineering Laboratory for Resource Development of Endangered Crude Drugs in Northwest China, The Key Laboratory of Medicinal Resources and Natural Pharmaceutical Chemistry, College of Life Sciences, Shaanxi Normal University, Xi'an, Shaanxi 710119, China

⁷ARC Centre of Excellence in Plant Cell Walls, School of BioSciences, The University of Melbourne, Parkville, VIC 3010, Australia

⁸Sino-Australia Plant Cell Wall Research Centre, State Key Laboratory of Subtropical Silviculture, School of Forestry and Biotechnology, Zhejiang A&F University, Lin'an 311300, China

⁹La Trobe Institute for Agriculture & Food, Department of Animal, Plant & Soil Sciences, La Trobe

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/TPJ.14921](https://doi.org/10.1111/TPJ.14921)

University, Bundoora, VIC 3083, Australia

[†]Present address: Biology Department, Brookhaven National Laboratory, Upton, New York 11973

*Corresponding author email: wuaimin@scau.edu.cn

Running title: AXS limits the RG-II-borate complex

Keywords: pectin, rhamnogalacturonan-II, apiose, AXS, boron, development

SUMMARY

Rhamnogalacturonan-II (RG-II) is structurally the most complex glycan in higher plants, containing 13 different sugars and 21 distinct glycosidic linkages. Two monomeric RG-II molecules can form a RG-II-borate diester dimer through the two apiosyl (Api) residues of side chain A to regulate crosslinking of pectin in the cell wall. But the relationship of Api biosynthesis and RGII dimer is still unclear. In this study we investigated the two homologous UDP-D--apiose/UDP-D-xylose synthases (AXSs) in *Arabidopsis thaliana* that synthesize UDP-D-*apiose* (UDP-Api). Both two AXSs are ubiquitously expressed while AXS2 has higher overall expression level than AXS1 in the tissues analyzed. Homozygous *axs* double mutant is lethal, while heterozygous *axs1/+ axs2* and *axs1 axs2/+* mutants display intermediate phenotypes. The *axs1/+ axs2* mutant plants are unable to set seed and die. By contrast, the *axs1 axs2/+* mutant plants exhibit loss of shoot and root apical dominance. UDP-Api content in *axs1 axs2/+* mutant is decreased by 83%. The cell wall of *axs1 axs2/+* mutant plants are thicker and contain less RG-II-borate complex content than wild-type Col-0. Taken together, these results provide direct evidence of the importance of AXSs for the UDP-Api and RG-II-borate complex formation in plant growth and development.

INTRODUCTION

Plant cell walls play a fundamental role in providing form and structure to plant cells, tissues, and organs. The primary cell wall is largely composed of the polysaccharides cellulose, hemicelluloses, and pectins as well as varying amounts of protein (Doblin *et al.*, 2010). Pectin is generally assumed to account for approximately one-third of primary cell wall macromolecules in dicot herbaceous cells and is involved in the cell wall matrix network and plant defense against pathogens (Mohnen, 2008, Malinovsky *et al.*, 2014). Pectin consists of three major types: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II). These three polysaccharides make up approximately 65% HG, 20-35% RG-I, and 10% RG-II of the total pectin content, and are likely covalently linked to each other in the cell

65 wall (Atmodjo *et al.*, 2013). Although RG-II constitutes a minor pectic component, it is the most complex
66 and structurally conserved of the pectic polysaccharides (Funakawa and Miwa, 2015). The RG-II backbone
67 is composed of linear 1, 4-linked α -D-GalpA residues similar to HG, but is decorated with four side chains
68 (designated A to D) containing 13 different sugars and more than 21 distinct glycosidic linkages (Funakawa
69 and Miwa, 2015). Recently, two *t-Araf* side chains are found in RG-II heretofore not established (Ndeh *et*
70 *al.*, 2017). Side chains C and D contain two sugar residues. However, side chains A and B are composed
71 of eight and seven to nine sugar residues, respectively, and are highly diverse (Pabst *et al.*, 2013). RG-II
72 forms a RG-II-borate diester dimer linked through the two apiosyl (Api) residues present in side chain A of
73 two RG-II monomers (Kobayashi *et al.*, 1996). The RG-II-borate diester dimer is highly conserved and it
74 appears that all cell wall borate occurs in the form of a RG-II-borate complex (Matoh *et al.*, 1996).

75 The RG-II-borate complex is crucial for plant development and the structural integrity of RG-II is
76 important for its ability to dimerize. The *Arabidopsis mur1* mutant lacks GDP-mannose-4,6-dehydratase
77 function and is unable to synthesize sufficient GDP-L-fucose (GDP-Fuc) for correct RG-II assembly and
78 subsequent cross-linking (Reiter *et al.*, 1993). The *mur1* mutant exhibits a dwarf phenotype with a brittle
79 stem when compared to the wild-type Col-0. Later characterization of *mur1* indicated that aerial parts of
80 the plant produce RG-II with no L-Fuc but instead contains a 3,4-linked α -L-galactose (L-Gal) residue in
81 side chain A and a terminal non-reducing 2-O-Me α -L-Gal residue in side chain B (O'Neill *et al.*, 2001).
82 However, more recently, it was suggested that RG-II in *mur1* plants simply possessed a truncated side chain
83 A (an absence of L-Fuc) rather than a substitution by L-Gal (Pabst *et al.*, 2013). Notably, *Arabidopsis* RNAi
84 plants defective in the Golgi localized GDP-Fuc transporter (GFT1) exhibit a *mur1*-like phenotype
85 indicating that reduced RG-II Fuc content might be the cause (Rautengarten *et al.*, 2016).
86 RHAMNOGALACTURONAN XYLOSYLTRANSFERASES (RGXTs) belong to a small
87 glycosyltransferase (GT) family with four members and have been shown to possess the ability to transfer
88 D-xylose to L-Fuc, a process required for the biosynthesis of side chain A (Egelund *et al.*, 2006, Egelund *et*
89 *al.*, 2008). While mutations in RGXT1 and RGXT2 do not reveal alterations in either RG-II structure or
90 plant morphology, the RGXT4/ MALE GAMETOPHYTE DEFECTIVE 4 (MGP4) mutant plants show
91 reduced pollen tube and root growth (Liu *et al.*, 2011). Interestingly, defects in *mur1* (O'Neill *et al.*, 2001)
92 and *mgp4* (Liu *et al.*, 2011) mutant plants could be restored through excessive supplementation with borate,
93 indicating that the phenotypic defect was indeed a consequence of the reduced formation of the RG-II-
94 borate complex.

95 Api is present in both side chains A and B, however only Api residues in side chain A are the sites of
96 borate diester bond formation. UDP-Api is synthesized by two isoforms of the UDP-Api/UDP-Xyl synthase
97 (AXS). AXS enzymes can form the two UDP-sugars, UDP-Api and UDP-xylose (UDP-Xyl) at the same

time via decarboxylation of UDP-D-glucuronate (UDP-GlcA) (Molhoj *et al.*, 2003). AXS is evolutionarily close to the UDP-Xyl synthase (UXS) that forms UDP-Xyl via decarboxylation of UDP-GlcA. It has been proposed that elimination of AXS activity results in the absence of the two major RG-II side chains and thereby deprives plants of their ability to form the developmentally important RG-II-borate diester dimer (Ahn *et al.*, 2006).

In this study, we investigate the expression, subcellular location, and protein interaction capabilities of the two *Arabidopsis* AXS genes, *AXS1* and *AXS2*. Then loss-of-function mutations in the AXS genes are described. Genetic and phenotypic characterization of the mutants show the central role of AXSs in the synthesis of UDP-Api and RG-II. Our research confirms the importance of RG-II-borate complex formation in plant growth and development and, more specifically, suggests its involvement in male gametophyte development and maintenance of apical dominance in *Arabidopsis*.

RESULTS

Expression patterns of *AXS1* and *AXS2* Genes

Because both *AXS1* and *AXS2* share high CDS sequence similarity (88.29%, Figure S1), from publicly available expression data (Schmid *et al.* 2005) we cannot distinguish levels of expression of each gene. Therefore, to assess the expression pattern of the two AXS genes, promoter-GUS staining and qRT-PCR were utilized. Using promoter-GUS analysis, both genes revealed expression patterns across all plant tissues and developmental stages although *AXS2pro:GUS* plants generally displayed more intense GUS staining than *AXS1pro:GUS* plants (Figure S2A to M). Our data are consistent with previous results of *AXS1* expression pattern (Molhoj *et al.* 2003), but stronger *AXS2* expression was particularly obvious in roots and dark-grown seedlings (Figure S2G and M). In addition, the expression data obtained by qRT-PCR confirmed that while the two AXS genes were ubiquitously expressed across all tissues and developmental stages, *AXS2* shows higher expression overall (Figure S2N).

