



Rice OsPEX1, an extensin-like protein, affects lignin biosynthesis and plant growth

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Received: 21 October 2018 / Accepted: 23 February 2019 / Published online: 6 March 2019
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Abstract

Key message Rice leucine-rich repeat extensin-like protein OsPEX1 mediates the intersection of lignin deposition and plant growth.

Abstract Lignin, a major structural component of secondary cell wall, is essential for normal plant growth and development. However, the molecular and genetic regulation of lignin biosynthesis is not fully understood in rice. Here we report the identification and characterization of a rice semi-dominant dwarf mutant (*pex1*) with stiff culm. Molecular and genetic analyses revealed that the *pex1* phenotype was caused by ectopic expression of a leucine-rich repeat extension-like gene, *OsPEX1*. Interestingly, the *pex1* mutant showed significantly higher lignin content and increased expression levels of lignin-related genes compared with wild type plants. Conversely, *OsPEX1*-suppressed transgenics displayed low lignin content and reduced transcriptional abundance of genes associated with lignin biosynthesis, indicating that the *OsPEX1* mediates lignin biosynthesis and/or deposition in rice. When *OsPEX1* was ectopically expressed in rice cultivars with tall stature that lacks the allele of *semi-dwarf 1*, well-known green revolution gene, the resulting transgenic plants displayed reduced height and enhanced lodging resistance. Our study uncovers a causative effect between the expression of *OsPEX1* and lignin deposition. Lastly, we demonstrated that modulating *OsPEX1* expression could provide a tool for improving rice lodging resistance.

Keywords Rice · Semi-dwarf · Lignin · Lodging resistance · Leucine-rich repeat extensin-like protein · *OsPEX1*

Shanwen Ke and Xin Luan contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11103-019-00849-3>) contains supplementary material, which is available to authorized users.

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Introduction

Rice, wheat, and maize are the world's three major crops. Of these three crops, rice is the most important food crop in Asian, especially in Southeast Asian. Lodging resistance is an important trait for increasing rice grain yield. Lodging resistance is closely related to plant height and culm composition. The broad use of dwarf rice varieties resulted in the Green Revolution in the 1960s. However, lodging resistance of rice with the *semi-dwarf 1* (*sd1*) gene alone is limited (Yano et al. 2015), and many varieties with *sd1* allele remain lodging-sensitive (Zhang et al. 2014). Another approach for improving lodging resistance is enhancing mechanical strength of the culm, which is determined by culm diameter, wall thickness and composition of cell wall at the basal internodes including third and fourth internodes from the tops of the plants. Secondary wall, which is primarily composed of cellulose, hemicellulose, and lignin, provides a major mechanical strength for rice culm (Zhong et al. 2011). The stiffness of the basal stem is positively correlated with the content of lignin in the secondary wall (Zhang et al. 2014).

Lignin is a major structural component of secondary cell walls in vascular plants; however, mechanisms underlying the regulation of lignin synthesis remain elusive, in spite of extensive research on lignin metabolism (Yoon et al. 2015).

Cell walls in plants protect and restrict cell movement and enlargement. As a result, strict regulation of cell wall expansion is crucial for plant growth and development. The primary cell wall is composed of cellulose and hemi-cellulose intermingled with xyloglucan. In addition to these structural cell wall components, plant cells have evolved a rich array of complex wall-associated proteins with diverse extracellular domains for the purpose of sensing, signaling, and cytoskeleton reconfiguration functions. At least three main classes of cell wall proteins have been described: proline-rich-proteins (PRP)s, glycine-rich-proteins (GRPs), and hydroxyproline-rich-glycoproteins (HRGPs). Extensins (EXTs) are a diverse plant-specific family of HRGPs characterized by the repeated occurrence of serine (Ser) followed by several consecutive prolines (Pro) or Tyr-X-Tyr motifs (X: any amino acid). They require several post-translational modifications (PTMs) including proline Hydroxylation that is catalyzed by prolyl 4-hydroxylases (P4Hs), hydroxyproline and serine O-glycosylation by glycosyltransferases, and tyrosine (Tyr) crosslinking by type-III peroxidases, before becoming functional in plant cell walls (Marzol et al. 2018). The Tyr crosslinking is responsible for intramolecular or intermolecular cross-linking with other EXT molecules. These cross-linking properties contribute to the extracellular matrix and play roles in plant development and defense mechanisms (Borassi et al. 2016).

LRXs (leucine-rich repeat extensins) are a class of chimeric EXTs which have signal peptides at the N terminus, followed by leucine-rich repeat (LRR) domains, and extensin domain (harboring Ser-Pro(3–5) repeated motif) near the C terminus, which is probably involved in cross-linking to cell wall components. Since LRR domain is known for protein–protein interactions, the LRXs are candidates for regulatory functions on the cell surface. In *Arabidopsis*, LRX proteins have been implicated in root hair morphogenesis, cell wall composition modifications, and plant growth (Baumberger et al. 2001, 2003; Draeger et al. 2015).

Plants have evolved cell wall signaling pathways to sense the state of the cell wall and transmit the information in plant growth and development and in response to environmental stress. In agreement with this notion, it is becoming clear that any disturbance in plant cell wall has a direct impact on cell membrane systems; and likewise, disruption of any intracellular component also could influence plant cell wall (Kohorn and Kohorn 2012; Borassi et al. 2016). Extensins are abundant structural proteins of the cell walls, but their relatively simple structure suggests that they have a role in cross linking components within the cell wall rather than signaling (Bedinger 2018).

Recently, several members of the *Catharanthus roseus* receptor-like kinase (CrRLK1L) subfamily such as FERONIA (FER) ANXURs (ANX1/ANX2) and BUDDHA'S PAPER SEAL1 and 2 (BUPS1/BUPS2) have been identified as potential cell wall sensors (Marzol et al. 2018). These members of the CrRLK1L subfamily are located at the interface between the apoplast and the cytoplasmic side of the plasma membrane and have pleiotropic functions in a variety of cellular processes, including root cell and pollen tube growth. FER functions as a receptor of RALF (RAPD ALKALINIZATION FACTOR) peptides to regulate cell expansion (Haruta et al. 2014). Recently, it was reported that the *Arabidopsis* cell-wall-localized LRX3/4/5 interact with RALF22/23, which in turn interact with the plasma membrane-localized receptor-like protein kinase FER (Zhao et al. 2018). In addition, pollen-expressed ANX1/2 and BUPS1/2 interact with AtRALF4/19 peptides, which show a physical interaction with AtLRX8/9 (Ge et al. 2017; Mecchia et al. 2017). Moreover, it was suggested that LRR domain of LRX4 physically interacts with the FER, suggesting a direct connection between the LRX LRR domain and the cell surface CrRLK1L sensors (Dunser et al. 2017). Together these observations suggest that LRX proteins might link the extensin network to the cell surface integrity sensors (Nissen et al. 2016; Marzol et al. 2018).

