

RESEARCH PAPER

# Mutation in xyloglucan 6-xylosyltransferase results in abnormal root hair development in *Oryza sativa*

Chuang Wang<sup>1,2,\*</sup>, Shuai Li<sup>1,\*</sup>, Sophia Ng<sup>2,3,\*</sup>, Baocai Zhang<sup>4</sup>, Yihua Zhou<sup>4</sup>, James Whelan<sup>2,5</sup>, Ping Wu<sup>1,2</sup> and Huixia Shou<sup>1,2,†</sup>

<sup>1</sup> State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou, 310058, P. R. China

<sup>2</sup> University of Western Australia-Zhejiang University Joint Research Laboratory in Genomics and Nutriomics, College of Life Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou, 310058, P. R. China

<sup>3</sup> ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, 35 Stirling Highway, Crawley, 6009 Western Australia, Australia

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

<sup>5</sup> Department of Botany, School of Life Science, Australian Research Council Centre of Excellence in Plant Energy Biology, La Trobe University, Bundara, Victoria 3086, Australia

\* These authors contributed equally to this work.

† To whom correspondence should be addressed. E-mail: [huixia@zju.edu.cn](mailto:huixia@zju.edu.cn)

Received 29 December 2013; Revised 1 April 2014; Accepted 2 April 2014

## Abstract

Root hairs are important for nutrient uptake, anchorage, and plant–microbe interactions. From a population of rice (*Oryza sativa*) mutagenized by ethyl methanesulfonate (EMS), a *short root hair2* (*srh2*) mutant was identified. In hydroponic culture, *srh2* seedlings were significantly reduced in root hair length. Bubble-like extrusions and irregular epidermal cells were observed at the tips of *srh2* root hairs when grown under acidic conditions, suggesting the possible reduction of the tensile strength of the cell wall in this mutant. Map-based cloning identified a mutation in the gene encoding xyloglucan (XyG) 6-xylosyltransferase (*OsXXT1*). *OsXXT1* displays more than 70% amino acid sequence identity with the previously characterized *Arabidopsis thaliana* XYG XYLOSYL TRANSFERASE 1 (*AtXXT1*) and XYG XYLOSYL TRANSFERASE 2 (*AtXXT2*), which catalyse the transfer of xylose onto  $\beta$ -1,4-glucan chains. Furthermore, expression of the full-length coding sequence of *OsXXT1* could complement the root hair defect, and slow growth and XyG synthesis in the *Arabidopsis xxt1 xxt2* double mutant. Transgenic plants expressing the  $\beta$ -glucuronidase (GUS) reporter under the control of the *OsXXT1* promoter displayed GUS expression in multiple tissues, most prominently in root epidermal cells. These results demonstrate the importance of *OsXXT1* in maintaining cell wall structure and tensile strength in rice, a typical grass species that contains relatively low XyG content in cell walls.

**Key words:** Rice, root hair, type I cell wall, type II cell wall, xyloglucan, xylosyltransferase.

## Introduction

Plant cell walls are composed of cellulose, hemicellulose, and pectin, in addition to a number of inorganic compounds. Based on the chemical structures, wall architecture, and cell

wall biosynthetic processes, primary cell walls of flowering plants are divided into two classes, type I and type II (Carpita and Gibeaut., 1993; Carpita, 1996; Carpita and McCann,

Abbreviations: dCAPS, derived cleaved amplified polymorphic sequence; GAX, glucuronoxarabinoxylans; GT34, glycosyltransferase family 34; GUS,  $\beta$ -glucuronidase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MLG, mixed linkage glucans; MS, Murashige and Skoog; *srh2*, short root hair 2; SSR, simple sequence repeat; Ubi-1, ubiquitin-1; XET/XTH, xyloglucan endotransglucosylase/hydrolase; XXT1, xyloglucan xylosyltransferase1; XyG, xyloglucan.

© The Author 2014. Published by Oxford University Press on behalf of the Society for Experimental Biology.

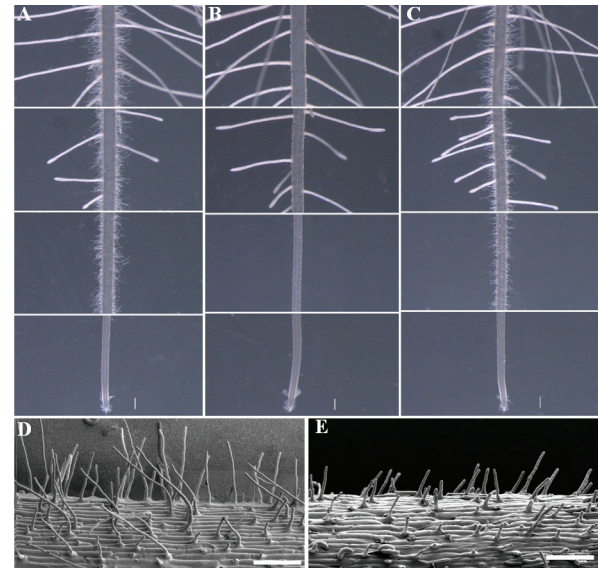
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

2000). Type I cell walls are typically found in dicots and non-commelinoid monocots. They are characterized by a cellulose–xyloglucan (XyG) network with high pectin and structural proteins content. Type II cell walls are found only in the commelinoid monocots (e.g. grasses, rushes, and gingers) and are composed of cellulose fibres encased in glucuronoarabinoxylans (GAX), high levels of hydroxycinnamates, and very low levels of pectin and structural proteins. Additionally, cell walls of grasses and some related families contain significant amounts of mixed linkage glucans (Carpita, 1996; Carpita and McCann, 2000; Vogel, 2008).