AXSs locate to cytosol and could form homomers or heteromers

In plants, nucleotide sugar interconverting enzymes can localize to either the cytosol, the Golgi apparatus, or in some instances both compartments (e.g. UXS). To determine the subcellular location of the two *Arabidopsis* AXS proteins we generated C-terminal Yellow Fluorescent Protein (YFP) fusions and transiently expressed these in *N. benthamiana*. Cytosol localized UXS3-CFP was expressed at the same time to be a reference. Our data show that both *Arabidopsis* AXSs

located in the cytosol (Figure S3A and B). This is consistent with previous results where *N. benthamiana* AXS1 localizes to the cytosol when using an *Arabidopsis* protoplast expression system (Ahn *et al.*, 2006). In addition, with yeast two-hybrid and Co-IP assays, we found that AXS1 and AXS2 could interact with each other (Figure S3C and D). According to these data, we speculate that AXS1 and AXS2 can specifically associate with themselves and with each other to form homomers or heteromers. However, AXS1 and AXS2 cannot interact with the cytosol-localized UXSs in yeast (Figure S4).

AXSs are essential for plant propagation

The *axs* single mutants exhibited no significant morphological differences when compared to wild-type Col-0 plants (Figure S5). Then we created two sets of double mutants (*axs1-1* and *axs2-1*, *axs1-2* and *axs2-2*). The zygosity of the *axs1-1* and *axs2-1* mutants was determined by genotyping 109 F₂ progeny seedlings using PCR. Our results indicated that no homozygous *axs1-1 axs2-1* double mutant could be identified (Table S1) and that *axs1-1/+ axs2-1* mutant plant occurs at a lower than expected ratio. We then tested about 60 F₂ progeny seedlings from the *axs1-1/+ axs2-1* cross and again could not identify any homozygous double mutants. The genetic cross of the *axs1-2* and *axs2-2* mutant had similar zygosity, no homozygous *axs1-2 axs2-2* double mutants and a lower than expected number of *axs1-2/+ axs2-2* mutant plants (Table S2).

To investigate whether plants without functional *AXS* transcript can survive, we used a dexamethasone (DEX)-inducible promoter to drive a hairpin RNAi fragment targeting *AXS2*, to generate *dex:RNAi-axs2* plants in the *axs1-1* single mutant background. When induced with DEX, the *dex:RNAi-axs2* seeds could germinate. However, the growth of *dex:RNAi-axs2* plants were arrested at the seedling stage (Figure 1A). After about 2 weeks, the *dex:RNAi-axs2/axs1-1* seedlings turned yellow and eventually died. RT-PCR analysis of these plants confirmed that *axs2* transcript was significantly reduced upon DEX induction (Figure 1B). We further induced seedlings 4 days post germination (4 dpg) with DEX, and obtained very similar results, *dex:RNAi-axs2/axs1-1* plants turn yellow and die after approximately 6 days (Figure 1C-D). Together, our analysis of the F₂ population of the *axs1*×*axs2* genetic cross and the DEX induction experiments strongly supports the conclusion that the *AXS* genes are functionally redundant, and that an *axs1 axs2* double mutant homozygous for both genes is lethal. These observations support previous data and hypotheses on *AXS* function in both *Arabidopsis* and *Nicotiana tabacum* that cell will die without synthesis of Api (Molhoj *et al.*, 2003, Ahn *et al.*, 2006).

Pollens without AXS gene are sterile

Siliques resulting from self-fertilization of *axs1 axs2/+* mutant plants are shorter and contain fewer seeds than the wild-type Col-0 (Figure 2A). Aborted ovules and seeds were observed in the *axs1-1 axs2-1/+* mutants (Figure 2B). From the PCR analysis results of the *axs1 axs2/+* mutant progeny, like previous analysis (Table S1 and S2), no homozygous *axs1 axs2* mutant was found. To determine which gamete caused the lethal phenotype, we generated reciprocal crosses of *axs1-1 axs2-1/+* mutants and the wild-type Col-0. When wild-type Col-0 pollen was used to fertilize *axs1-1 axs2-1/+* plants, the progeny showed the expected results that about half of the progenies were able to detect T-DNA insertion of *AXS2* (Table 1). This suggested that transmission through the female gametophyte was not affected. However, when mutant pollen was used to fertilize wild-type Col-0 plants, only the *axs1-1/+ AXS2* offspring was detected (Table 1). These data clearly indicate that pollen carrying both insertions (*axs1-1 axs2-1*) was not viable. Thus, impairment of gametogenesis in *axs1-1 axs2-1* pollen is likely the reason for lethality observed in the homozygous double mutant.

To investigate how *AXS* mutants affect male gametophytic function, pollen from wild-type Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* mutants were examined. Although wild-type Col-0 morphology of all pollen grains appeared normal (Figure 2C and F), many abnormal and collapsed pollen grains were visible in two mutants (Figure 2D and E). We then tested the *in vitro* germination of pollen grains from wild-type Col-0 and *axs1-1 axs2-1/+* mutant plants. The *axs1-1 axs2-1/+* mutant plants produced two types of pollen grains: 50% *axs1-1 axs2-1*, and the remaining 50% *axs1-1 AXS2*. In *in vitro* cultures, the germination rate of pollen grains from the *axs1-1 axs2-1/+* mutant showed an approximately 50% reduction compared to pollen originating from the wild-type Col-0 (Figure 2I and J). We also investigated whether pollen tube length was affected in the mutant. However, measurements at 8 hours after germination revealed no significant difference in the length of mutant and wild-type Col-0 pollen tubes (Figure 2K). Taken together, these results indicate that partial pollens from mutant plants cannot develop normally and pollens without *AXS* gene are sterile.

Disruption of *AXS*s compromise apical dominance

Interestingly, two combinations of mutants homozygous for one *AXS* gene and heterozygous for the other, namely *axs1/+ axs2* and *axs1 axs2/+*, exhibited intermediate phenotypes. While 5-week-old plants carrying the homozygous *axs1* mutation in the heterozygous *axs2/+* background (*axs1-1 axs2-1/+*) were significantly smaller than wild-type Col-0 plants (Figure 3A), the reverse combination (*axs1-1/+ axs2-1*) exhibited a more pronounced morphological phenotype with plants severely small and chlorotic, and eventually dying (Figure 3A). The 5-week-old *axs1-1 axs2-1/+* mutants showed growth defects resembling that of the *mur1* mutant, with shorter petioles and smaller leaves (Figure 3A and Figure S6). Furthermore, 3-week-old *axs1-1 axs2-1/+* mutants grown on MS medium formed more than 4 shoots simultaneously

(Figure 3B). An examination of longitudinal sections indicated that the 2-week-old *axs1-1 axs2-1/+* mutants form several lateral meristems at the leaf axis with an abnormally large apical meristem (Figure 3C). These lateral meristems form more than ten compact embryonic shoots (axillary buds) at the base of the rosette leaves and only a part of these axillary buds produced reproductive shoots, transitioned to the flowering stage and subsequently carried siliques with very short pedicels (Figure 3D). The first one or two emerging shoots were also very short and had several flowers resulting in compact siliques (Figure 3E and F). On the other hand, >20 shoots were able to elongate till maturity with comparably larger stems, however, these were smaller than those of wild-type Col-0 plants (Figure 3G). Collectively, these mutant phenotypes suggest that the *axs1-1 axs2-1/+* mutants had lost shoot apical dominance. In addition, we noticed a cracking stem phenotype in all *axs1-1 axs2-1/+* mutant plants (Figure 3H to L). The combination of another two T-DNA insertion alleles *axs1-2* and *axs2-2* can produce similar phenotypes as the combination of *axs1-1* and *axs2-1*, except *axs1-2 axs2-2/+* has more severely dwarf phenotypes on growth comparing with *axs1-1 axs2-1/+* (Figure 3G). The reason might be the T-DNA insertion of *axs1-2* and *axs2-2* close to the N-terminal of genes to split smaller fragments.

The *axs1 axs2/+* mutants were also impaired in root development when germinated on MS medium. Compared to the wild-type Col-0, the 12 dpg *axs1 axs2/+* seedlings exhibited significantly shorter roots and more lateral root growth (Figure 4A to C). The root elongation zone of *axs1 axs2/+* mutants was stunted and the root hair density was dramatically increased (Figure 4D to F). The meristem size of *axs1 axs2/+* mutants was smaller and meristematic cortex cell number was less than wild type Col-0 seedlings (Figure 4G to I). The root cell morphology of the *axs1 axs2/+* mutants was also altered; cells appeared extremely swollen (Figure 4J). Moreover, root of *axs1-1 axs2-1/+* mutants exhibited a thicker cell wall than wild-type Col-0 (Figure 4K).