According to a recent large-scale genome survey, LRXs have been identified in all flowering plants, with eudicot genomes encoding more LRXs in general (Liu et al. 2016). Several LRXs in *Arabidopsis* have been studied in detail (Baumberger et al. 2001, 2003; Draeger et al. 2015; Mecchia et al. 2017; Fabrice et al. 2018; Sede et al. 2018; Wang et al. 2018). The rice genome encodes at least eight LRX proteins (Baumberger et al. 2003a, b), compared to 11 proteins encoded by the *Arabidopsis* genome (Baumberger et al. 2003a, b; Liu et al. 2016). Here we report the characterization of a rice mutant that affects plant stature and lodging resistance due to ectopic expression of the rice LRX gene *OsPEX1*. Our study also uncovered an important role of *OsPEX1* in lignin biosynthesis and deposition in rice.

Materials and methods

Plant materials and growth conditions

Rice cultivar 'Zhonghua 11' was used as the wild type. The *pex1* mutant described in this paper was derived from an activator/dissociator (Ac/Ds) transposon-tagging population in the *japonica* rice variety Zhonghua 11 (Liu et al. 2007). All rice seeds in this study were propagated in the paddy field in Guangzhou, China.

Isolation of the *Ds* flanking sequences

Genomic sequences flanking the *Ds* insertions in *pex1* were identified by TAIL-PCR (Liu and Whittier 1995). Specific tertiary PCR fragments were sequenced. The presence of the *Ds* insertion was verified according to the methods described previously (Zhang et al. 2016). The primers used for TAIL-PCR were listed in Supplementary Table S1.

Molecular cloning and rice transformation

To suppress *OsPEX1* expression in rice plants, rice *OsPEX1* cDNA fragment was amplified with the primers LRX-RNAiF and LRX-RNAiR (Supplementary Table S1), and cloned into a binary pCUBi1390 vector in antisense orientation under the control of the maize (*Zea mays*) Ubiquitin promoter. To produce *OsPEX1* overexpression transgenic plants, the full-length *OsPEX1* coding sequence was amplified with the primers LRX-1BF and LRX-4200SR. The amplified sequence was cloned into the binary vector pCUBi1390 for *OsPEX1* overexpression in rice. For each construct, about twenty transgenic lines were produced by Agrobacterium-mediated transformation (strain EHA105) and their transgenic seeds were planted individually to obtain T₁ generation.

Reverse transcription (RT)-PCR, quantitative RT-PCR

Total RNA was extracted from frozen samples with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Purified RNA was pre-treated with DNase I, and first-strand cDNA was generated using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative RT-PCRs were performed using a SYBR Premix Ex Taq™ RT-PCR kit (Takara) following the manufacturer's instructions. The relative expression level of a target gene was normalized to that of rice *ACTIN1*. The primers used for RT-PCR were listed in Supplementary Table S1.

Lignin staining and autofluorescence of rice stems

The internodes of each sample were numbered consecutively from the top to the base of the stem. Sections were cut at the center of each internode and stained with either phloroglucine-HCl according to the procedure described by Hirano et al. (2012) or directly examined for autofluorescence. Sections were viewed and photographed with a light microscope (Zeiss Axioimager with UV excitation).

Measurement of lignin content

Lignin contents were determined using the method of Moreira-Vilar et al (Moreira-Vilar et al. 2014) with

modifications and expressed as a percentage of the cell wall residue (%). Briefly, samples were ground to fine powder using a pestle and mortar containing liquid nitrogen and dried in an oven at 30 °C until a constant weight was achieved. Dry samples (20 mg) were placed into screw-cap centrifuge tubes containing 2 ml of 25% acetyl bromide (v/v in glacial acetic acid) and incubated at 70 °C for 30 min. After complete digestion, partial samples (0.2 ml) were mixed with 0.45 ml of 2 M NaOH, 0.05 ml of 7.5 M hydroxylamine-HCl, and 3.3 ml of glacial acetic acid. After centrifugation (1400×g, 5 min), the absorbance of the supernatant was measured at 280 nm. The results were expressed as mg lignin g⁻¹ dry samples.

Measurement of physical properties of rice stems

Breaking force was referred to as the force required for breaking a culm segment. The breaking force of the third internode of rice culms 20 days after heading was measured using a single column testing machine (HDE-500N). The bending load at breaking was measured at a distance of 5 cm between two supporting points.