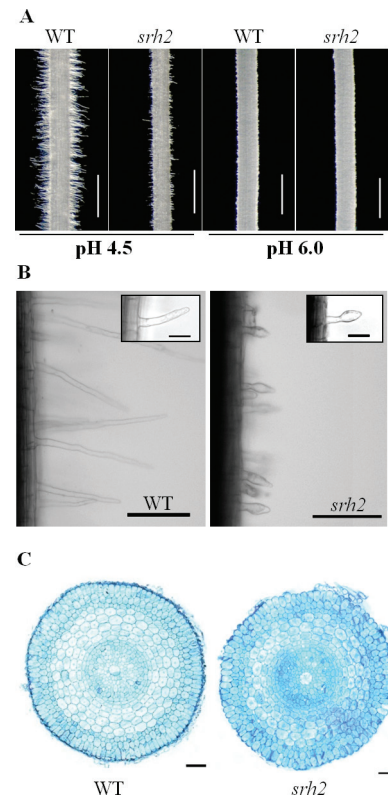
XyG is the most abundant hemicellulosic polysaccharide in type I cell walls, which comprises ~20–25% dry mass of cell walls (Obel *et al.*, 2007; Vogel, 2008). XyG consists of a  $\beta$ -1,4-glucan backbone with  $\alpha$ -D-xylose substitution on the oxygen-6 position in a regular pattern. There are two general types of XyG: the poly-XXXG and poly-XXGG; approximately 75% and 50% of their backbone residues are branched, respectively (Vincken *et al.*, 1997). These xylosyl residues can be further substituted at the oxygen-2 position with either a single  $\beta$ -D-galactose or an  $\alpha$ -L-fucose-(1,2)- $\beta$ -D-galactose dimer, depending on the plant species (Obel *et al.*, 2007). The substructure of XyG has been investigated in detail using  $\beta$ -1,4-endoglucanase to digest the non-substituted glucose units in the polymer (Hoffman *et al.*, 2005). Hydrolysed *Arabidopsis* XyG released XXXG, XLXG, XXFG, XLLG, and XLFG subunits (see Figure 1 and 2 in Obel *et al.* (2007) for a description of XyG nomenclature). In contrast to the observations made in *Arabidopsis*, the hemicelluloses of primary cell walls in graminaceous monocots are mainly comprised of xylans and mixed linkage glucans. Only ~1–5% of XyG is found in cereals or grasses with a low degree of substitution (Carpita, 1996; Hoffman *et al.*, 2005; Sims *et al.*, 2000).

Although the cell wall composition differs between type I and type II cell walls, many cell wall-related genes are conserved between species with both types, presumably to maintain the basic structure of cell walls (Penning *et al.*, 2009; Yokoyama and Nishitani, 2004). The  $\beta$ -glucan backbone of XyG is synthesized by a cellulose synthase-like C family protein, named CSLC4 in *Nasturtium* and *Arabidopsis* (Cocuron *et al.*, 2007). AtXXT1, AtXXT2, and AtXXT5 are involved in linking xylose to the  $\beta$ -glucan backbone at different positions (Cavalier and Keegstra, 2006; Cavalier *et al.*, 2008; Faik *et al.*, 2002; Vuttipongchaikij *et al.*, 2012; Zabolina *et al.*, 2008; Zabolina *et al.*, 2012). The galactosyltransferase MUR3 and fucosyltransferase MUR2 further catalyse the UDP–galactose or UDP–fucose to the side chain of a XyG oligosaccharide block (Madson *et al.*, 2003; Vanzin *et al.*, 2002). Plants with mutations affecting XyG structure or content exhibited collapsed trichome papillae or abnormal root hairs (Cavalier *et al.*, 2008; Madson *et al.*, 2003; Pena *et al.*, 2012; Vanzin *et al.*, 2002; Zabolina *et al.*, 2008), and *Arabidopsis* XyG mutants had reduced tensile strength in primary cell walls (Cavalier *et al.*, 2008; Park and Cosgrove, 2012a; Pena *et al.*, 2004).

Grass cell walls are a major source of dietary fibre for animals and a significant source of renewable energy (Ragauskas *et al.*, 2006). Cell walls in grass species contain significant



**Fig. 1.** Phenotype of root hairs from wild type (WT, cv Kasalath), *srh2*, and *srh2* complemented by *Ubiquitin-1<sub>promoter</sub>::OsXXT1*. (A and D) root hairs from the WT; (B and E) root hairs from *srh2*; (C) root hairs from transgenic seedling of *srh2* overexpressing *OsXXT1*. Seedlings of A, B, and C were grown under the same pots with nutrient solution for seven days. Bar=1 cm. Seedlings of D and E were grown for three days on Murashige and Skoog medium (pH 5.5) and examined under an electron microscope examination. Bar=200  $\mu$ m.



**Fig. 2.** Root hair morphology of rice seedlings grown in different media with various pH. (A) Root hairs of three-day-old seedlings of wild-type (WT) and *srh2* grow on Murashige and Skoog medium at pH 4.5 and 6.0. Bar=1 mm. (B) The root hairs of the WT and *srh2* examined under a microscope (pH 4.5). Bar=100  $\mu$ m. (C) Cross section of root tip from WT and *srh2* seedlings grown at pH 4.5. The section was stained with toluidine blue (TBO). Bar=20  $\mu$ m. (This figure is available in colour at JXB online.)

amounts of GAX and mixed linkage glucans, but relatively low amounts of XyG. It is commonly accepted that XyG is a less important component in type II cell walls compared with type I cell walls (Vogel, 2008). The function and genetic controls of synthesis of XyG is unknown in type II cell walls.

In this study, we isolated and characterized the rice *short root hair2* (*srh2*) mutant and provide evidence for the importance of XyG in cell wall structure and root hair tip growth in a grass species.

## Materials and methods

### Plant materials and growth conditions

The rice *srh2* mutant was identified in an EMS-mutagenized population from the rice cultivar Kasalath. For all experiments, the *srh2* mutant and wild-type seeds were germinated in distilled water for two days. The seedlings were then grown in a hydroponic solution (Yoshida *et al.*, 1976). Seedlings were grown in a growth chamber at 30 °C/22 °C day/night temperatures with a 12h light/12h dark regime (450 μmol photons m<sup>-2</sup>s<sup>-1</sup>). For the pH treatment experiment, seeds were surface sterilized with 95% (v/v) ethanol for 2 min and 15% (v/v) bleach for 20 min. After rinsing in distilled water, seeds were germinated and grown in test tubes (15 cm×3 cm) containing Murashige and Skoog medium, 0.059% (w/v) 2-(*N*-morpholino) ethanesulfonic acid (MES), 1% (w/v) sucrose and 0.3% (w/v) phytigel (Sigma, US). The basic medium contained 2.0 mM NH<sub>4</sub>NO<sub>3</sub>, 1.9 mM KNO<sub>3</sub>, 0.3 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.15 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 μM KI, 25 μM H<sub>3</sub>BO<sub>3</sub>, 0.1 mM MnSO<sub>4</sub>·H<sub>2</sub>O, 0.3 mM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1 μM Na<sub>2</sub>MO<sub>4</sub>·2H<sub>2</sub>O, 0.1 μM CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.1 μM CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.1 mM FeSO<sub>4</sub>·7H<sub>2</sub>O, and 0.1 mM Na<sub>2</sub>EDTA·2H<sub>2</sub>O.