***AXS* mutants contain reduced UDP-Api, Api, B and RG-II content**

To examine the impact of plants only possessing a single *AXS* allele, we examined a range of parameters in the *AXS* mutants related to Api metabolism. In *axs1 axs2/+* mutant plants, UDP-Api was decreased by approximately 83% compared to the wild-type Col-0 (Figure 5A). The strong decrease in UDP-Api in the mutant was not compensated by changes to other nucleotide sugars which further support the UDP-Api synthase function of *AXS* genes (Table 2). We next measured the Api content in cell wall preparations (alcohol insoluble residue (AIR)) prepared from mutant and wild-type Col-0 plants. The cell wall Api content of the two *axs1 axs2/+* mutant lines was decreased by ~31% and ~37% in the *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* mutants, respectively (Figure 5B). We then analyzed the B content of AIR extracted from the mutants and wild-type Col-0. Our data showed that the proportions of cell wall B were reduced in the two *axs1 axs2/+* mutants (Figure 5C), which was consistent with a reduction in Api content. Reductions in

UDP-Api, Api, and B content were likely to affect cell wall RG-II content and composition. To verify this, we digested AIR with endo-polygalacturonase and analyzed the RG-II content of the two *axs1 axs2/+* mutants using PAGE (Chormova *et al.*, 2014) and size-exclusion chromatography (SEC). The results, as shown in Figure 5D and E, suggested that the RG-II content in the *axs1 axs2/+* mutant was reduced.

To determine if the composition of the RG-II structure was affected, RG-II-enriched preparations were made from wild-type Col-0 and the *axs1 axs2/+* mutant plants and monosaccharides analyzed as their alditol acetates. The monosaccharides Api, MeXyl, and MeFuc specific to RG-II showed no significant difference between wild-type Col-0 and the two *axs1 axs2/+* mutants (Table S3). However, the content of Gal and Ara which mainly link to the end of RG-II chains A and B was reduced (Table S3). These results deduced that the integrality of side chains A and B of RG-II structure might be affected in *axs1 axs2/+* mutants.

Mutants with defected RG-II, such as *mur1* and *mgp4* could be restored with exogenous B (O'Neill *et al.*, 2001, Liu *et al.*, 2011). To check whether the *axs1-1 axs2-1/+* mutants could be restored with B as well, the mutant seeds were germinated on plates containing 0.5, 2, 10, 100, 1000 and 5000 μ M boric acid, respectively (Figure S7). The root growth defect of *axs* mutants was not complemented by exogenous B. In addition, we tried to complement the short root phenotype with exogenous Api but failed (data not shown), indicating the lack of an Api C-1 kinase and pyrophosphorylase required to convert Api to UDP-Api.

Lastly, we analyzed the total monosaccharide composition of wild-type Col-0, the two *axs1 axs2/+* mutants and the *axs1/+ axs2* mutant by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The *axs1/+ axs2* sample was a mixture of *axs1-1/+ axs2-1* and *axs1-2/+ axs2-2* because of the low sample amount obtained from these mutants. All mutant lines showed reduced Rha and GalA content which represent the main pectic sugars (Table 3). Considering RG-II only constitutes about 10% of the pectin polymers (Atmodjo *et al.*, 2013) and the *axs1/+ axs2* mutant showed a considerable reduction of pectic sugars, this indicates that an impairment in RG-II synthesis also confers restraints to other pectic sugars.

Impairment of RG-II-borate formation leads to changes in gene expression related to auxin and the cell wall modification

To better understand changes in the *axs1 axs2/+* mutants, we performed an analysis of mutant roots using microarrays. RNA was prepared from the roots of 12 dpg seedlings of *axs1-1 axs2-1/+* mutants and wild-type Col-0 plants and subjected to transcriptional profiling. Our analysis revealed that compared to the wild-type Col-0, 768 genes were up-regulated and 614 genes were down-regulated two-fold or more in the

axs1-1 axs2-1/+ mutant (Supplemental Data Set 1). A functional classification of differentially expressed genes was performed using GO::TermFinder (Boyle *et al.*, 2004) (Supplemental Data Set 2). Among the genes that showed altered expression, GO biological process categories of cell wall loosening, response to auxin, lignin catabolic process, cell wall organization, pectin catabolic process, and cell wall macromolecule catabolic process were significantly overrepresented (Figure 6A). The differentially regulated genes were mostly associated with the extracellular region and cell wall processes (Figure 6B).

A total of 94 cell wall related genes showed altered expression, including polysaccharide acting genes, oxide-reductase genes, and cell wall structure genes (Supplemental Data Set 3). All of the detected 8 expansion (*EXP*) genes and 3 xyloglucan endotransglycosylase/hydrolase (*XTH*) genes showed obvious higher expression. 2 polygalacturonase (*PG*) genes (1 up-regulated and 1 down-regulated), 2 PG inhibitor protein (*PGIP*) genes (both up-regulated), 5 pectin lyase (*PL*) genes (3 up-regulated and 2 down-regulated), 9 pectin methylesterase (*PME*) genes (6 up-regulated and 3 down-regulated) and 4 PME inhibitor (*PMEI*) genes (2 up-regulated and 2 down-regulated) were observed. Among them, the pairs of *PG* and *PGIP*, *PME* and *PMEI* functioned opposite, respectively. So many pectin-related genes were differentially expressed in *axs1 axs2/+* mutant which hints the possibility of other pectic composition changes in addition to RG-II. Besides, this study also detected 2 up-regulated cellulose synthesis-like genes (*CSLB04* and *CSLB05*), 27 oxido-reductase genes (21 up-regulated and 6 down-regulated) and 28 cell wall structural protein genes (18 up-regulated and 10 down-regulated) affected.

Moreover, 48 auxin related genes were detected, including genes implicated in auxin synthesis, signaling, and transport (Supplemental Data Set 4). To provide information on the overlap in transcripts in this study with those identified by other published microarray experiments, we performed a meta-analysis with previously published data which showed the genes responsive to auxin treatment (Vanneste *et al.*, 2005). A total of 244 (17.7%) genes that we found in our analysis were also present in this published dataset (Figure 6C, Supplemental Data Set 5). This result provided evidence that normal auxin distribution might be affected in the *axs1 axs2/+* mutants.

DISCUSSION

Despite the essentiality of RG-II for growth and development, it was not known how the content of UDP-Api impacts cell wall composition and plant organ formation. Elimination of *AXS* expression leads to collapsed and sterile pollen which indicates the essential role of *AXS* for pollen formation and germination. Notably, with one copy of *AXS* gene, the plants were viable. Interestingly, the two different double mutants containing deduced UDP-Api and RG-II-borate complex displayed various phenotypes. The *axs1/+ axs2*

mutant exhibited a more serious defect than observed for the *axs1 axs2/+* mutant (Figure 3A). AXS1 and AXS2 protein were duplicated with high sequence identity (95.63%, Figure S8) which implied their similar enzymatic function in the production of UDP-Api. Expression analysis of the two *AXS* genes revealed that *AXS2* was highly expressed than *AXS1* across all tissues and developmental stages (Figure S2). And it is likely that differential expression intensities and patterns were responsible for the inordinate phenotype of the *axs1/+ axs2* and *axs1 axs2/+* double mutants. Therefore, by controlling the UDP-Api level, the two *AXS* genes regulate RG-II-borate complex content to affect plant development. *AXS1* and *AXS2* genes function redundancy in *Arabidopsis* while *AXS2* has an overall higher expression level than *AXS1*.

Intriguingly, the *axs1 axs2/+* mutants showed reduced apical dominance (Figure 3 and 4). Another RG-II related mutant, *mur1*, also showed reduced apical dominance (Reiter *et al.*, 1993). The *mur1* mutant contained more monomeric RG-II (Figure 5D) as previously reported (O'Neill *et al.*, 2001). PAGE and SEC analysis confirmed the reduction of RG-II in the two *axs1 axs2/+* mutants (Figure 5D and E). Moreover, both PAGE and SEC analysis showed a reduced amount of monomeric RG-II in the two *axs1 axs2/+* mutants. This suggests that the balance between the RG-II dimer and monomer has been disrupted and that all available RG-II is immediately used for dimer formation. With this aspect, it looks like under our growth condition the B supply for *axs1 axs2/+* mutants is plentiful. However, the *axs1 axs2/+* mutant plants showed B-deficient phenotype that of reduced apical dominance (Figure 3 and 4), swollen root cell (Figure 4J) and thick cell wall (Figure 4K).