Gene accession number

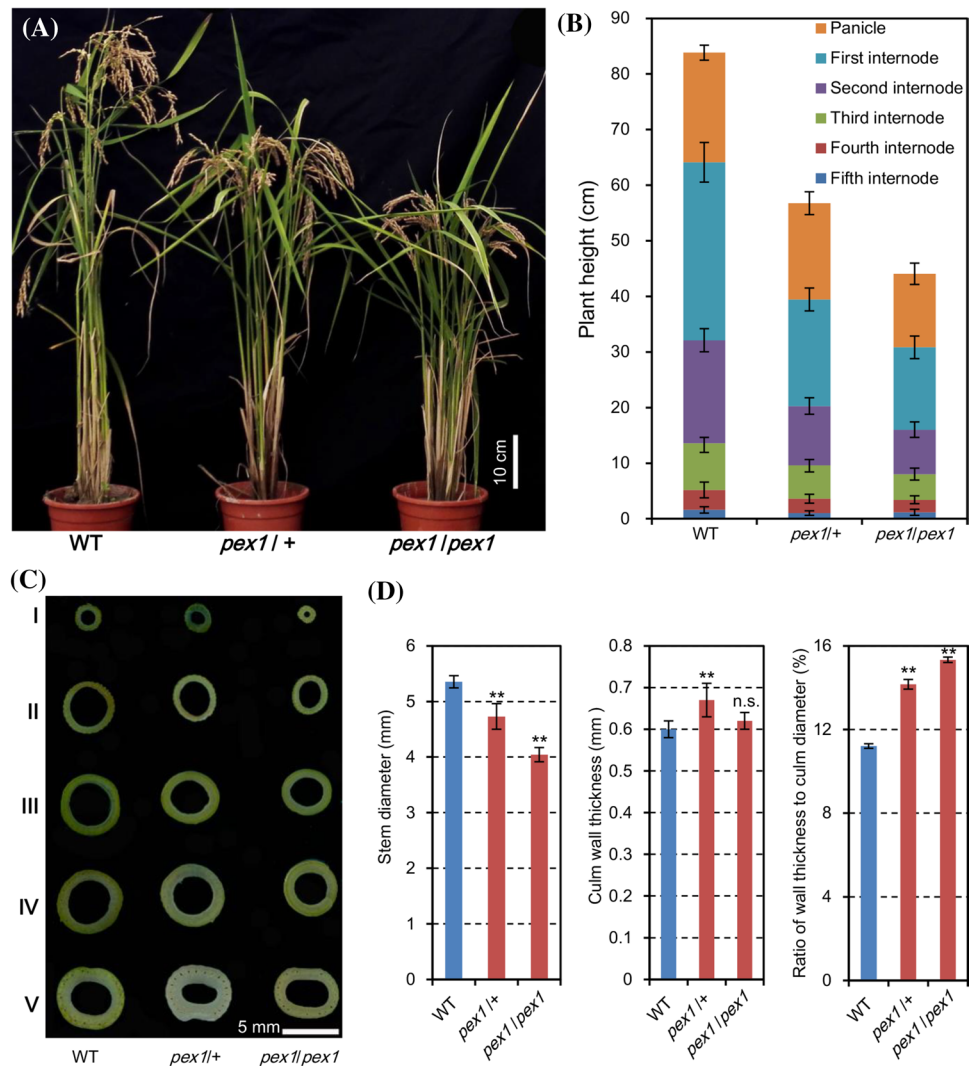
LOC_Os11g43640 (*OsPEX1*), LOC_Os06g04090 (*OsSWN1*), LOC_Os08g01330 (*OsSWN3*), LOC_Os06g01480 (*OsSWN7*), LOC_Os04g43800 (*OsPAL6*), LOC_Os05g35290 (*OsPAL7*), LOC_Os09g08720 (*OsCCR*), LOC_Os02g09490 (*OsCAD2*), LOC_Os12g02080 (*OsPRX1*), LOC_Os11g02130 (*OsPRX2*).

Results

Isolation and characterization of a rice semi-dominant dwarf mutant

A dwarf mutant, designated as *pex1*, was obtained from a two-component activator/dissociation (*Ac/Ds*) transposon-tagging population in the *japonica* rice variety Zhonghua 11 (Liu et al. 2007). To determine the inheritance of *pex1*, we examined the phenotypes of 135 progeny of self-pollinated *pex1* plants. Three distinct phenotypes were observed. Among them, 40 were normal looking (wild type) plants, 65 were semi-dwarf, and 30 exhibited dwarf phenotype (Fig. 1a). The ratio of wild type to semi-dwarf to dwarf plants was 1:1.63:0.75 (for 1:2:1 ratio, $\chi^2 = 1.67$, $p > 0.05$). In addition, 35 F₁ plants derived from the cross between *pex1* and Zhonghua11 showed a near 1:1 ratio of wild-type vs semi-dwarf progeny (Data not shown). These results demonstrated that the *pex1* mutant is a heterozygous allele controlled by a single, semi-dominant locus.

Fig. 1 Phenotypes of the *pex1* mutant. **a** Mature plant phenotype of wild type (left), *pex1* heterozygote (middle), and homozygote (right). **b** Shortened internodes resulted in semi-dwarfism in *pex1*. The length of the upper five internodes of the main culms was proportionally diminished compared with WT. **c** Cross-sections of the first (I, panicle-neck internode) down to the fifth (V, basal internode) of the WT and *pex1* mutants. In addition to dwarfism throughout its growth, the *pex1* mutant exhibited the dramatic reduction of stem diameters. **d** Comparison of culm diameter and culm thickness and the ratio of wall thickness to culm diameter of the 3rd internode in WT and *pex1* mutants at 20 days after heading. Each column represents mean \pm SD ($n=6$). Asterisks represent significant difference determined by Student's *t*-test at $**p$ value <0.01 , not significant (n.s.)



Thus, the semi-dwarf progeny is referred to as the *pex1* heterozygotes (*pex1*/+), and the dwarf progeny as *pex1* homozygotes (*pex1*/*pex1*).

The reduced height of *pex1* was the result of uniformly shortened internodes in the mutant culms, determined by comparing the length of each internode between mutant and wild-type plants (Fig. 1b). In addition, the *pex1* mutant showed decreased culm diameter, but with an increased ratio of wall thickness to culm diameter compared with WT (Fig. 1c, d). Interestingly, in spite of slightly shortened panicle length (Supplementary Fig. S1a), average grain number per panicle/plant in heterozygous *pex1* is comparable to that in wild type plants, whereas homozygous *pex1* has a significantly reduced grain number due to reduced panicle length (Supplementary Fig. S1b). Moreover, tiller number of the *pex1* is slightly increased (Supplementary Fig. S1b). However, other agronomic traits, such as heading date, blade length of flag leaf and blade width

of flag leaf, in the *pex1* mutant are similar to wide-type plants (Supplementary Fig. S1b, c).