### Map-based cloning and genetic complementation

An F<sub>2</sub> mapping population was generated from crosses between homozygous *srh2* mutant plants and the Japonica cultivar Nipponbare. The *SRH2* gene was mapped to chromosome 3 between simple sequence repeat (SSR) markers RM232 and RM3280 using 1800 F<sub>2</sub> mutant plants. SSR markers were obtained from NCBI database (<http://www.ncbi.nlm.nih.gov/unists>). The mutation was further mapped to a 36-kb region between STS274-04 and STS274-04-06 using nine newly developed SSR markers. Based on the phenotype of *srh2*, the *OsXXT1* gene was selected as a candidate gene. A derived cleaved amplified polymorphic sequences (dCAPS) marker was developed using the dCAPS finder 2.0 program (<http://helix.wustl.edu/dcaps/dcaps.html>) to further confirm the mapping result. The genes were amplified by PCR from genomic DNA isolated from *srh2* and wild-type plants and sequenced to identify the mutation in the genomic sequence.

For complementation, the full-length open reading frame of *OsXXT1* was amplified by reverse transcription PCR and inserted into the modified binary vector pTF101-ubi (Zheng *et al.*, 2010) between the maize *Ubiquitin-1* promoter and a nopaline synthase terminator. The resulting transformation plasmid, pXXT1-Oe, was used for the *Agrobacterium*-mediated rice transformation of *srh2* mutant as described (Nishimura *et al.*, 2006).

For the complementation of *Arabidopsis xxt1 xxt2* double mutant, the full length coding sequence of *OsXXT1* was PCR amplified and cloned into the binary vector (pH7WG2D). The expression of *OsXXT1* was driven by the constitutive 35S CaMV promoter. This binary vector was then transformed into *Agrobacterium tumefaciens* GV3130 strain. Floral dipping was performed with an inoculum medium containing 10% (w/v) sucrose and 0.05% (v/v) Silwet-77 (Clough, 1998). T1 transformants were screened on hygromycin (50 mg l<sup>-1</sup>) (Harrison, 2006). Successful transformants, were age matched and Columbia-0 and *Arabidopsis xxt1 xxt2* double mutant plants were used for imaging using a Nikon Eclipse 80i Microscope (Nikon, Japan).

### Microscopic analysis

The root hairs were examined by a Leica MZ95 stereomicroscope with a colour CCD camera (Leica Instrument, Nussloch, Germany). For cryo-scanning electron microscopy seeds were plated on Murashige and Skoog medium and grown for three days (pH 5.7). Root samples were placed on moist nitrocellulose paper mounted on a stub and immersed in liquid nitrogen slush, then transported under vacuum to a cryo preparation chamber. Ice was sublimed at -90 °C and the specimens were sputter-coated with gold and observed using a Hitachi S-3000N scanning electron microscope (Hitachi, Naka, Japan) with a Gatan Alto 2100 cryo preparation system (Gatan UK, Abingdon, UK).

For light microscopic analysis, root tips were fixed overnight in 2.5% (v/v) glutaraldehyde with 0.1 M sodium phosphate buffer (pH 7.2) and washed three times for 30 min in the same buffer. Root samples were then re-fixed for 4 h in 1% (v/v) OsO<sub>4</sub> with 0.1 M sodium phosphate buffer (pH 7.2) and washed for 30 min in the same buffer. The samples were dehydrated in a gradient ethanol and embedded in Spurr resin. Semi-thin sections (2 μm) were made using glass knives on a Power Tome XL (RMC-Boeckeler Instruments, Arizona, USA) microtome and stained in 0.1% (w/v) methylene blue for 3 min at 70 °C. The samples were rinsed with distilled water and visualized with a Zeiss Axiovert 200 microscope (Zeiss, Jena, Germany).

For GUS staining, tissues samples were fixed overnight in FAA (constituted of 5% (v/v) formalin, 5% (v/v) acetic acid, 75% (v/v) alcohol) and washed twice for 30 min in 70% (v/v) ethanol. The tissues were dehydrated in gradient acetone and embedded in Spurr resin. The sections and visualizations were carried out as described above.

### Sequence alignments and phylogenetic analysis

The putative XXT sequences for alignment were extracted from NCBI (<http://www.ncbi.nlm.nih.gov>). Multiple sequence alignment of XXT proteins was conducted using the ClustalX 1.83 program (Thompson *et al.*, 1997) with default multiple alignment parameters and viewed by GeneDoc 3.2. A phylogenetic tree of the gene family was constructed using the Neighbor-Joining method by MEGA5.

### Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of xyloglucan oligosaccharides

Cell wall was extracted from leaves that were ground into powder in liquid nitrogen. The homogenate was washed three times with hot 70% (v/v) ethanol and extracted with a mixture of chloroform and methanol (1:1). The pellet was suspended in acetone and air-dried overnight. The alcohol-insoluble residues (AIRs) were de-starched with α-amylase (*Bacillus* sp). The XyG enriched KOH-soluble fraction was prepared by neutralizing 50 mg of de-starched AIRs in 4 M KOH solution, samples were then dialysed and finally lyophilized. Then, 0.5 mg of AIRs or KOH fraction was incubated in 100 ml of 50 mM ammonium formate, pH 5.0, with one unit of xyloglucanase (EXEGP; Megazyme) for 18 h at 37 °C. The supernatants were recovered, and 1 ml of aqueous sample plus 10 ng xylopentaose was spotted with an equal volume of matrix solution (10 mg ml<sup>-1</sup> 2,5-dihydroxybenzoic acid). After being dried on the MALDI target plate, spectra were analysed on a Bruker Autoflex MALDI-TOF mass spectrometry instrument (Bruker) in the positive reflection mode with an acceleration voltage of 20 kV. The relative height of each generated oligosaccharide ion peak was counted to determine their relative abundance as described previously (Zhang *et al.*, 2012).

### Generation of *OsXXT1::GUS* transgenic lines

The 1.8-kb region upstream of the start codon of *OsXXT1* gene was amplified from the genomic DNA of Kasalath using primers shown in Table S1. The PCR product was then cloned into the



pBIGUS-plus vector, in which the original *GUS* gene in binary vector pBI101.3 was replaced by a GUS-Plus sequence from pCAMBIA1305.1. The resultant vector was introduced into Nipponbare rice using *Agrobacterium*-mediated rice transformation as described (Nishimura *et al.*, 2006).