B requirements of flowering plants are correlated with the amounts of RG-II present in the cell wall (Matoh *et al.*, 1996). B is found in cell wall RG-II-borate complexes, presumably a range of molecular complexes and as free B in the cytoplasm (Power and Woods, 1997). Although there is evidence for possible roles of B in membrane structures, auxin transport, membrane protein stability and wall-membrane attachment (Blevins and Lukaszewski, 1998, Camacho-Cristobal *et al.*, 2008, Li *et al.*, 2015, Matthes and Torres-Ruiz, 2016), its function has only been demonstrated mechanistically in RG-II-borate complexes in primary cell walls (Funakawa and Miwa, 2015). The effects of B deficiency on cell wall ultrastructure have been well investigated. Several reports have shown that under B deficiency condition, cell wall thickening will be observed (Hirsch and Torrey, 1980, Loomis and Durst, 1992, Hu and Brown, 1994). And the formation of RG-II-borate complex was considered the determination of pumpkin tissue cell wall thickness under B treatment (Ishii *et al.*, 2001). Fleischer *et al.* (1999) reported the significance of B for cell wall porosity. Furthermore, RG-II-borate complex was found to contribute significantly to the tensile strength of cell walls (Ryden *et al.*, 2003). In this study, the reason for swelling root cells, thick cell wall and collapsed pollen of *axs1 axs2/+* mutants might be attributed to altered cell wall ultrastructure and mechanical property. Furthermore, the *axs1 axs2/+* mutants displayed more interesting phenotype, loss of apical dominance. It

is widely known that the extensibility of cell walls and the turgor pressure determines plant cell growth. Recently, with atomic force microscopy method, pectin demethylesterification was found to increase the elasticity of shoot apical meristems (SAM) (Peaucelle *et al.*, 2008, Peaucelle *et al.*, 2011). More fundamentally, the cell wall properties of SAM are distinct from their mature descendants (Yang *et al.*, 2016). The primary effect of diminished RG-II-borate complexes will trigger cell wall ultrastructure alteration such as cell wall thickening (Figure 4K). This contributes to the change of SAM cell wall elasticity and likely lead to the loss of apical dominance of *axs1 axs2/+* mutants, to some extent, similar to B deficiency which also give rise to thick cell wall and loss of apical dominance (Sutinen *et al.*, 2006, Sutinen *et al.*, 2007, Camacho-Cristobal *et al.*, 2008).

One of the more obvious phenotypic alterations that we observed in the *axs1 axs2/+* mutants compared to the wild-type Col-0 was cracking stems (Figure 3H to L). This is a phenotype often seen in crops such as *Brassica napus* (Hirai and Tamai, 1956), coconut (Broschat, 2007) and tomato (Liebisch *et al.*, 2009) grown under low B conditions. Tissues of plants grown on low B levels were brittle or fragile, while with plenty of B supplement the plant tissues were flexible or resilient (Loomis and Durst, 1992). The presence of cracking stems in the *axs1 axs2/+* mutants provides evidence that the cracking symptoms under B starvation are probably caused by the reduced amount of cell wall RG-II-borate complex. However, in *Arabidopsis* pectin mutants, the cracking stems are exclusively observed in *axs1 axs2/+* mutants. *mur1* mutant has not shown any evident crack under our growth conditions and no description of this phenotype in previous reported pectin mutants was found. The explanation might be the discrepancy of cell wall property conferred by various mutants. The middle lamella cements the primary cell wall of two adjoining plant cells. Cell plates, the precursor of middle lamella, was examined the distribution of RG-II which imply the linkage function of RG-II in cell plate (Zhou *et al.*, 2017). Both calcium cross-links and pectin esters are required for cell-cell adhesion in fresh sugar-beet root parenchyma (Marry *et al.*, 2006). However, in poplar wood, RG-I, but not HG, is a determinant of cell-cell adhesion (Yang *et al.*, 2020). RG-II is regarded as decorated HG. Therefore, the cell-cell adhesion function of RG-II-borate complex may be different between woody plants and grass plants.

In consideration of the low content of RG-II in plant cell wall (Matsunaga *et al.*, 2004), it is reasonable that pectin sugar, Rha and GalA, were reduced slightly in *axs1 axs2/+* mutants (Table 3). However, the Rha content in *axs1/+ axs2* mutants was dropped by about a half compared with wild-type Col-0 (Table 3). This data offers a hint that cell wall RG-II content might affect the deposition of the other pectic composition, such as RG-I. On the contrary, the other two RG-II deficient mutants, *mur1* and *mgp4*, showed no obvious change of pectic sugar (Liu *et al.*, 2011, Voxeur *et al.*, 2017). Nevertheless, the altered lignification was determined in *mur1* mutant (Voxeur *et al.*, 2017). This study also observed that number of genes related to

cell wall formation and auxin pathway were differentially expressed in *axs1-1 axs2-1/+* mutant. Likewise, genes involved in lignified tissue formation and jasmonic acid signaling were overexpressed in *mur1* mutant (Voxeur *et al.*, 2017). Thus, the drastic decrease of cell wall Rha might be also resulted from the transcription regulation. The altered transcription feature may be conferred by the changed cell wall composition directly, or by reduced cell wall tensile strength and cell-cell adhesion.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *Arabidopsis thaliana* wild-type Col-0 and four T-DNA insertion lines (*axs1-1*, SALK_038986; *axs2-1*, SALK_029018; *axs1-2*, SALK_000016; *axs2-2*, SALKseq_077913.1) (Arabidopsis Biological Resource Center, ABRC) were used in this study. Homozygous T-DNA insertion mutants were identified by PCR with genomic DNA as template. Primers used for PCR are listed in Table S4. For seed germination, sterilized seeds were plated on MS medium and incubated at 4°C for 3 days. Seedlings were grown under a 16 h light/8 h dark regime in a growth cabinet except if noted differently. For the morphological analysis of mature plants, seedlings were transferred to soil 10 days after germination.

qRT-PCR

Plant RNA was extracted from 5-day-old seedlings, 2-week-old roots, 3-week-old rosette leaves, full-opened flowers, 10-day-old siliques and 7-week-old stems (up, middle, down) using the Trizol (Life Technologies) method according to the manufacturer's protocol. First-strand cDNA was synthesized with a TAKARA cDNA synthesis kit according to the manufacturer's protocol. qRT-PCR was conducted using a Roche 480 instrument. The qRT-PCR primers are listed in Table S4. For absolute qRT-PCR, a formula for quantifying the transcript copy numbers per unit weight of total RNA was derived according to the procedure described by Suzuki *et al.* (2006). Then the transcript copy number of the two *AXS* genes in different tissue were calculated. The relative qRT-PCR was performed according to the method of Schmittgen and Livak (2008) using *PP2A* as the reference.

Promoter GUS assays

The 1352 bp *AXS1* promoter and 1486 bp *AXS2* promoter upstream of the start codon sequences were cloned into the *pBI121* vector replacing the *35S* promoter. Primers used are listed in Table S4. The promoter fragments were obtained via PCR amplification and subcloned into the *HindIII/SmaI* sites of *pBI121* vector using Gibson assembly method (NEB) (Gibson *et al.*, 2009). The constructions were validated by DNA sequencing and transformed into *Agrobacterium tumefaciens* GV3101 by freeze-thaw method. Floral dip method was used to introduce the constructions into *Arabidopsis* Col-0 plants (Clough and Bent, 1998).

The T2 generation was analyzed for GUS staining according to the method published by Jefferson *et al.* (1987)).

DEX-induced RNAi of AXS2

The CDS sequence of *AXS2* gene was amplified as two reverse complement fragments using PCR with primers listed in Table S4. The PDK-intron was amplified from pHANNIBAL (Helliwell and Waterhouse, 2005). Then the three sequences were sub-cloned into a DEX-inducible vector kindly supplied by Hao Wang (Zhuang *et al.*, 2013). The vector was cut with *XhoI* and *SpeI*. The resultant constructs were validated by DNA sequencing, transformed into *axs1-1* mutant plants and subsequently selected on MS medium containing 20 $\mu\text{g mL}^{-1}$ hygromycin. The T3 generation was analyzed using RT-PCR. 5 dpg seedlings were transferred to MS plates with or without 30 μM DEX.

Yeast two-hybrid assay

The CDS sequence of *AXS1*, *AXS2*, *UXS3*, *UXS5* and *UXS6* gene were amplified with primers listed in Table S4. *pB42AD* and *pLexA* plasmids were cut with *EcoRI* and *XhoI*. Then the sequences were sub-cloned into the corresponding vectors using Gibson assembly method (NEB) (Gibson *et al.*, 2009). The resultant constructs were validated by DNA sequencing. The respective combinations of *pLexA* and *pB42AD* fusion vectors were co-transformed into yeast EGY48 containing *p8op-LacZ* plasmid. Transformants were selected and grown on SD/-His-Trp-Ura dropout plates at 30°C. For color development, the transformants were transformed to SD/-His-Trp-Ura dropout plates containing 80 mg/L X-gal.