Molecular cloning of *PEX1*

To determine the *Ds* insertion site in the *pex1* mutant, we isolated genomic flanking sequences of the *Ds* insertion by thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995). Analysis of flanking sequences revealed that the *Ds* element is inserted into the promoter of the *OsPEX1* (LOC_Os11g43640) gene on rice chromosome 11, generating an 8-bp duplication at the target site expected for a *Ds* insertion (Fig. 2a, b). All plants showing the mutant phenotype were heterozygous or homozygous for the *Ds* insertion (Fig. 2c). We next investigated the expression level of *OsPEX1*. Compared with wild-type stems, *OsPEX1* showed elevated expression in *pex1*/+ and *pex1*/*pex1* stems (Fig. 2d,

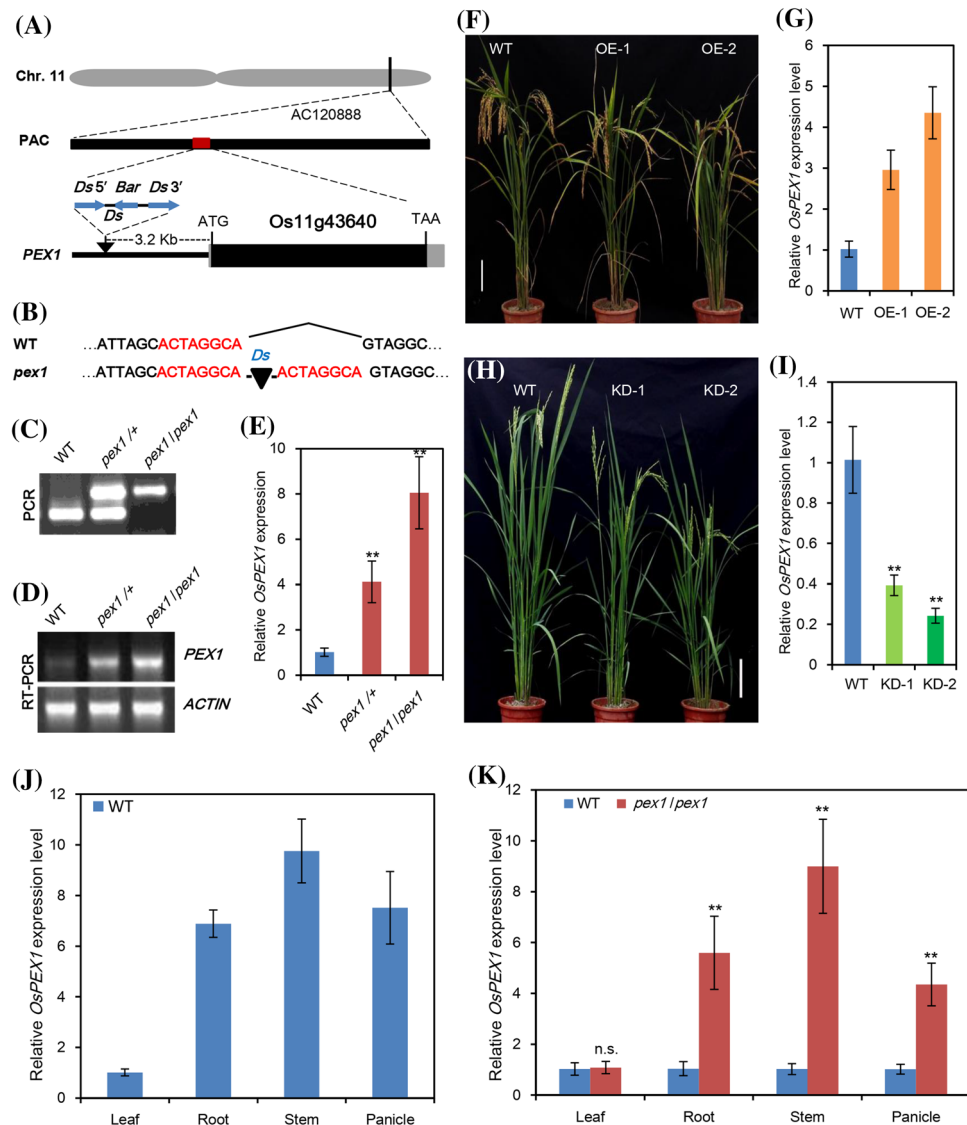


Fig. 2 Molecular cloning of the *OsPEX1* gene. **a** Location of *Ds* insertion and the schematic structure of *OsPEX1*. The *Ds* insertion was mapped to rice genomic PAC clone AC120888 on chromosome 11. The *Ds* element was inserted about 3.2 kb upstream of the *OsPEX1* gene. The untranslated regions are shown in gray rectangles, the exons in black rectangles. *Ds* 5' and *Ds* 3', the 5' and 3' end of the *Ds* transposon, respectively; *Bar*, the *BASTA* resistant gene. **b** Identification of *Ds* insertion footprint in the *pex1* mutant. The 8 base-pairs (bp) sequence, ACTAGGCA (in red), is duplicated upon *Ds* insertion. **c** PCR-based analysis on the genomic DNAs of the wild-type control, the *pex1* heterozygous (*pex1/+*) and homozygous (*pex1/pex1*) mutants. Expression analysis of *OsPEX1* by reverse transcription-PCR (RT-PCR, **d**) and real-time reverse transcription-PCR (qRT-PCR, **e**) in the wild type (WT), *pex1* heterozygous (*pex1/+*) and homozygous (*pex1/pex1*) mutants. **f** Morphology of WT, and

transgenic plants overexpressing *OsPEX1* (OE). Bar = 10 cm. **g** qRT-PCR analysis showing the transcript levels of *OsPEX1* in overexpressing transgenic plants. **h** Morphology of transgenic rice plants with antisense-mediated knock-down of *OsPEX1* expression (KD). Bar = 10 cm. **i** qRT-PCR analysis showing the transcript levels of *OsPEX1* in the knock-down transgenic plants. **j** Expression pattern of *OsPEX1* in wild-type plants. **k** Comparison of *OsPEX1* expression in various tissues between wild-type and *pex1* plants. For qRT-PCR analysis, the samples in **g** and **i** were derived from the second internode of rice stem at heading stage; Total RNA in **j** and **k** was isolated from roots, leaf blade, the second internode of rice stem and panicle at heading stage. Values are means \pm SD of three biological replicates. Asterisks represent significant difference determined by Student's *t*-test at ***p* value < 0.01, not significant (n.s.)

e). The results suggested that *OsPEX1* overexpression in *pex1* plants is due to the *Ds* insertion.

Analysis of amino acids sequences revealed that *OsPEX1* protein includes a C-terminal extensin-like domain

containing numerous copies of Ser-(Pro)₂₋₄ repeats (Supplementary Fig. S2). Analysis of the N terminus of *OsPEX1* by Petersen et al method (Petersen et al. 2011) confirmed the presence of a signal sequence, suggesting that the protein

is secreted. In addition to the extensin-like domain, it has a distinct N-terminal domain containing eight LRR repeats (LRRs). LRRs are frequently implicated in protein–protein interactions, pathogen recognition and defense in plants (Borassi et al. 2016). The most prevalent amino acids in this domain are leucine, phenylalanine, and aspartic acid (10.6%, 9.7%, and 8.2%, respectively). By contrast, the extensin-like domain has an entirely different composition of amino acids, with a very high ratio (> 40%) of proline.