#### *GUS* histochemical analysis

Histochemical GUS staining was performed as described in Jefferson *et al.* (1987). Plant tissues from GUS transgenic lines were immediately submerged in GUS staining solution after harvest and placed under a vacuum for 10 min. The samples were incubated overnight in darkness at 37 °C. Chlorophyll was removed by submerging the stained tissues in 70% (*v/v*) ethanol. Plant material was placed on glass slides using 20% chloral (*w/v*) in 25% glycerol (*v/v*) for 10 min. GUS staining was visualized using a Leica MZ95 stereomicroscope with a colour CCD camera (Leica Instrument, Nussloch, Germany). The GUS staining solution contained 100 mM sodium phosphate buffer (pH 7.0), 10 mM Na<sub>2</sub>EDTA, 1 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], 1 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 0.5% (*v/v*) TritonX-100, 20% (*v/v*) methanol, and 0.5 mg ml<sup>-1</sup> 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc).

#### Expression analysis

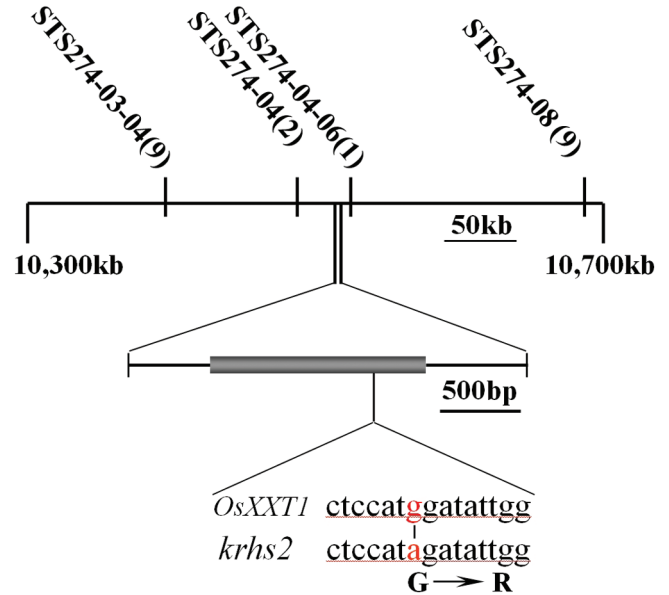
Publicly available Affymetrix *Arabidopsis* and rice microarray CEL files were downloaded from the Gene Expression Omnibus within the National Centre for Biotechnology Information database or from the MIAME ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress/>). The CEL files were imported and quantile normalized together using Partek Genomics Suite version 6.5 (St. Louis, Missouri, USA) as carried out in previous studies (Narsai *et al.*, 2011). The accession numbers for the *Arabidopsis* studies were GSE30223 and E-AFMX-9, and for rice several were combined, including E-MEXP-1766, E-MEXP-2267, GSE6908, GSE11966, GSE7951, and GSE6893.

## Results

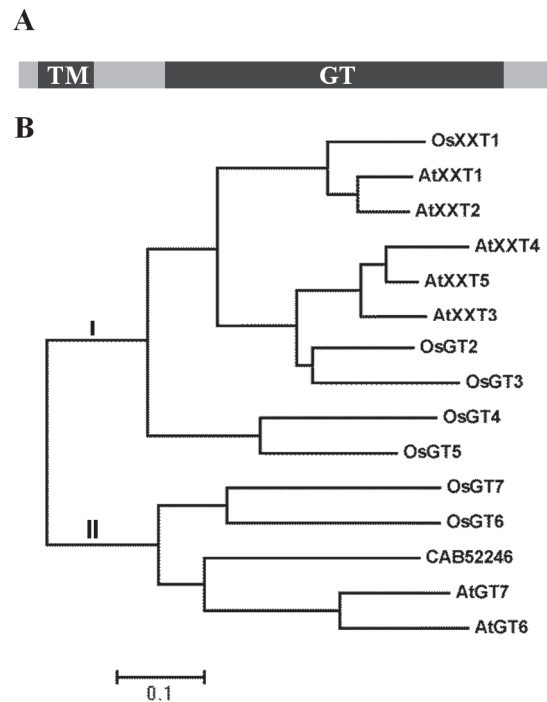
### Isolation of the *srh2* mutant

Seeds from the M<sub>2</sub> generation of an EMS-mutagenized population of Indica cultivar Kasalath, were germinated and grown in nutrient solution to screen for mutants with abnormal root hair phenotype. Seven days after germination, a mutant with significantly reduced length in root hairs was identified (Fig. 1A, B). No obvious difference was observed in leaf or root growth between wild type and mutant (Fig. S1). The mutant was designated as *srh2*. Scanning electron microscopy of the wild-type and *srh2* root surface showed that the length of the root hairs of *srh2* were shorter than those of wild type. However, no significant difference was found in root hair density or distribution pattern between *srh2* and wild type (Fig. 1D, E).

It is reported that extracellular pH can regulate root hair growth by modification to cell wall rigidity (Monshausen *et al.*, 2007). To investigate whether the root hair growth of *srh2* is affected by extracellular pH, wild-type and mutant seeds were germinated on Murashige and Skoog medium under pH 4.5 and 6.0. The medium pH was stabilised by MES. At pH 6.0, root hair growth in both wild type and the *srh2* mutant was significantly inhibited (Fig. 2A). The acidic conditions (pH 4.5) induced root hair growth in wild-type seedlings, but not in the



**Fig. 3.** Molecular identification of *srh2* by positional cloning. Physical map of the chromosomal region encompassing the *srh2* gene was defined by high-resolution mapping. *srh2* was mapped between the simple sequence repeat (SSR) markers STS274-04 and STS274-04-06 with the number of recombinants given in parentheses. (This figure is available in colour at JXB online.)



**Fig. 4.** Schematic diagram of protein domain structure and phylogenetic analysis of OsXXT1. (A) Predicted schematic of OsXXT1 protein domain structure. TM, predicted transmembrane domain; GT, predicted glycosyltransferase domain. (B) Neighbor-joining phylogenetic tree of putative xylosyltransferases in *Arabidopsis* and rice using MEGA5 program. CAB52246 is accession number of fenugreek α-(1,6) galactosyltransferase. The gene locus of rice GT34 genes are: *OsXXT1*, LOC\_Os03g18820; *OsGT2*, LOC\_Os02g32750; *OsGT3*, LOC\_Os12g05380; *OsGT4*, LOC\_Os03g19310; *OsGT5*, LOC\_Os03g19330; *OsGT6*, LOC\_Os11g34390; *OsGT7*, LOC\_Os02g49140.

*srh2* mutant (Fig. 2A). Bubble-like extrusions were observed at the tip of the hairs in the *srh2* mutants grown under acidic conditions (Fig. 2B). Transverse section of the wild-type and mutant roots showed that the shape of epidermal cells in the *srh2* root meristem zone was irregular (Fig. 2C).