Subcellular location of AXS

Gateway primers (Table S4) containing *attB* site were designed for the amplification of *AXS* and *UXS* genes. The PCR products without stop codons were cloned into the *pDONR207* vector via BP reaction (ThermoFisher, USA) and then cloned to the *pEarleyGate101* or *pEarleyGate102* destination vector (Earley *et al.*, 2006) via LR reaction (ThermoFisher, USA). The constructions were validated by DNA sequencing and used for transient expression assays in *Nicotiana benthamiana* (Sparkes *et al.*, 2006). The YFP or CFP signals were imaged using confocal laser scanning microscopy.

Characterization of pollen grains and pollen tubes

Pollen grains were observed using a scanning electron microscope. Released pollen grains were mounted on aluminum stubs (Hitachi Ion Sputter E-1010, HITACHI), sputter-coated with 40 nm of gold-palladium and viewed using a Hitachi S-4800 FESEM. *In vitro* germination of pollen grains was performed as described Rodriguez-Enriquez *et al.* (2013). *In vitro* growth of pollen tubes was assessed as described in Jia *et al.* (2009)).

Nucleotide sugar extraction, chromatographic separation and detection

The shoots of 3-week-old plants were harvested and ground with a mortar and pestle in liquid N₂. Nucleotide sugars were extracted from 50 mg of fresh weight as previously described (Ito *et al.*, 2014). The samples were chromatographically separated using porous graphitic carbon as the stationary phase on an 1100 series HPLC system (Agilent Technologies) and detected using a 4000 QTRAP LC-MS/MS system (AB Sciex) equipped with a TurbolonSpray ion source using methods previously described (Rautengarten *et al.*, 2014).

Transmission electron microscopy

1 cm root hair zone segments were cut from 4dpg seedlings then vacuum infiltrated in 4%(v/v) glutaraldehyde in PBS (33 mM Na₂HPO₄, 1.8 mM NaH₂PO₄, and 140 mM NaCl [pH 7.2]) before being fixed in 2% glutaraldehyde in PBS at 4°C overnight. After fixation, samples were post-fixed in 1% (w/v) osmium tetroxide, then dehydrated through a gradient of ethanol and embedded in Spurr's resin. Sections (70 – 100 nm) were cut and stained with uranyl acetate and lead citrate and visualized using an FEI Tecnai 12 transmission electron microscope.

RG-II isolation

RG-II isolation was performed as described earlier with some modifications (Liu *et al.*, 2011; Chormova *et al.*, 2014). Cell wall preparations, as alcohol insoluble residues (AIR), were prepared by extraction of the sample with 96% (v/v) ethanol at 70°C for 30 min. The AIR was saponified with 1 M Na₂CO₃. After 16 h incubation at 4°C, the saponified AIR was washed three or more times with water and freeze-dried. Endo-polygalacturonase (10 U mL⁻¹, Megazyme, <http://www.megazyme.com>) was added to the saponified AIR (50 µL mg⁻¹) and incubated at 37 °C for 12 h. After centrifuge at 20, 000 × g for 10 min, the supernatant was filtered through a 0.2 µm filter. The filtrate was taken for electrophoresis or SEC.

Chemical analyses of cell wall composition

For B measurements, material of both 3-week-old dried leaves and AIR extracted from 3-week-old dried leaves was subjected separately to microwave-assisted nitric acid digestion. The resultant solution was used to determine the B concentration using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7900) (Ishii *et al.*, 2001). The monosaccharide composition of cell wall AIR was analyzed as described by Ebert *et al.* (2015). Briefly, AIR materials were hydrolyzed in 2 N trifluoroacetic acid for 1 h at 120 °C. After centrifuge at 20, 000 × g for 10 min, the supernatants were freeze-dried. The pellets were dissolved with water for analysis of sugars except Api. Sugars were separated and quantified with high performance anion exchange chromatography with pulsed amperometric detection as on an ICS 3000 (Dionex) using a CarboPac PA1 anion exchange column (3 × 150 mm, Dionex). The Api content of leaf was quantified by

Nirupama Samanmalie Jayasinghe in The University of Melbourne using gas chromatography coupled with triple quadrupole mass spectrometry (GC-QqQ-MS). The pellets resulting from trifluoroacetic acid were subjected to the analysis as described (Jayasinghe *et al.*, 2018).

PAGE analysis of RG-II

PAGE analysis of RG-II was performed as described by Chormova *et al.* (2014). Briefly, samples were loaded to 26.4% polyacrylamide gel. Electrophoresis was conducted at 200 V for 30 min. The gel was fixed in ethanol/acetic acid/water (50 mL, 4:1:5) for 30 min. After 3 times wash with water, the gel was treated with sodium thiosulphate (50 mL, 0.1 g/L) for 1 min and immediately wash 3 times. Then freshly prepared silver nitrate solution (50 mL, 1 g/L, containing 50 µL formaldehyde) was added to the gel and incubated for 20 min with gently shaking. After 3 times quick wash with water, sodium carbonate solution (50 mL, 30 g/L, containing 50 µL formaldehyde) was added to develop color.

SEC analysis of RG-II

SEC analysis of RG-II was performed as described (Liu *et al.*, 2011). Briefly, the filtered digest solution was dialyzed for 2 d and freeze-dried. The pellets were dissolved in 50 mM ammonium formate (pH5.0). RG-II was purified with a Superdex 75 10/300 GL column (GE Healthcare, <http://www.gehealthcare.com>). The monomeric and dimeric RG-II fractions were collected at the same time. Then RG-II fractions were desalted by repeated freeze-drying. The composition of RG-II was analyzed by GC-MS.

Microarray and data analyses

For the microarray experiments, seedlings were grown on vertical MS plates under long-day condition. Roots of 12 dpg seedlings were cut and about 30 roots were pooled as one biological replicates. Total RNA was extracted with the RNeasy Plant Kit (Qiagen) from three independent biological replicates. Total RNA was quantified by the NanoDrop ND-2000 (Thermo Scientific). RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). cDNA and cRNA synthesis, cRNA labeling and fragmentation were performed and Arabidopsis (V4) Gene Expression Microarray (4x44K) was used for the hybridization. After washing, the arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies) in Oebiotech (<https://www.oebiotech.com>). The raw data were got from array images by Feature Extraction software (version 10.7.1.1, Agilent Technologies) and analyzed primarily by Genespring (version 13.1, Agilent Technologies). Differentially expressed genes were identified under two criteria, $|\log_2\text{fold change}| \geq 1.0$ and a P value ≤ 0.05 . Gene annotation were applied on TAIR (<https://www.arabidopsis.org>) and KEGG analysis were performed to get their biological roles. The raw data of the transcript profiles were deposited in the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>) under the accession code GSE151256.

DATA AVAILABILITY STATEMENT

All summary data are included in the article or in supporting information online at the journal website. Contact the corresponding author for access to original data files.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31670670, 31870653 and 31811530009), and the Guangdong Province Science and Technology Projects (2015A050502045 and 2016A010104012) to AMW. The work was supported in part by an Australian Research Council Discovery Project (DP180102630) to JLH. BE is supported by a Future Fellowship from the Australian Research Council (FT160100276). WZ and AB acknowledge the support of an Australian Research Council grant to the ARC Centre of Excellence in Plant Cell Walls (CE110001007).

AUTHOR CONTRIBUTIONS

X. Z, J. L. H and A-M. W conceived the project and designed the experiments. X. Z, B. E, B. Z, H. L, Y. Z, W. Z, C. R performed the experiments. H. L, X. C, A. B, G. W, S. M, Y. Z assisted the research. X. Z, B. E, J. L. H, T. B and A-M. W analyzed data and wrote the article. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Alignment of coding sequences of *Arabidopsis* AXS genes using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>).

Figure S2. The expression patterns of *AXS1* and *AXS2*.

Figure S3. The subcellular location and interaction of *AXS1* and *AXS2*.

Figure S4. *AXS1* and *AXS2* could not interact with the cytosol-localized UXs in yeast.

Figure S5. T-DNA insertion lines for the two *AXS* genes.

Figure S6. Leaves of *axs1 axs2*/+ double mutants.

Figure S7. The roots of *axs* mutant seedlings grown on plates containing various levels of boric acid.

Figure S8. Alignment of amino acid sequences of *Arabidopsis* AXS proteins using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>).