To confirm that overexpression of *OsPEX1* is responsible for the developmental defects in *pex1*, we overexpressed *OsPEX1* in wild type plant. All positive transformants displayed dwarf phenotypes characteristic of the *pex1* mutant plants (Fig. 2f, g). Thus, we concluded that the abnormal phenotype in the *pex1* mutant results from overexpression of *OsPEX1*. Interestingly, specific knock-down of *OsPEX1* in wild type plants (Supplementary Fig. S3) also resulted in dwarf phenotype with uniformly shortened internodes (Fig. 2h, i, and Supplementary Fig. S4a, b), which resulted from reduced cell size as in the *pex1* mutants (Supplementary Fig. S5). In addition, the KD (knock-down) plants showed decreased stem diameter and culm wall thickness compared with WT (Supplementary Fig. S4c, d). These results suggest that *OsPEX1* gene is required for normal plant growth.

In wild-type plants, *OsPEX1* transcripts are detected in roots, leaf blades, stems and panicles, with expression level varies significantly among tissues examined (Fig. 2j). For example, compared to leaf, *OsPEX1* expression is significantly elevated in root, stem, and panicle tissues, highlighting a special requirement of its activity in these tissues. Interestingly, the expression of *OsPEX1* is even further induced in *pex1* mutant compared to wild-type in roots, stems, and panicles, but not in leaves (Fig. 2k). This is consistent with the *pex1* mutant exhibiting normal leaf morphology (Fig. 1 and Supplementary Fig. S1c).

Lignin content increase in *pex1* stem

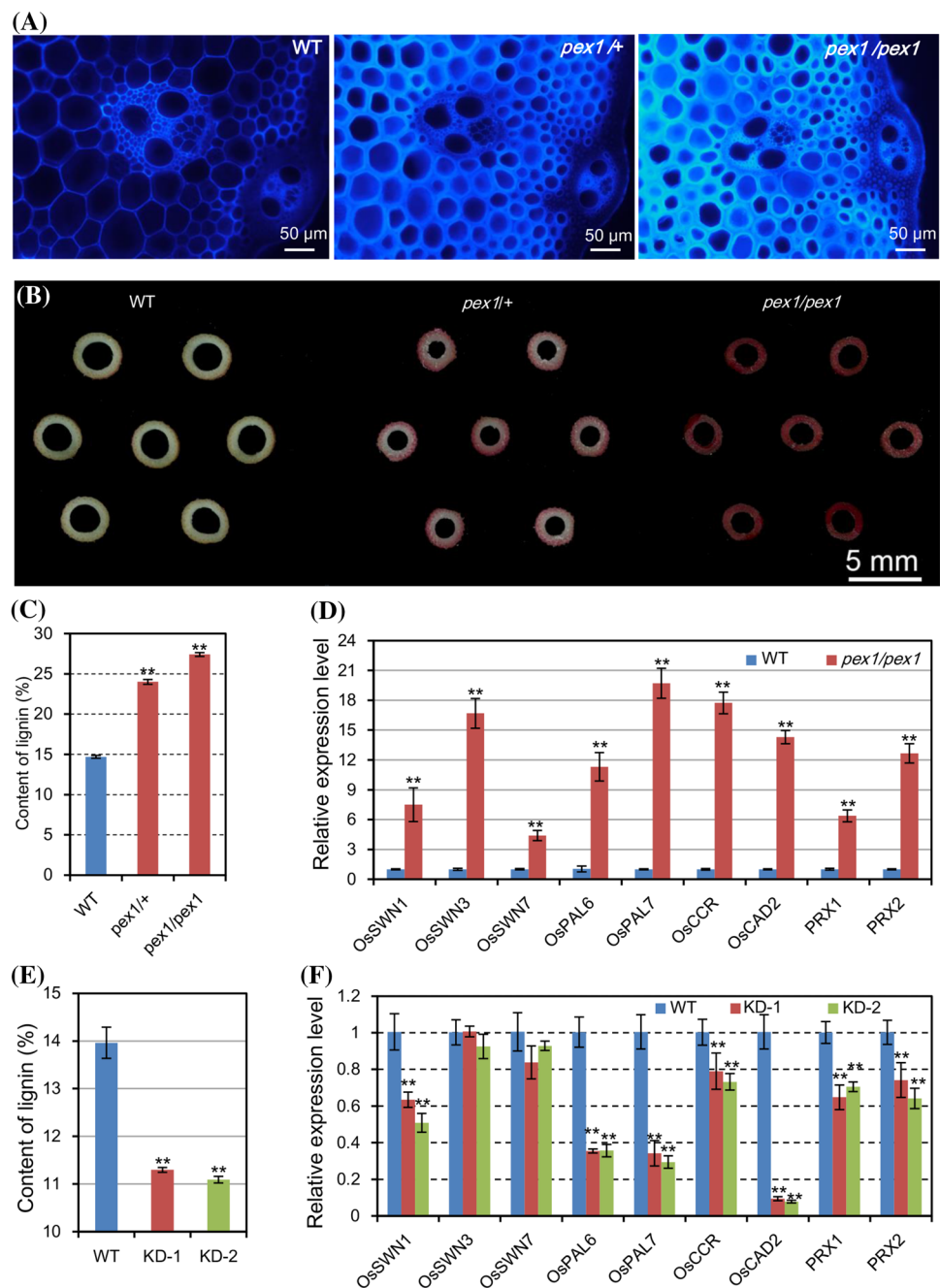
Autofluorescence examination under UV light excitation is a sensitive and effective technique for total lignin distribution in plant cell wall (De Micco and Aronne 2007). As shown in Fig. 3a, autofluorescence was clearly visible in cell walls of wild-type stems. By contrast, enhanced autofluorescence was observed in parenchyma cells of the *pex1*, which have smaller cell size with thickened cell wall compared with those of WT. Moreover, phloroglucinol staining for lignin showed that strong phloroglucinol staining (a red–violet colour) were detected in *pex1* compared to WT (Fig. 3b and Supplementary Fig. S6), suggesting elevated lignin contents in *pex1* mutant. We quantified the total lignin content of stems of wild-type and *pex1* plants using the acetyl-bromide method (Moreira-Vilar et al. 2014). Stem from the *pex1*