#### Gene cloning of the *srh2* mutant

Genetic analysis showed that a single recessive gene was responsible for the mutant phenotype. In 1800 mutant seedlings from the F<sub>2</sub> population derived from a cross between the mutant (from the Indica cultivar Kasalath) and the Japonica cultivar Nipponbare, the roots hairs of 1362 seedlings grew normally, whereas 438 seedlings showed short root hairs. Using this population, the mutation was mapped to a 36-kb region between SSR markers STS274-04 and STS274-04-06 on chromosome 3 (Fig. 3). This region contains seven open reading frames, including a putative *OsXXT1* gene (LOC\_Os03g18820). Altered XyG structure or content of cell walls in *Arabidopsis* caused collapsed trichome papillae or a short root hair phenotype (Cavalier *et al.*, 2008; Madson *et al.*, 2003; Vanzin *et al.*, 2002; Zobotina *et al.*, 2012). Therefore, the phenotype of *srh2* suggested that the *OsXXT1* gene is a tentative candidate gene for the mutation. To confirm this, the full-length cDNA sequence of the *OsXXT1* gene was amplified from both the wild-type and *srh2* mutant genomic DNA. Comparison of these two sequences revealed the presence of a single point mutation (G to A) at the nucleotide position 1009 bp from the start codon of *OsXXT1* (Fig. 3). The nucleotide substitution of the *srh2* mutant sequence resulted in an amino acid change of a glycine (G) into an arginine (R) (Fig. 3). To further verify the positional cloning of the mutant,

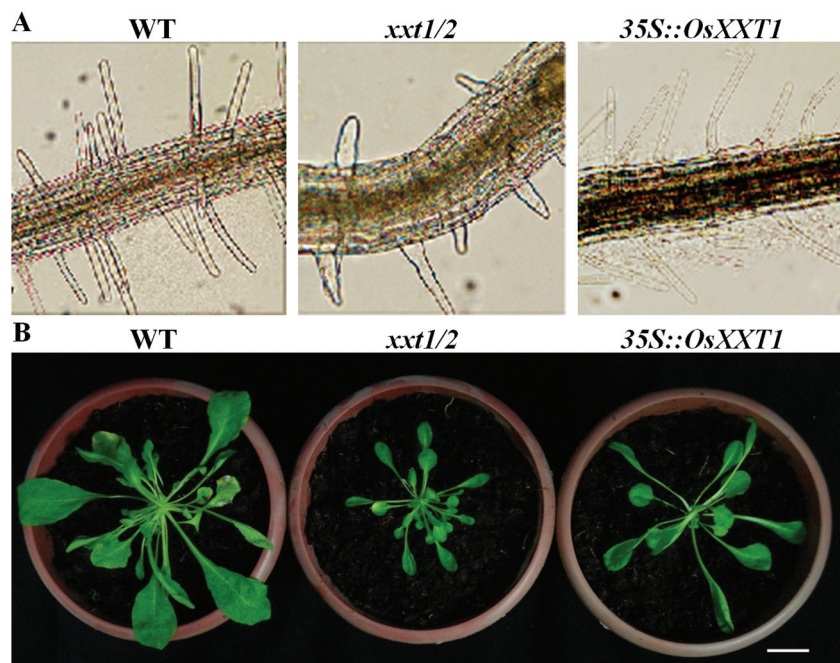
a dCAPS marker was developed using the *NcoI* restriction endonuclease (Fig. S2). The enzyme cuts the PCR product of the wild-type *OsXXT1* gene into four fragments: 456 bp, 73 bp, 244 bp, and 1210 bp. In contrast, the PCR product amplified from the *srh2* mutant produced only three fragments when digested with *NcoI*: 529 bp, 244 bp, and 1210 bp (Fig. S2).

A genetic complementation test was carried out to confirm that the point mutation in *OsXXT1* was responsible for the mutant phenotype. The full-length open reading frame of the wild-type *OsXXT1* gene was inserted into the binary vector pTF101.1 under the control of a maize *Ubiquitin-1* promoter. The resulting construct was used to introduce a full-length *OsXXT1* gene into the *srh2* mutant genome via *Agrobacterium*-mediated transformation. Four positive transgenic lines were identified. The root hair from the T<sub>2</sub> transgenic seedlings displayed normal root hair growth (Fig. 1C), indicating overexpression of the *OsXXT1* gene could complement the mutant phenotype completely.

#### Protein structure and phylogenetic analysis of *OsXXT1*

The *OsXXT1* gene encodes a predicted protein of 448 amino acids that has been classified as a member of glycosyltransferase family 34 (GT34) in CAZy (<http://www.cazy.org>). The *OsXXT1* protein is predicted to have a transmembrane domain near the N-terminus and a glycosyltransferase domain near the C-terminus (Fig. 4A). The base substitution of the *srh2* mutant sequence resulted in an amino acid change of a glycine to an arginine in the highly conserved glycosyltransferase domain (Fig. S3).

Phylogenetic analysis of 14 putative xylosyltransferases in *Arabidopsis* and rice revealed that these genes could be



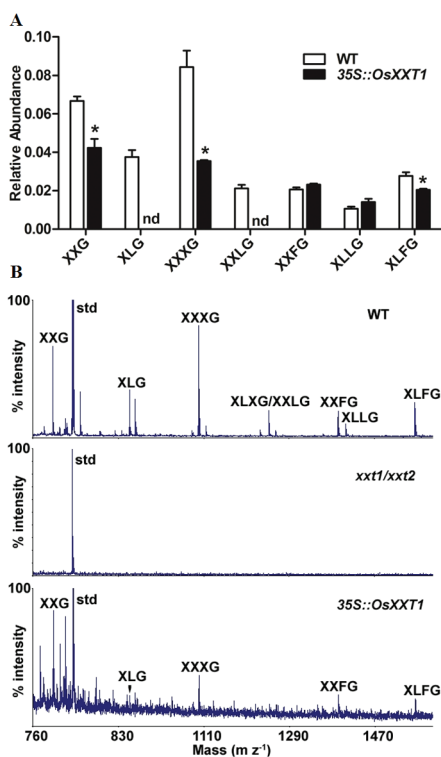
**Fig. 5.** Complementation of root hair and slow growth phenotype of *Arabidopsis xxt1 xxt2* double mutant with *OsXXT1* in ten-day-old seedlings. Representative examples of root hair and growth are shown. (A) Roots hairs of wild type (WT, Columbia-0), *Arabidopsis xxt1 xxt2* double mutant, and complemented *Arabidopsis xxt1 xxt2* double mutant with *35S::OsXXT1* (*35S::OsXXT1*). Bars=100  $\mu$ m. (B) Seedlings of wild type (WT, Columbia-0), *Arabidopsis xxt1xxt2* double mutant and *35S::OsXXT1*. Bars=2 cm. (This figure is available in colour at JXB online.)

divided into two classes (Fig. 4B). Five genes from rice, and five genes from *Arabidopsis*—AtXXT1 to AtXXT5, which have demonstrated XXT activity—are grouped into class I. Class II contains two *Arabidopsis* genes (*AtGT6* and *AtGT7*) and two rice genes (*OsGT6* and *OsGT7*), which are clustered with the fenugreek  $\alpha$ -(1,6) galactosyltransferase (*CAB52246*). Although there are 8, 10, and 18 RNA variants encoding GT34 protein members in *Arabidopsis*, rice, and maize (Penning et al., 2009), respectively, only class I proteins contained both the transmembrane and glycosyltransferase domains in *Arabidopsis* and rice (Fig. S3, and Fig. 4B).