-
- Table S1.** Genetic analysis of *axs1-1* × *axs2-1* F2 progeny.
- Table S2.** Genetic analysis of *axs1-2* × *axs2-2* F2 progeny.
- Table S3.** Sugar composition of RG-II isolated from Col-0, *axs1-1* *axs2-1*/+ and *axs1-2* *axs2-2*/+ double mutants.
- Table S4.** Primers list.
- Supplemental Data Set 1.** List of the 1382 significant genes.
- Supplemental Data Set 2.** GO enrichment analysis of the significant genes.
- Supplemental Data Set 3.** Genes acting on carbohydrates.
- Supplemental Data Set 4.** Genes related to auxin synthesis, signaling and transport.
- Supplemental Data Set 5.** Overlapping genes between the present study and that of Vanneste et al. (2005).
- References**
- Ahn, J.W., Verma, R., Kim, M., Lee, J.Y., Kim, Y.K., Bang, J.W., Reiter, W.D. and Pai, H.S. (2006) Depletion of UDP-D-apiose/UDP-D-xylose synthases results in rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. *J. Biol. Chem.* **281**, 13708-13716.
- Atmodjo, M.A., Hao, Z. and Mohnen, D. (2013) Evolving views of pectin biosynthesis. *Annu. Rev. Plant Biol.* **64**, 747-779.
- Blevins, D.G. and Lukaszewski, K.M. (1998) Boron in Plant Structure and Function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 481-500.
- Boyle, E.I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J.M. and Sherlock, G. (2004) GO::TermFinder--open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics*, **20**, 3710-3715.
- Broschat, T.K. (2007) Boron deficiency symptoms in palms. *Palms*, 115-126.
- Camacho-Cristobal, J.J., Rexach, J. and Gonzalez-Fontes, A. (2008) Boron in plants: deficiency and toxicity. *J. Integr. Plant Biol.* **50**, 1247-1255.
- Chormova, D., Messenger, D.J. and Fry, S.C. (2014) Boron bridging of rhamnogalacturonan-II, monitored by gel electrophoresis, occurs during polysaccharide synthesis and secretion but not post-secretion. *Plant J.* **77**, 534-546.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.
- Doblin, M.S., Pettolino, F. and Bacic, A. (2010) Plant cell walls: the skeleton of the plant world. *Funct. Plant Biol.* **37**,

357-381.

Earley, K.W., Haag, J.R., Pontes, O., Oppen, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616-629.

Ebert, B., Rautengarten, C., Guo, X., Xiong, G., Stonebloom, S., Smith-Moritz, A.M., Herter, T., Chan, L.J., Adams, P.D., Petzold, C.J., Pauly, M., Willats, W.G., Heazlewood, J.L. and Scheller, H.V. (2015) Identification and Characterization of a Golgi-Localized UDP-Xylose Transporter Family from Arabidopsis. *Plant Cell*, **27**, 1218-1227.

Egelund, J., Damager, I., Faber, K., Olsen, C.E., Ulvskov, P. and Petersen, B.L. (2008) Functional characterisation of a putative rhamnogalacturonan II specific xylosyltransferase. *FEBS Lett.* **582**, 3217-3222.

Egelund, J., Petersen, B.L., Motawia, M.S., Damager, I., Faik, A., Olsen, C.E., Ishii, T., Clausen, H., Ulvskov, P. and Geshi, N. (2006) Arabidopsis thaliana RGXT1 and RGXT2 encode Golgi-localized (1,3)-alpha-D-xylosyltransferases involved in the synthesis of pectic rhamnogalacturonan-II. *Plant Cell*, **18**, 2593-2607.

Fleischer, A., O'Neill, M.A. and Ehwald, R. (1999) The Pore Size of Non-Graminaceous Plant Cell Walls Is Rapidly Decreased by Borate Ester Cross-Linking of the Pectic Polysaccharide Rhamnogalacturonan II. *Plant physiol.* **121**, 829-838.

Funakawa, H. and Miwa, K. (2015) Synthesis of borate cross-linked rhamnogalacturonan II. *Front. Plant Sci.* **6**, 223.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A. and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods*, **6**, 343-U341.

Helliwell, C.A. and Waterhouse, P.M. (2005) Constructs and methods for hairpin RNA-mediated gene silencing in plants. *Meth. Enzym.* **392**, 24-35.

Hirai, K. and Tamai, M. (1956) Studies on the boron deficiency in field crops (Part I) boron deficiency symptoms in rape plants. *Soil Sci. Plant Nutr.* **2**, 201-205.

Hirsch, A.M. and Torrey, J.G. (1980) Ultrastructural-Changes in Sunflower Root-Cells in Relation to Boron Deficiency and Added Auxin. *Can. J. Bot.* **58**, 856-866.

Hu, H. and Brown, P.H. (1994) Localization of Boron in Cell Walls of Squash and Tobacco and Its Association with Pectin (Evidence for a Structural Role of Boron in the Cell Wall). *Plant physiol.* **105**, 681-689.

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

Ito, J., Herter, T., Baidoo, E.E., Lao, J., Vega-Sanchez, M.E., Michelle Smith-Moritz, A., Adams, P.D., Keasling, J.D., Usadel, B., Petzold, C.J. and Heazlewood, J.L. (2014) Analysis of plant nucleotide sugars by hydrophilic interaction liquid chromatography and tandem mass spectrometry. *Anal. Biochem.* **448**, 14-22.

Jayasinghe, N.S., Mendis, H., Roessner, U. and Dias, D.A. (2018) Quantification of Sugars and Organic Acids in Biological Matrices Using GC-QqQ-MS. *Methods Mol. Biol.* **1778**, 207-223.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

-
- Jia, D.J., Cao, X., Wang, W., Tan, X.Y., Zhang, X.Q., Chen, L.Q. and Ye, D. (2009) GNOM-LIKE 2, encoding an adenosine diphosphate-ribosylation factor-guanine nucleotide exchange factor protein homologous to GNOM and GNL1, is essential for pollen germination in Arabidopsis. *J. Integr. Plant Biol.* **51**, 762-773.
- Kobayashi, M., Matcho, T. and Azuma, J. (1996) Two Chains of Rhamnogalacturonan II Are Cross-Linked by Borate-Diol Ester Bonds in Higher Plant Cell Walls. *Plant Physiol.* **110**, 1017-1020.
- Li, K., Kamiya, T. and Fujiwara, T. (2015) Differential Roles of PIN1 and PIN2 in Root Meristem Maintenance Under Low-B Conditions in Arabidopsis thaliana. *Plant Cell Physiol.* **56**, 1205-1214.
- Liebisch, F., Jfj, M., Heine, G. and Horst, W.J. (2009) Blossom-end rot and fruit cracking of tomato grown in net-covered greenhouses in Central Thailand can partly be corrected by calcium and boron sprays. *J. Plant Nutr. Soil Sci.* **172**, 140–150.
- Liu, X.L., Liu, L., Niu, Q.K., Xia, C., Yang, K.Z., Li, R., Chen, L.Q., Zhang, X.Q., Zhou, Y. and Ye, D. (2011) Male gametophyte defective 4 encodes a rhamnogalacturonan II xylosyltransferase and is important for growth of pollen tubes and roots in Arabidopsis. *Plant J.* **65**, 647-660.
- Loomis, W.D. and Durst, R.W. (1992) Chemistry and biology of boron. *Biofactors*, **3**, 229-239.
- Malinovsky, F.G., Fangel, J.U. and Willats, W.G. (2014) The role of the cell wall in plant immunity. *Front. Plant Sci.* **5**, 178.
- Marry, M., Roberts, K., Jopson, S.J., Huxham, I.M., Jarvis, M.C., Corsar, J., Robertson, E. and McCann, M.C. (2006) Cell-cell adhesion in fresh sugar-beet root parenchyma requires both pectin esters and calcium cross-links. *Physiol. Plant.* **126**, 243-256.
- Matcho, T., Kawaguchi, S. and Kobayashi, M. (1996) Ubiquity of a Borate-Rhamnogalacturonan II Complex in the Cell Walls of Higher Plants. *Plant Cell Physiol.* **37**, 636-640.
- Matsunaga, T., Ishii, T., Matsumoto, S., Higuchi, M., Darvill, A., Albersheim, P. and O'Neill, M.A. (2004) Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes. Implications for the evolution of vascular plants. *Plant Physiol.* **134**, 339-351.
- Matthes, M. and Torres-Ruiz, R.A. (2016) Boronic acids induce monopteros phenocopies by affecting PIN1 membrane stability and polar auxin transport in Arabidopsis thaliana embryos. *Development*, **143**, 4053.
- Mohnen, D. (2008) Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* **11**, 266-277.
- Molhoj, M., Verma, R. and Reiter, W.D. (2003) The biosynthesis of the branched-chain sugar d-apiose in plants: functional cloning and characterization of a UDP-d-apiose/UDP-d-xylose synthase from Arabidopsis. *Plant J.* **35**, 693-703.
- Ndeh, D., Rogowski, A., Cartmell, A., Luis, A.S., Basle, A., Gray, J., Venditto, I., Briggs, J., Zhang, X., Labourel, A., Terrapon, N., Buffetto, F., Nepogodiev, S., Xiao, Y., Field, R.A., Zhu, Y., O'Neil, M.A., Urbanowicz, B.R., York, W.S., Davies, G.J., Abbott, D.W., Ralet, M.C., Martens, E.C., Henrissat, B. and Gilbert, H.J. (2017) Complex pectin metabolism by gut bacteria reveals novel catalytic functions. *Nature*, **544**, 65-70.
- O'Neill, M.A., Eberhard, S., Albersheim, P. and Darvill, A.G. (2001) Requirement of borate cross-linking of cell wall