plants have higher lignin content (% dry cell wall residues) than the WT (Supplementary Table S2) and the difference is more profound in older stems (Fig. 3c). We also observed a slight reduction in cellulose content in *pex1* plants, which might be a consequence due to their increased lignin content (Supplementary Fig. S7). We next analyzed the expression of lignin pathway genes in the second internode at 10 days before heading (Fig. 3d). Compared to wild-type plants, the *pex1/pex1* showed increased expression in all the lignin biosynthesis genes examined, including *PHENYLALANINE AMMONIA-LYASE* (*OsPAL6*, *OsPAL7*), *CINNAMOYL-CoA REDUCTASE* (*OsCCR*), *CINNAMYL ALCOHOL DEHYDROGENASE* (*OsCAD2*), *PEROXIDASE* (*OsPRX1* and *OsPRX2*), which showed at least sixfold increase in abundance. The rice secondary wall-associated NAC (*NAM*, *ΔTAF* and *CUC2*) transcription factors (namely the *OsSWNs*) are master transcriptional activators of the secondary wall biosynthetic program (Zhong et al. 2011). The expression of *OsSWNs* (*OsSWN1*, *OsSWN3*, and *OsSWN7*) in the *pex1/pex1* mutants is induced more than fourfold compared to wild-type plants, consistent with elevated transcriptional levels of lignin biosynthesis genes in the *pex1* mutants. In contrast to the *pex1* mutants, *OsPEX1*-knockdown (KD) transgenic lines showed a substantial reduction in lignin content and down-regulation of lignin-associated genes expression (Fig. 3e, f and Supplementary Fig. S8). These findings provide empirical evidence that modulating *OsPEX1* expression affects lignin biosynthesis in rice, either directly or indirectly.

OsPEX1 is involved in lignin accumulation in developing rice internodes

The rice internode is a suitable organ for studying secondary cell wall formation, because after cell elongation has ceased, the secondary cell wall accumulates at the maturation zone, resulting in a continuing developmental stages of secondary wall thickening from the lower (the young stage) to the upper (the developed stage) part of the internode (Hirano et al. 2013). To further understand the relationship between *OsPEX1* expression and lignin metabolism in a natural physiological tissue, we analyzed the lignin deposition and *OsPEX1* expression in different section of at the second internode from wild-types plants at 10 days before heading. The second internode was divided into five sections from the bottom up (stage I to V, representing from younger to more developed stage) (Fig. 4a). Wiesner staining with phloroglucinol-HCl revealed that lignin deposition gradually increased in the internode from the bottom up (Fig. 4b). Gene expression analysis showed that transcript abundance of *OsPEX1* gradually increased from section 1 to 5 (Fig. 4c). This is concomitant with a rapid increase in lignin accumulation toward upper sections of internode (Fig. 4d). These

Fig. 3 *OsPEX1* promotes lignin deposition. **a** Autofluorescence micrographs of transverse stem sections of the third internode under UV light for visualizing lignin distribution in cell walls of wild type and *pex1* stems. **b** Phloroglucinol staining of cross sections of the third internode from the WT and *pex1* plants at 20 days after heading. **c** Lignin content of stem from the plants of WT and *pex1* at 20 days after heading. **d** Relative expressive levels of lignin-related genes in the second internodes of wild-type and *pex1/pex1* plants at 10 days before heading. **e** Lignin content of stem in WT and knock-down transgenic plants (KD) at 20 days after heading. **f** qRT-PCR analysis of lignin-related genes in the second internodes of WT and KD plants at 10 days before heading. Each column represents the means of three biological samples \pm SD. Asterisks represent significant difference determined by Student's *t*-test at ***p* value < 0.01, not significant (n.s.)



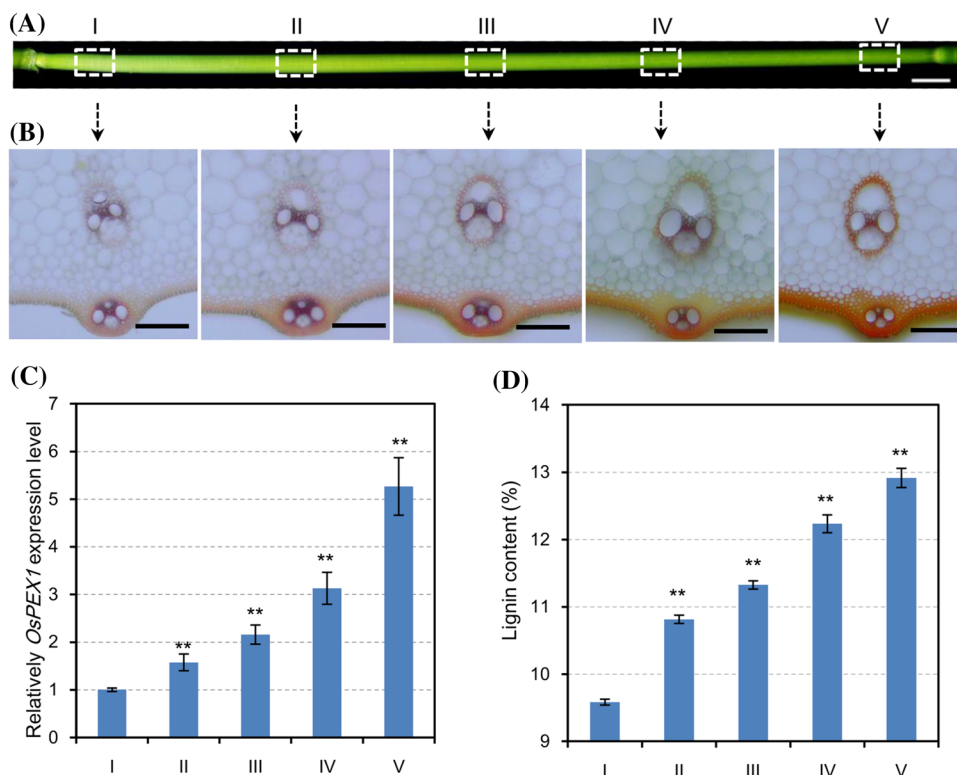
results and our earlier observations support that *OsPEX1* may function in lignin biosynthesis and deposition.