#### Functional verification of *OsXXT1* by complementation of the *Arabidopsis* *xxt1 xxt2* double mutant

The amino acid sequence of *OsXXT1* is 70% and 72% identical to that of AtXXT1 and AtXXT2 in *Arabidopsis*,

respectively. This suggests that *OsXXT1* functions as an XXT gene in rice, as *AtXXT1* and *AtXXT2* function in *Arabidopsis*. To test if *OsXXT1* possesses xyloglucan 6-xylosyltransferase activity, a functional complementation test of the *Arabidopsis* *xxt1 xxt2* double mutant, which displays a root hair development phenotype (Cavalier et al., 2008), was carried out. Expression of *OsXXT1* under the 35S CaMV promoter was measured by reverse-transcription PCR (RT-PCR) in complemented transgenic lines (*35S::OsXXT1*). Two transgenic lines showed a high expression level of *OsXXT1* (Fig. S4), and in these two lines the short root hair growth was restored (Fig. 5A). Furthermore, the expression of *OsXXT1* also partially complemented the slow growth phenotype of the *Arabidopsis* *xxt1 xxt2* double mutant (Fig. 5B). Thus, *OsXXT1* may have xyloglucan 6-xylosyltransferase similar to AtXXT1 and AtXXT2. To measure the effect of *OsXXT1* on XyG synthesis, cell walls from the leaves of wild type, *xxt1 xxt2* double mutant and *35S::OsXXT1*-complemented *Arabidopsis* plants were digested with a XyG-specific endoglucanase (XEG) followed by extraction with 4M KOH. Xyloglucan oligosaccharides released after XEG digestion were analysed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Fig. 6B). As previously reported, we could not detect any signature of XyG fragment in the *Arabidopsis* *xxt1 xxt2* mutant (Fig. 6B). In the *35S::OsXXT1*-complemented *Arabidopsis* lines, we detected several XyG oligosaccharides, including normal wild-type levels of XXFG, XLLG, and XLFG, and relatively lower level of XXG, XLG, XXXG, and XXLG compared with wild-type plants (Fig. 6A, B).



**Fig. 6.** MALDI-TOF mass spectrometry analysis of the relative abundance of xyloglucan oligosaccharides released by xyloglucan-specific endoglucanase (XEG). (A) Relative proportions of xyloglucan subunits generated from cell wall preparations of wild-type (WT, white bar) and *35S::OsXXT1* *Arabidopsis* (black bar) leaves digested with XEG. Results are expressed as the percentile of the areas of the corresponding peaks for each subunit on high-performance anion exchange chromatography (HPAEC). *Arabidopsis* were grown in growth chamber for two months and leaves of each of the plants were sampled as a biological replicate. All analyses were performed on three plant preparations. Significant differences are indicated with an asterisk. nd, not detected. (B) MALDI-TOF mass spectrometry of XEG-generated xyloglucan fragments from the hemicellulosic fractions of wild-type (WT, Columbia-0), *Arabidopsis* *xxt1 xxt2* double mutant, and complemented *Arabidopsis* *xxt1 xxt2* double mutant with *35S::OsXXT1* (*35S::OsXXT1*). Xyloglucan subunit is described from the nomenclature introduced by Fry et al. (1993). WT (top), *Arabidopsis* *xxt1 xxt2* (middle) and *35S::OsXXT1* (bottom). Xylopentaose was used as a standard control (std).

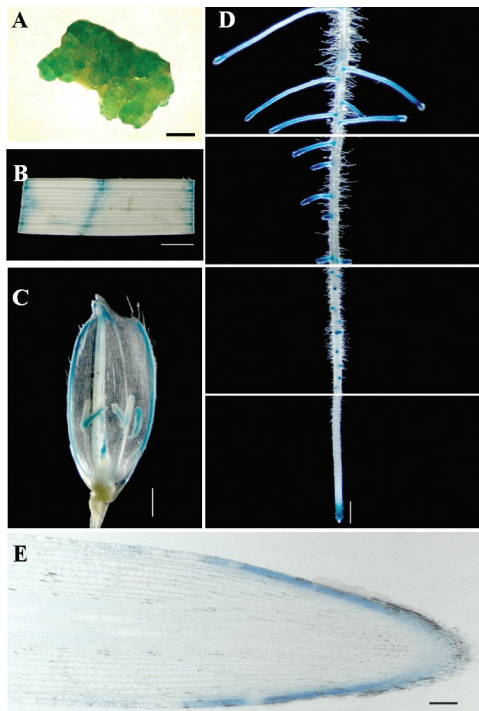
#### Expression pattern of *OsXXT1*

Expression patterns of rice XXT subfamily genes were determined by examining expression across development using publically available microarray datasets. The expression data are colour coded according to the RNA expression level (Fig. 7). *OsXXT1* and *OsGT2* have similar expression patterns and are expressed across a variety of tissues. In contrast, other members of this family (*OsGT3* to *OSGT5*) display a more restricted expression pattern, suggesting that *OsXXT1* and *OsGT2* are the predominantly expressed genes (Fig. 7A). A comparison to the expression in *Arabidopsis* displays a similar pattern, with *AtXXT1* and *AtXXT2* being predominantly expressed, followed by *AtXXT5* (Fig. 7B).

To further analyse the expression of *OsXXT1*, a 1.8-kb fragment upstream of the *OsXXT1* start codon was amplified and fused to a GUS reporter gene to make the *OsXXT1::GUS* construct. Using *Agrobacterium*-mediated rice transformation, six independent transgenic lines were generated. Analysis of the T<sub>1</sub> plants of these transgenic lines showed a similar GUS expression patterns. Expression of GUS was observed in most tissues, including callus, leaves, panicles, inflorescences, and root tips (Fig. 8A–D). Strong GUS staining was observed in the initiating lateral roots (Fig. 8D). Upon development of the lateral roots, the GUS staining was progressively restricted to the tip area (Fig. 8D). Similar expression patterns could be observed in adventitious roots. No GUS staining was detected







**Fig. 8.** GUS reporter gene expression in transgenic plants expressing the *OsXXT1* promoter-GUS constructs. (A) Callus; (B) Leaf (2-week-old seedlings); (C) Flower (3-month-old seedlings); (D) Root (2-week-old seedlings); (E) Longitudinal section of root tip (2-week-old seedlings). (A–D) Bar=1 mm; (E) Bar=20  $\mu$ m.