-
- rhamnogalacturonan II for Arabidopsis growth. *Science*, **294**, 846-849.
- Pabst, M., Fischl, R.M., Brecker, L., Morelle, W., Fauland, A., Kofeler, H., Altmann, F. and Leonard, R.** (2013) Rhamnogalacturonan II structure shows variation in the side chains monosaccharide composition and methylation status within and across different plant species. *Plant J.* **76**, 61-72.
- Peaucelle, A., Braybrook, S.A., Le Guillou, L., Bron, E., Kuhlemeier, C. and Hofte, H.** (2011) Pectin-induced changes in cell wall mechanics underlie organ initiation in Arabidopsis. *Curr. Biol.* **21**, 1720-1726.
- Peaucelle, A., Louvet, R., Johansen, J.N., Höfte, H., Laufs, P., Pelloux, J. and Mouille, G.** (2008) Arabidopsis Phyllotaxis Is Controlled by the Methyl-Esterification Status of Cell-Wall Pectins. *Curr. Biol.* **18**, 1943-1948.
- Power, P.P. and Woods, W.G.** (1997) The chemistry of boron and its speciation in plants. *Plant Soil*, **193**, 1-13.
- Rautengarten, C., Ebert, B., Liu, L., Stonebloom, S., Smith-Moritz, A.M., Pauly, M., Orellana, A., Scheller, H.V. and Heazlewood, J.L.** (2016) The Arabidopsis Golgi-localized GDP-L-fucose transporter is required for plant development. *Nat. Commun.* **7**, 12119.
- Rautengarten, C., Ebert, B., Moreno, I., Temple, H., Herter, T., Link, B., Donas-Cofre, D., Moreno, A., Saez-Aguayo, S., Blanco, F., Mortimer, J.C., Schultink, A., Reiter, W.D., Dupree, P., Pauly, M., Heazlewood, J.L., Scheller, H.V. and Orellana, A.** (2014) The Golgi localized bifunctional UDP-rhamnose/UDP-galactose transporter family of Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **111**, 11563-11568.
- Reiter, W.D., Chapple, C.C. and Somerville, C.R.** (1993) Altered growth and cell walls in a fucose-deficient mutant of Arabidopsis. *Science*, **261**, 1032-1035.
- Rodriguez-Enriquez, M.J., Mehdi, S., Dickinson, H.G. and Grant-Downton, R.T.** (2013) A novel method for efficient in vitro germination and tube growth of Arabidopsis thaliana pollen. *New Phytol.* **197**, 668-679.
- Ryden, P., Sugimoto-Shirasu, K., Smith, A.C., Findlay, K., Reiter, W.D. and McCann, M.C.** (2003) Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. *Plant physiol.* **132**, 1033-1040.
- Schmittgen, T.D. and Livak, K.J.** (2008) Analyzing real-time PCR data by the comparative C-T method. *Nat. Protoc.* **3**, 1101-1108.
- Sparkes, I.A., Runions, J., Kearns, A. and Hawes, C.** (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* **1**, 2019-2025.
- Sutinen, S., Aphalo, P.J. and Lehto, T.** (2007) Does timing of boron application affect needle and bud structure in Scots pine and Norway spruce seedlings? *Trees*, **21**, 661-670.
- Sutinen, S., Vuorinen, M. and Rikala, R.** (2006) Developmental disorders in buds and needles of mature Norway spruce, *Picea abies* (L.) Karst., in relation to needle boron concentrations. *Trees*, **20**, 559-570.
- Suzuki, S., Li, L.G., Sun, Y.H. and Chiang, V.L.** (2006) The cellulose synthase gene superfamily and biochemical functions of xylem-specific cellulose synthase-like genes in *Populus trichocarpa*. *Plant Physiol.* **142**, 1233-1245.
- Vanneste, S., De Rybel, B., Beemster, G.T., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Grussem,**

- W., Tasaka, M., Inze, D., Fukaki, H. and Beeckman, T. (2005) Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in *Arabidopsis thaliana*. *Plant Cell*, **17**, 3035-3050.
- Voxeur, A., Soubigou-Taconnat, L., Legee, F., Sakai, K., Antelme, S., Durand-Tardif, M., Lapierre, C. and Sibout, R. (2017) Altered lignification in *mur1-1* a mutant deficient in GDP-L-fucose synthesis with reduced RG-II cross linking. *PLoS One*, **12**, e0184820.
- Yang, H., Benatti, M.R., Karve, R.A., Fox, A., Meilan, R., Carpita, N.C. and McCann, M.C. (2020) Rhamnogalacturonan-I is a determinant of cell-cell adhesion in poplar wood. *Plant Biotechnol J.* **18**, 1027-1040.
- Yang, W., Schuster, C., Beahan, C.T., Charoensawan, V., Peaucelle, A., Bacic, A., Doblin, M.S., Wightman, R. and Meyerowitz, E.M. (2016) Regulation of Meristem Morphogenesis by Cell Wall Synthases in *Arabidopsis*. *Curr. Biol.* **26**, 1404-1415.
- Zhou, Y., Awano, T., Kobayashi, M., Matoh, T. and Takabe, K. (2017) Immunocytochemical detection of rhamnogalacturonan II on forming cell plates in cultured tobacco cells. *Biosci. Biotech. Bioch.* **81**, 899-905.
- Zhuang, X., Wang, H., Lam, S.K., Gao, C., Wang, X., Cai, Y. and Jiang, L. (2013) A BAR-domain protein SH3P2, which binds to phosphatidylinositol 3-phosphate and ATG8, regulates autophagosome formation in *Arabidopsis*. *Plant Cell*, **25**, 4596-4615.

Figure legends

Figure 1. The complete suppression of *AXS1* and *AXS2* gene expression leads to plant death.

(A) and (B) Phenotype of *AXS2* RNAi knockdown plants and corresponding transcription levels confirmed by RT-PCR analysis. The *axs1-1* single mutant and *dex:RNAi-axs2* with *axs1-1* background plants were grown on MS plates containing DEX for 4 days. *Act2* transcripts are used as loading controls. Bars = 1 cm. (C) and (D) Downregulation of *AXS2* causes ceased growth and death. *axs1-1* single mutant and *dex:RNAi-axs2* with *axs1-1* background plants were grown on MS plates for 4 days and followed with/without DEX treatment for another 5 days. The corresponding transcription levels were confirmed by RT-PCR analysis. *Act2* transcripts are used as loading controls.

Figure 2. *AXS1* and *AXS2* loss of function results in aberrant pollen grain morphology and germination.

(A) The siliques of Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* plants. Arrowheads indicate unnatural intervals. (B) The aborted ovules (arrowheads) and aborted seed (arrow) of *axs1-1 axs2-1/+* mutant silique. (C) to (H) SEMs of pollen grains from Col-0 (C and F), *axs1-1 axs2-1/+* (D, G and H) and *axs1-2 axs2-2/+* (E) plants. (G) and (H) indicate the normal and collapsed pollen form *axs1-1 axs2-1/+* mutant. Arrows indicate normal pollens and arrowheads indicate collapsed pollens. Bars = 50 μ m (C) to (E), 10 μ m (F) to (H). (I) Pollen tubes of wild type Col-0 and *axs1-1 axs2-1/+* mutant. Bars = 100 μ m. (J) and (K) Average pollen germination and pollen tube length from wild type Col-0 and *axs1-2 axs2-2/+* mutant plants. Error bars indicate SD. (J) $n \geq 200$, (K) $n \geq 100$, ** $P < 0.01$, t test.

Figure 3. The phenotypes of *axs* double mutants.

(A) 5-week-old rosette leaves of Col-0, *mur1*, *axs1-1/+ axs2-1*, *axs1-1 axs2-1/+*, *axs1-2/+ axs2-2* and *axs1-2 axs2-2/+* plants. The *axs1-1/+ axs2-1* and *axs1-2/+ axs2-2* mutants were ~5 mm in size and died after about one more week. Bar = 2 cm. (B) The 3-week-old Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* plants grown on MS medium. The *axs1 axs2/+* mutants produce more than one shoot. Arrows indicate shoots. Bar = 1 cm. (C) The longitudinal sections of 2-week-old Col-0, *axs1-1 axs2-1/+* plants. Arrows indicate shoots. Bar = 0.5 mm. (D) To (F) 6-week-old (D), 7-week-old (E) and 8-week-old (F) *axs1-1axs2-1/+* mutants exhibiting a bushy stature. Bars = 2 cm. (G) 8-week-old Col-0, *mur1*, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* plants. Bars = 2 cm. (H) 7-week-old *axs1-1 axs2-1/+* mutant plants. Bar = 2 cm. (I) The wound on the stem of the 7-week-old *axs1-1 axs2-1/+* mutant plants. Bar = 1 mm. (J) to (L) Sections of the up (J), middle (K) and lower (L) sites of the stem of the 7-week-old *axs1-1 axs2-1/+* mutant plants. Bar = 100 μ m.