***OsPEX1* overexpression confers rice dwarfism and lodging resistance**

Lodging resistance is an agronomically important trait for crop productivity, which is closely related to plant height and culm strength. The loss-of-function mutations in GA 20-oxidase, namely *sd1* alleles known as “Green Revolution Gene”, have been widely used in rice dwarfism breeding.

Thus, the use of semi-dwarf trait to increase lodging resistance by reducing plant height has been effectively exploited and is proven beneficial to a certain degree (Yano et al. 2015). Another potential strategy for improving lodging resistance is to explore traits with enhanced culm strength. It has been reported that lignin content positively correlated with lodging resistance (Peng et al. 2014). Given the fact that *OsPEX1* play a crucial role in lignin deposition in rice stem, we examined the effect of *OsPEX1* overexpression on rice lodging resistance. The entire *OsPEX1* coding sequence driven by a maize Ubiquitin promoter was introduced into

Fig. 4 Effects of *OsPEX1* expression on lignin accumulation in developing rice internodes. **a** Schematic structure of the second internode at 10 days before heading, tentatively divided into five zones (from lower to upper, with I being the youngest stage). Bar = 1 cm. **b** Fresh hand cut cross-sections of the segments in **a** stained with phloroglucine-HCl. Bar = 100 μ m. **c** Relatively *OsPEX1* expressive level in the five segments of the second internode. The expression levels of *OsPEX1* in segment I was assigned value of 1. **d** Lignin content of the five segments of the second internode. Values are means \pm SD. (n = 3). Asterisks represent significant difference determined by Student's *t*-test at ***p* value < 0.01



two representative cultivars (*japonica* variety Nanyangzhan, W10; *indica* variety Basmati 370, W11) with functional *SD1* allele encoding gibberellin 20-oxidase (*OsGA20ox2*), a key enzyme in the gibberellin biosynthesis pathway. We found that the transgenic plants W10-T and W11-T showed reduced plant height (Fig. 5a), reduced diameter of culm (Fig. 5b), elevated transcriptional level of *OsPEX1* (Fig. 5c) and increased lignin content (Fig. 5d), resulting in high stem breaking resistance (Fig. 5e), indicating increased lodging resistance. The findings suggest that overexpression of *OsPEX1* can significantly decrease the risk of rice lodging by increasing culm strength and reducing plant height independent of the *sd1* allele.

Discussion

Ds insertion induced ectopic expression of the *pex1* allele

In this study, we characterized a rice semi-dominant dwarf mutant line caused by a *Ds* insertion in the promoter of the rice LRX gene *OsPEX1*. Our analyses revealed that the semi-dominant dwarf phenotype was a result of *OsPEX1* ectopic expression induced by the *Ds* insertion. Ectopic expression of the mutant allele could be caused by a loss of negative regulation of *OsPEX1* transcription due to the *Ds* insertion. Alternatively, *Ds* transposon insertion could

provide new cis-elements and alter the transcriptional regulation of the inserted genes. The latter is particularly evident in maize where many *KNOX* genes were originally identified as dominant or semi-dominant mutants where ectopic expression of the transposon-inserted genes is responsible for the mutant phenotypes (Hake et al. 1989; Vollbrecht et al. 1991; Schneeberger et al. 1995; Muehlbauer et al. 1999). Furthermore, a rice mutant allele, *OSH6-Ds*, was generated by inserting a *Ds* element into the *OSH6* gene (an ortholog of the maize *Liguleless3 KNOX* gene) also resulted in ectopic expression of a truncated novel transcript caused by the *Ds* insertion (Park et al. 2007). Based on these reports, we favor the model that ectopic *OsPEX1* expression in *pex1* is likely due to transposon induced new transcriptional regulation (Lisch 2013).

OsPEX1 affects rice lodging resistance

Increasing evidences have suggested that a hierarchical transcriptional network is involved in the coordinated regulation of the biosynthesis of secondary walls, in which several NACs (*NAM*, *ATAF*, and *CUC2*) are located at the top tier of regulatory hierarchy and orchestrate a cascade of downstream transcription factors, leading to the activation of secondary wall biosynthetic genes (Zhong and Ye 2007; Demura and Ye 2010; Huang et al. 2015). It has been shown that three rice secondary wall-associated NACs (namely *OsSWN1*, *OsSWN3* and *OsSWN7*) are master transcriptional