*OsXXT1* exhibited a similar short root hair phenotype as the *Arabidopsis xxt1 xxt2* double mutant. Moreover, constitutive expression of *OsXXT1* in the *Arabidopsis xxt1 xxt2* double mutant partially complemented the growth phenotype and XyG synthesis, which demonstrates that *OsXXT1* possess xyloglucan 6-xylosyltransferase activity. However, the *Arabidopsis xxt1 xxt2* double mutant complemented with a *35S::OsXXT1* transgenic line contained a relatively low abundance of XyG oligosaccharides, especially lacking galactose modified XyG (XLG and XXLG). This result suggests that the xyloglucan 6-xylosyltransferase activity of *OsXXT1* may not be exactly the same as *AtXXT1* and *AtXXT2*.

Although the proportions and structure of XyG vary between type I and type II cell walls, all flowering plants studied to date contain XyG in their primary cell walls (Hsieh and Harris, 2009). It has been observed that the XET activity of epidermal cells in root elongation zones and trichoblasts of diverse species of vascular plants is high (Vissenberg *et al.*, 2003). It is possible that the active form of XET is involved in the restructuring of XyG to regulate root hair development in all vascular plants. *OsXXT1* is preferentially expressed in epidermal cells of primary, adventitious, and lateral roots (Fig. 7E, S3). The expression pattern supports the hypothesis that XyG synthesis and modification in root epidermal cells is critical for cell wall development in the root hairs of all plants. Recently, a root hair specific galacturonic acid containing XyG has been discovered in *Arabidopsis* and the lack of this galacturonic acid containing XyG resulted in a short hair phenotype (Pena *et al.*, 2012). Therefore, XyG is a

pivotal component in the regulation of root hair tip growth in both *Arabidopsis* and rice.

XyG is the most abundant hemicellulose in type I primary cell walls. This polysaccharide has been depicted as a binding surface of cellulose microfibrils, forming a load-bearing network (Thompson, 2005). However, the important role of XyG in this model has been challenged by the discovery that a lack of XyG in *Arabidopsis* manifests as a short root hair phenotype and an ability to grow normally, despite the defect in root hair morphology (Cavalier *et al.*, 2008). Based on the biomechanical properties of the *Arabidopsis xxt1 xxt2* double mutant and wild-type *Arabidopsis*, a revised architecture of primary cell wall was proposed: a minor XyG component was found in wall mechanics (Park and Cosgrove, 2012b). The analysis of the microarray data and GUS expression patterns under the *OsXXT1* promoter suggest that *OsXXT1* is expressed in a variety of tissues, yet like in *Arabidopsis* (Cavalier *et al.*, 2008; Vuttipongchaikij *et al.*, 2012), a phenotypic defect is only observed in root hair development. If only a minor, inaccessible XyG component works as a load-bearing connection between microfibrils in type I cell walls, it is reasonable to suggest that a relatively low abundance of XyG has a similar function in type II cell walls. Thus, it seems that XyG plays a similar role in the cell walls of both plants with type I and type II cell walls, and it seems that under normal situations, this role is only observed to be non-redundant in root hair development.

## Supplementary data

Supplementary data are available at *JXB* online

**Figure S1.** Growth performance of *srh2* mutant and wild-type (cv Kasalath, *kas*) plants. The seedlings were growth at nutrient solution (pH 5.5) for 7 d and examined under an electron microscope.

**Figure S2.** Confirmation the single nucleotide mutation of *srh2* by dCAPS marker. A, The PCR fragment of WT contained three *Nco*I site, whereas mutation of *srh2* eliminate one *Nco*I site (red colour). B, Electrophoresis of *Nco*I-digested PCR product. The red arrows indicated specific digested fragment of wild-type and mutant samples.

**Figure S3.** Protein sequence alignment of putative xyloglucan 6-xylosyltransferase in *Arabidopsis* and rice. The transmembrane and glycosyltransferase domains were indicated by red box and black line, respectively.

**Figure S4.** The expression of *OsXXT1* in complementation *Arabidopsis* by RT-PCR.

**Figure S5.** A, Transverse section of root mature region of *OsXXT1::promoter* GUS plants. Bar=20  $\mu$ m.

**Table S1.** Primers used in this research.

## Acknowledgements

This work was supported by the Key Basic Research Special Foundation of China (2011CB100303), National Science Foundation of China (31172024, 31201675), the Ministry of Science and Technology of China (2010DFA31080, 2014ZX08009328-002), and an Australian Research Council Centre of Excellence grants to JW. (CEO561495 and CE140100008). We would like to thank Reena Narsai for help with the digital expression



analysis. The *xxt1 xxt2* double mutant was obtained from the *Arabidopsis* Biological Resource Center (ABRC).