Figure 4. Roots of *axs1 axs2/+* mutants are abnormal.

(A) 12 dpg seedlings of Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+*. Bars = 5 mm. (B) and (C) Root length (B) and lateral root number (C) of 12 dpg Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* seedlings. Error bars indicate SD. $n = 26$, ** $P < 0.01$, t test. (D) Roots of 4 dpg Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* seedlings. The first root hairs are indicated with arrows. Bar = 1 mm. (E) and (F) Distance between root tip and first root hair (E) and root hair density (F) of Col-0,

axs1-1 axs2-1/+ and *axs1-2 axs2-2/+* seedlings. Error bars indicate SD. $n \geq 6$, $**P < 0.01$, t test. (G) Root tips of 4 dpg Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* seedlings. Arrows indicate the start site of the elongation zone (Cell length > Cell width). Arrowheads indicate the quiescent centers (QC). Bar = 0.1 mm. (H) and (I) Meristem size and meristematic cortex cells of Col-0, *axs1-1 axs2-1/+* and *axs1-2 axs2-2/+* seedlings. Error bars indicate SD. $n \geq 6$, $**P < 0.01$, t test. (J) Root hair zones of 4 dpg Col-0 and *axs1-1 axs2-1/+* seedlings. Bar = 0.1 mm. (K) Representative SEM image showing cell wall of root hair zones of 4 dpg Col and *axs1-1 axs2-1/+* seedlings. Bar = 2 μ m.

Figure 5. The *axs1 axs2/+* mutants show reduced UDP-API, API, B and RG-II content.

(A) UDP-API content from 3-week-old plant leaves. Error bars indicate SD. $n = 6$, $**P < 0.01$, t test. (B) API content of AIR extracts from 3-week-old plant leaves. Error bars indicate SD. $n = 6$, $**P < 0.01$, t test. (C) B content of AIR extracts from 3-week-old leaves. Data are % of total dry weight. Error bars indicate SD. $n = 6$, $**P < 0.01$, t test. (D) Characterization of RG-II preparations using PAGE. AIR extracts from 3-week-old leaves digested with endo-polygalacturonase. Samples were loaded as serial dilutions. RG-II was visualized by silver staining. The *mur1* mutant was used as control. Dimeric RG-II (**), monomeric RG-II (*). (E) Characterization of RG-II preparations by SEC. The *mur1* mutant was used as a control. The peaks corresponding to eluting RGI, RG-II dimer and monomer, and oligogalacturonides (OGAs) are labeled.

Figure 6. Microarray analysis of the *axs1-1 axs2-1/+* double mutant.

(A) and (B) Enrichment of selected categories of GO biological process and GO cellular component in genes up-regulated and down-regulated. The P values calculated based on their relative abundance in the wild-type Col-0 genome were labeled on the right. For a complete list of GO terms, see Supplemental Data Sets online. (C) Overlap between genes in the microarray we conducted and of Vanneste *et al.* (2005). (D) qRT-PCR verification of microarray results of the cell wall related genes. RNA was prepared from the roots of 12 dpg seedlings of *axs1-1 axs2-1/+* mutant and Col-0. Error bars indicate SD ($n=3$).

Tables

Table 1. Genetic analysis of *axs1-1 axs2-1/+* gametophytes.

Classes	Observed(<i>o</i>)	Expected(<i>e</i>)	Deviation <i>d(o-e)</i>	<i>d</i> ²	<i>X</i> ² (<i>d</i> ² / <i>e</i>)
Back cross of ♀ <i>axs1-1 axs2-1/+</i> × ♂Col-0 ^a					
<i>axs1-1/+ axs2-1/+</i>	15	16	-1	1	0.0625
<i>axs1-1/+ AXS2</i>	17	16	1	1	0.0625
Back cross of ♀Col-0 × ♂ <i>axs1-1 axs2-1/+</i> ^b					
<i>axs1-1/+ axs2-1/+</i>	0	14	-14	196	14
<i>axs1-1/+ AXS2</i>	28	14	14	196	14

^a*n*=32, *X*²=0.125, no significance. ^b*n*=28, *X*²=28, *P*<0.001

Reciprocal crosses of *axs1-1 axs2-1/+* and the Col-0 parent were generated, and the progenies were PCR genotyped. PCR score was subjected to a *X*² test. *n*, sample number; *P*, statistical significance.

Table 2. Nucleotide sugars analysis of *axs* double mutants. The amounts are presented as mol % ± SD of three biological replicates. For all data, values significantly different from the Col-0 plants are marked with asterisks (**P*<0.05, ***P*<0.01, *t* test).

	UDP-Glc	UDP-GlcA	UDP-GalA	UDP-Rha	UDP-Gal	UDP-Xyl	UDP-Arap	GDP-Man	UDP-Araf	GDP-Fuc	UDP-Api
Col-0	65.2±2.5	3.8±0.3	2.2±0.1	4.4±0.6	15.0±1.3	4.0±0.2	2.7±0.1	1.8±0.5	0.38±0.09	0.45±0.08	0.12±0.02
<i>axs1-1 axs2-1/+</i>	64.9±2.0	4.6±0.6	2.4±0.2	4.3±0.4	13.5±1.1	4.2±0.4	2.8±0.3	2.4±0.4	0.40±0.04	0.53±0.04	0.02±0.01
<i>axs1-2 axs2-2/+</i>	65.3±0.8	4.5±0.4	2.3±0.3	4.6±0.3	13.1±0.7	4.1±0.3	2.8±0.2	2.4±0.4	0.35±0.03	0.54±0.05	0.02±0.01

Table 3. Cell wall analysis as AIR of *axs* double mutants. Monosaccharide composition of Col-0, *axs1-1 axs2-1/+*, *axs1-2 axs2-2/+*, *axs1/+ axs2* plants were analyzed by HPAEC PAD. The shoots of 3-week-old plants were pooled and *n*=6 technical replicates were analyzed. The amounts are presented as mol % ± SD of three biological replicates. For all data, values significantly different from the Col-0 plants are marked with asterisks (**P*<0.05, ***P*<0.01, *t* test).

	Fucose	Rhamnose	Arabinose	Galactose	Xylose	GalA	GlcA
Col-0	1.8±0.1	9.8±0.3	14.9±0.1	19.7±0.2	11.9±0.3	36.3±0.6	5.6±0.3
<i>axs1-1 axs2-1/+</i>	1.7±0.0	8.2±0.1**	17.2±0.2	20.1±0.1	13.5±0.1	34.0±0.1**	5.4±0.2
<i>axs1-2 axs2-2/+</i>	1.8±0.0	8.3±0.2**	18.3±0.2	20.3±0.1	13.3±0.1	33.2±0.2**	5.0±0.2**
<i>axs1/+ axs2</i>	1.5±0.0	5.0±0.0**	20.0±0.1	23.5±0.1	20.8±0.1	28.8±0.3**	0.9±0.3**

757

tpj_14921_f1.tif

tpj_14921_f2.tif

tpj_14921_f3.tif

tpj_14921_f4.tif

tpj_14921_f5.tif

tpj_14921_f6.tif

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Zhao, X;Ebert, B;Zhang, B;Liu, H;Zhang, Y;Zeng, W;Rautengarten, C;Li, H;Chen, X;Bacic, A;Wang, G;Men, S;Zhou, Y;Heazlewood, JL;Wu, A-M

Title:

UDP-Api/UDP-Xyl synthases affect plant development by controlling the content of UDP-Api to regulate the RG-II-borate complex

Date:

2020-09

Citation:

Zhao, X., Ebert, B., Zhang, B., Liu, H., Zhang, Y., Zeng, W., Rautengarten, C., Li, H., Chen, X., Bacic, A., Wang, G., Men, S., Zhou, Y., Heazlewood, J. L. & Wu, A. -M. (2020). UDP-Api/UDP-Xyl synthases affect plant development by controlling the content of UDP-Api to regulate the RG-II-borate complex. PLANT JOURNAL, 104 (1), pp.252-267. <https://doi.org/10.1111/tpj.14921>.

Persistent Link:

<http://hdl.handle.net/11343/276010>