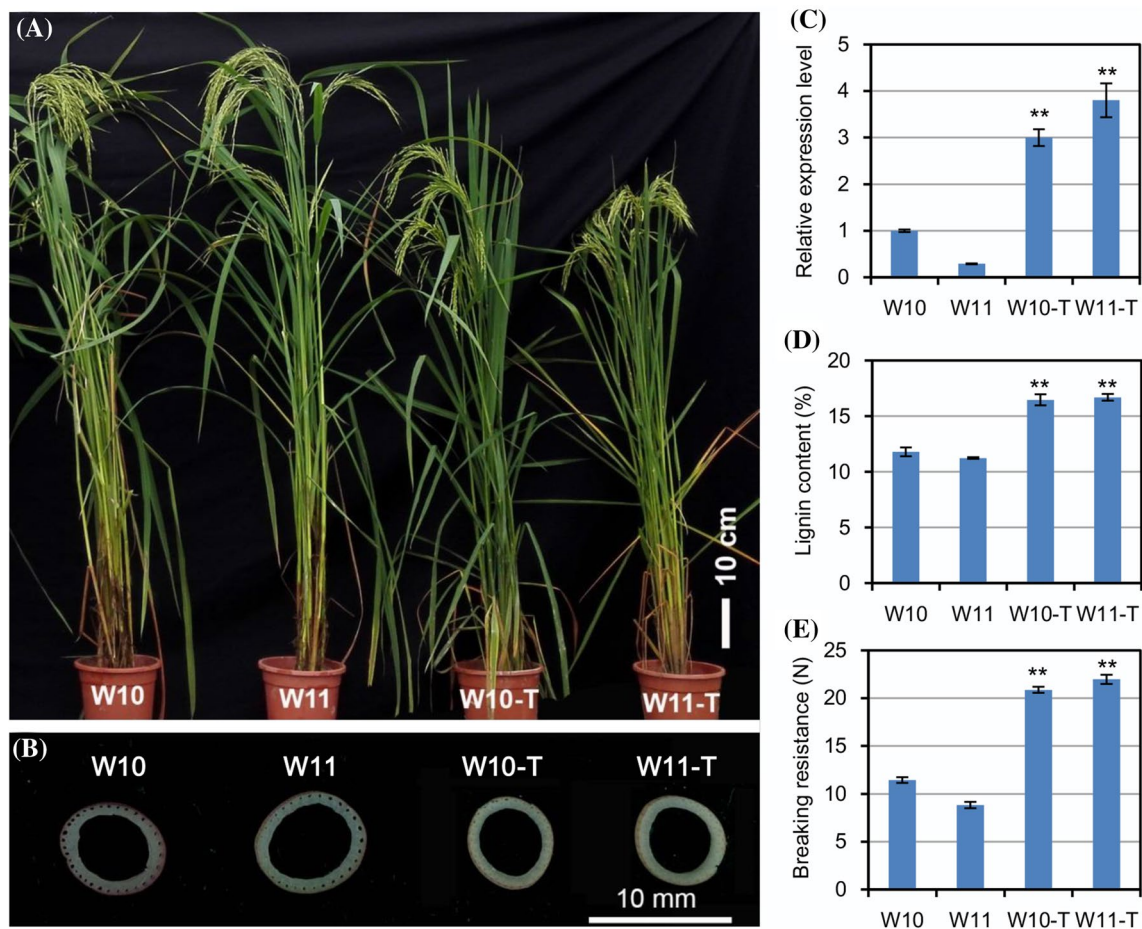


Fig. 5 Effects of overexpressing *OsPEX1* on rice lodging resistance. **a** Morphological characteristics of transgenic plants overexpression *OsPEX1* gene at 20 days after heading. **b** Transverse sections of the fourth internodes 20 days after heading from corresponding cultivars in **a**. **c** Relative *OsPEX1* expressive level of the second internode in the corresponding cultivars at 10 days before heading. The expression level of *OsPEX1* in W10 was assigned as the value of 1. **d** Lignin content of the fourth internodes 20 days after heading from corresponding cultivars in **a**. **e** Breaking resistance of fourth internodes 20 days after heading from corresponding cultivars in **a**. W10 (*japonica* variety Nanyangzhan) and W11 (*indica* variety Basmati 370) were two representative cultivars with functional *SD1* allele encoding *gibberellin 20-oxidase* (*OsGA20ox2*); W10-T and W11-T represent the resulting transgenic plants overexpression *OsPEX1* gene. Each column represents mean \pm SD ($n=3$). ** $p < 0.01$ based on Student's *t*-test

activators of the secondary wall biosynthetic program (Zhong et al. 2011). When overexpressed in *Arabidopsis*, *OsSWNs* can activate a number of secondary wall-associated transcription factors and biosynthetic genes, and ectopic deposition of cellulose, xylan and lignin (Zhong et al. 2011). In this study, we found that in *OsPEX1* overexpressing lines, the expression of the rice *SWNs* were substantially up-regulated, while knockdown of *OsPEX1* significantly reduced their expression (Fig. 3), consistent with the alterations of lignin content in the *peX1* mutants.

Significantly, overexpression of *OsPEX1* can substantially decrease the risk of lodging occurrence not only by altering the plant height but also by increasing the physical strength of the culm internode through altering the lignin accumulation (Fig. 5), suggesting that *OsPEX1* could be a promising candidate gene for novel breeding strategy.

spending cultivars in **a**. **e** Breaking resistance of fourth internodes 20 days after heading from corresponding cultivars in **a**. W10 (*japonica* variety Nanyangzhan) and W11 (*indica* variety Basmati 370) were two representative cultivars with functional *SD1* allele encoding *gibberellin 20-oxidase* (*OsGA20ox2*); W10-T and W11-T represent the resulting transgenic plants overexpression *OsPEX1* gene. Each column represents mean \pm SD ($n=3$). ** $p < 0.01$ based on Student's *t*-test

Coordinated regulation of growth and lignin deposition

The formation of cell wall is necessary for plant growth and development. It is well known that the processes of cell expansion and elongation are tightly correlated with the regulation of lignification (Rogers and Campbell 2004). As shown in Fig. 4, during cell elongation, the mRNA level of *OsPEX1* is lower, thus avoiding excessive accumulation of lignin and permitting cell expansion and elongation. By contrast, at the mature zone the expression of *OsPEX1* is up-regulated, promoting lignin deposition and enhancing the physical strength of stem. Therefore, it is proposed that *OsPEX1* is involved in the regulation of secondary cell wall biosynthesis in response to plant growth and development.

Cell expansion cannot occur smoothly unless cell wall is sufficiently softened to allow cell expansion but rigid enough to resist turgor pressure. Lignin is a major component of secondary cell wall and the deposition of lignin is closely correlated with cell expansion (Rogers and Campbell 2004). Not surprisingly, given the fact that *OsPEX1* plays a vital role in lignin deposition, overexpression and down-regulation of *OsPEX1* could lead to a significant change in lignin deposition and results in diminished plant growth. In fact, it has been reported that mutation of lignin-related genes leads to a significant change in lignin deposition as well as reduced plant growth (Rogers and Campbell 2004; Yoon et al. 2015), supporting the idea that proper lignin deposition is essential for appropriate cell expansion and plant development.

Our results indicate that *OsPEX1* plays an important role in lignin deposition, thus affecting cell expansion and altering plant stature in rice. However, the regulatory mechanisms of lignin biosynthesis and deposition by *OsPEX1* remain to be elucidated. Increasing evidences suggest that LRXs could potentially be involved in the regulation of cell wall expansion in response to signals (Baumberger et al. 2001; Borassi et al. 2016). More recently, it has been shown that *Arabidopsis* LRX8/9, an orthologue of *OsPEX1*, can bind small peptides, which function as signal molecules (Mecchia et al. 2017). Thus, it is possible that rice *OsPEX1* may also have a signaling capacity in regulating lignin biosynthesis and deposition.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant Nos. 30900884, 31622041, 31471788 and 31671594); by Natural Science Foundation of Guangdong Province, China (Grant Nos. 2014A030313457 and 2015A020209118); by the Hatch Project of National Institute of Food and Agriculture, U.S.D.A (Grant No. 02413).

Author contributions SK and XL performed experiments and conducted fieldwork. JL and YHH worked on the transgenic lines. TFH and XQZ designed the experiments and analyzed the data; TFH supervised and complemented the writing; XQZ conceived the project and wrote the article with contributions of all the authors.

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