## References

- Carpita NC, Gibeaut DM.** 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**, 1–30.
- Carpita NC.** 1996. Structure and biogenesis of the cell walls of grasses. *Annual Review of Plant Physiology Plant Molecular Biology* **47**, 445–476.
- Carpita NC, McCann M.** 2000. The cell wall. In: Buchanan BB, Wilhelm G, Jones RL, eds. *In Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, 52–108.
- Cavalier DM, Keegstra K.** 2006. Two xyloglucan xylosyltransferases catalyze the addition of multiple xylosyl residues to cellohexaose. *The Journal of Biology Chemistry* **281**, 34197–34207.
- Cavalier DM, Lerouxel O, Neumetzler L et al.** 2008. Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *The Plant Cell* **20**, 1519–1537.
- Clough SJ, Bent AF.** 1998. Floral dip. A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.
- Cocuron JC, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, Raikhel N, Wilkerson CG.** 2007. A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. *Proceedings of the National Academy of Sciences, USA* **104**, 8550–8555.
- Faik A, Price NJ, Raikhel NV, Keegstra K.** 2002. An *Arabidopsis* gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. *Proceedings of the National Academy of Sciences, USA* **99**, 7797–7802.
- Fry SC, York WS, Albersheim P et al.** 1993. An unambiguous nomenclature for xyloglucan derived oligosaccharides. *Physiology Plant* **89**, 1–3.
- Hara Y, Yokoyama R, Osakabe K, Toki S, Nishitani K.** 2014. Function of xyloglucan endotransglucosylase/hydrolases in rice. *Annals of Botany* (in press).
- Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC, Cottage A.** 2006. A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods* **2**, 19.
- Hoffman M, Jia Z, Pena MJ, Cash M, Harper A, Blackburn AR 2nd, Darvill A, York WS.** 2005. Structural analysis of xyloglucans in the primary cell walls of plants in the subclass Asteridae. *Carbohydrate Research* **340**, 1826–1840.
- Hsieh YS, Harris PJ.** 2009. Xyloglucans of monocotyledons have diverse structures. *Molecular Plant* **2**, 943–965.
- Jefferson RA, Kavanagh TA, Bevan MW.** 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6**, 3901–3907.
- Madson M, Dunand C, Li X, Verma R, Vanzin GF, Caplan J, Shoue DA, Carpita NC, Reiter WD.** 2003. The *MUR3* gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. *The Plant Cell* **15**, 1662–1670.
- Monshausen GB, Bibikova TN, Messerli MA, Shi C, Gilroy S.** 2007. Oscillations in extracellular pH and reactive oxygen species modulate tip growth of *Arabidopsis* root hairs. *Proceedings of the National Academy of Sciences, USA* **104**, 20996–21001.
- Narsai R, Rocha M, Geigenberger P, Whelan J, van Dongen JT.** 2011. Comparative analysis between plant species of transcriptional and metabolic responses to hypoxia. *New Phytologist* **190**, 472–487.
- Nishimura A, Aichi I, Matsuoka M.** 2006. A protocol for *Agrobacterium*-mediated transformation in rice. *Nature Protocols* **1**, 2796–2802.
- Obel N, Neumetzler L, Pauly M.** 2007. *Hemicellulose and cell expansion*. Berlin: Springer-Verlag.
- Park YB, Cosgrove DJ.** 2012a. Changes in cell wall biomechanical properties in the xyloglucan-deficient *xxt1/xxt2* mutant of *Arabidopsis*. *Plant Physiology* **158**, 465–475.
- Park YB, Cosgrove DJ.** 2012b. A revised architecture of primary cell walls based on biomechanical changes induced by substrate-specific endoglucanases. *Plant Physiology* **158**, 1933–1943.
- Pena MJ, Ryden P, Madson M, Smith AC, Carpita NC.** 2004. The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in *Arabidopsis* during growth. *Plant Physiology* **134**, 443–451.
- Pena MJ, Kong Y, York WS, O'Neill MA.** 2012. A galacturonic acid-containing xyloglucan is involved in *Arabidopsis* root hair tip growth. *The Plant Cell* **24**, 4511–4524.
- Penning BW, Hunter CT, Tayengwa R et al.** 2009. Genetic resources for maize cell wall biology. *Plant Physiology* **151**, 1703–1728.
- Ragauskas AJ, Williams CK, Davison BH et al.** 2006. The path forward for biofuels and biomaterials. *Science* **311**, 484–489.
- Sims IM, Middleton K, Lane AG, Cairns AJ, Bacic A.** 2000. Characterisation of extracellular polysaccharides from suspension cultures of members of the Poaceae. *Planta* **210**, 261–268.
- Strohmeier M, Hrmova M, Fischer M, Harvey AJ, Fincher GB, Pleiss J.** 2004. Molecular modeling of family GH16 glycoside hydrolases: potential roles for xyloglucan transglucosylases/hydrolases in cell wall modification in the poaceae. *Protein Science* **13**, 3200–3213.
- Thompson DS.** 2005. How do cell walls regulate plant growth? *Journal of Experimental Botany* **56**, 2275–2285.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.** 1997. The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876–4882.
- Vanzin GF, Madson M, Carpita NC, Raikhel NV, Keegstra K, Reiter WD.** 2002. The *mur2* mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proceedings of the National Academy of Sciences, USA* **99**, 3340–3345.
- Vincken JP, York WS, Beldman G, Voragen AG.** 1997. Two general branching patterns of xyloglucan, XXXG and XXGG. *Plant Physiology* **114**, 9–13.
- Vissenberg K, Van Sandt V, Fry SC, Verbelen JP.** 2003. Xyloglucan endotransglucosylase action is high in the root elongation zone and in the trichoblasts of all vascular plants from *Selaginella* to *Zea mays*. *Journal of Experimental Botany* **54**, 335–344.
- Vogel J.** 2008. Unique aspects of the grass cell wall. *Current Opinion in Plant Biology* **11**, 301–307.
- Vuttipongchaikij S, Brocklehurst D, Steele-King C, Ashford DA, Gomez LD, McQueen-Mason SJ.** 2012. *Arabidopsis* GT34 family contains five xyloglucan alpha-1,6-xylosyltransferases. *New Phytologist* **195**, 585–595.
- Yokoyama R, Nishitani K.** 2004. Genomic basis for cell-wall diversity in plants. A comparative approach to gene families in rice and *Arabidopsis*. *Plant Cell Physiology* **45**, 1111–1121.
- Yokoyama R, Rose JK, Nishitani K.** 2004. A surprising diversity and abundance of xyloglucan endotransglucosylase/hydrolases in rice. Classification and expression analysis. *Plant Physiology* **134**, 1088–1099.
- Yoshida S, Forno DA, Cock JH, KA G.** 1976. *Laboratory manual for physiological studies of rice*, Ed 3. Manila: The International Rice Research Institute.
- Zabotina OA, van de Ven WT, Freshour G, Drakakaki G, Cavalier D, Mouille G, Hahn MG, Keegstra K, Raikhel NV.** 2008. *Arabidopsis* *XXT5* gene encodes a putative alpha-1,6-xylosyltransferase that is involved in xyloglucan biosynthesis. *The Plant Journal* **56**, 101–115.
- Zabotina OA, Avci U, Cavalier D, Pattathil S, Chou YH, Eberhard S, Danhof L, Keegstra K, Hahn MG.** 2012. Mutations in multiple *XXT* genes of *Arabidopsis* reveal the complexity of xyloglucan biosynthesis. *Plant Physiology* **159**, 1367–1384.
- Zhang SJ, Song XQ, Yu BS, Zhang BC, Sun CQ, Knox JP, Zhou YH.** 2012. Identification of quantitative trait loci affecting hemicellulose characteristics based on cell wall composition in a wild and cultivated rice species. *Molecular Plant* **5**, 162–175.
- Zheng L, Cheng Z, Ai C et al.** 2010. Nicotianamine, a novel enhancer of rice iron bioavailability to humans. *PLoS One* **5**, e10190.