OsPEX1, a leucine-rich repeat extensin protein, plays an important role in the regulation of caryopsis development in rice

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Abstract

Rice caryopses are enclosed by the outside glumes. It is long been acknowledged that the size and dimension of the outer glume is the main determinant that dictates the caryopsis size. However, it is unclear whether the development of caryopsis is completely dependent on the size of the glume, or it can grow and expand autonomously in spite of the constraint of glume enclosure. Here we report the identification of a mutant line that produces normal-sized glumes with smaller mature caryopsis that do not fill up the entire glume cavity. The mutant phenotype is caused by ectopic expression of a leucine-rice repeat extensin gene, OsPEX1 . The caryopsis phenotype in pex1 was caused by a reduction in cell size. OsPEX1 is highly expressed in the developing caryopsis. Over expression of the OsPEX1 gene driven by a constitutive promoter recapitulates the mutant phenotype, confirming that the small caryopsis phenotype is caused by ectopic expression of the OsPEX1 gene. Our results suggest that caryopsis development can be genetically uncoupled from maternally controlled glume development.

Background

Rice is one of the most important staple food crops in the world, and is primarily consumed by human as polished white or unpolished brown rice. Grain size, usually measured by grain length, width, thickness and weight, is an important quantitative trait for rice because it affects the yield and the quality of rice; and has attracted considerable attention as one major topic for research and breeding. The rice grain is derived from a floret with caryopsis that is encased by the glume (lemma and palea). The caryopsis consists of the diploid maternal tissues (pericarp, testa, and nucellus), the triploid endosperm, and the diploid embryo. During flower development, the glume expands its volume and grows to maximum size prior to anthesis. Inside the mature glume, the caryopsis starts to grow and increase in size after fertilization, and reaches its final size around twenty days after fertilization (Yoshida and Nagato, 2011). Although the glume and caryopsis grow and develop at different time points, the mature caryopses perfectly fill the inner chamber enclosed by the glumes. Thus, it has long been accepted that the size and dimension of the outer glume is the main determinant that dictates the caryopsis size.

Recent studies have begun to shed light into the regulatory mechanism of glume development through identification and functional characterization of genes associated with grain size and yield. To date, nearly one hundred genes associated with grain shape and weight have been isolated in rice (Huang et al., 2013; Li et al., 2019). However, almost all of the genes identified so far affect glume size. For example, natural mutants of GWs (grain width) produce wider glumes and widened caryopses. Other genes affecting glume size that have been studied influence glume size either by affecting cell number and size in lemmas and palea, or cell cycle regulation (Abe et al., 2010; Kitagawa et al., 2010; Heang and Sassa, 2012; Nakagawa et al., 2012; Qi et al., 2012; Segami et al., 2012; Zhang et al., 2012; Luo et al., 2013).

With respect to the regulatory mechanism of glume development, phytohormone and G-protein signaling have been shown to play important roles in regulating grain size and yield in rice. For instance, several dwarf mutants in brassinosteroid (BR) hormone biosynthesis and signaling pathways affect grain and

panicle sizes, including *d61*, *d2, d11, sg1* and *RAV6* (Yamamuro et al., 2000; Mori et al., 2002; Hong et al., 2003; Tanabe et al., 2005; Nakagawa et al., 2012; Zhang et al., 2015). Mutations in the *Dwart1* (*D1*), also known as the rice heterotrimeric G protein alpha subunit (*RGA1*), affect multiple signaling pathways such as BR and GA, and have pleiotropic effects on organ growth and reduction in glum and seed size (Ashikari et al., 1999; Oki et al., 2009; Oki et al., 2009). Furthermore, *GW6,* encoding a GAST family protein, has been identified that control grain size through gibberellin pathway (Shi et al., 2020). Moreover, recent studies indicated that ubiquitin-proteasome pathway and mitogen-activated protein kinase (MAPK) signaling are also involved in the regulation of glume size (Li et al., 2019). However, little is known about how caryopsis size is controlled and regulated. In addition, it is unclear whether the development of caryopsis is completely dependent on the size of the glume, or it can grow and expand autonomously, independent of the constraint of glume enclosure.

Mutation in *OsKinesin-13A* (*sar1*) causes small glumes and rounded grains due to defects in cell elongation. Although glumes and caryopses were both reduced in length, *sar1* specifically affects glume elongation and length and the reduction in caryopses size was an indirect effect of the reduced space of the shortened glumes as the *sar1* caryopses can grow to the WT length after removal of the glume height constrain (Deng et al., 2015). This observation suggests a model that the cell length in glume and caryopsis can be regulated independently. However, very little genetic evidence has been uncovered to support such model.

Leucine-rich repeat extensins (LRXs) are a class of cell wall-localized chimeric proteins with leucine-rich repeat (LRR) domain and extensin domain containing Ser-Pro (3-5) repeated modules near the C terminus (Liu et al., 2016). Several LRXs in *Arabidopsis* have been studied in detail (Baumberger et al., 2001; Baumberger et al., 2003; Draeger et al., 2015). The rice genome encodes 8 LRXs proteins (Liu et al., 2016). However, little is known about the functions of LRXs protein in rice. Here we reported the identification of a rice mutant that affects caryopsis size, instead of the development of glume. The mutant phenotype is caused by ectopic expression of the *OsPEX1* gene, encoding an LRX protein with LRR domains and extensin domain. Our result provides a novel example that caryopsis growth and cell expansion can be separated from the constraint of pre-determined glume size.

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 Ac/Ds transposon-tagging population in the *japonica* rice variety Zhonghua 11 (Liu et al., 2007; Ke et al., 2019). All rice seeds in this study were propagated in the paddy field in Guangzhou, China.

Plasmid Construction and Rice Transformation

To produce *PEX1* overexpression transgenic plants, the full-length *PEX1* coding sequences was amplified from cDNA derived from rice ‘Zhonghua 11’ using corresponding primer pairs listed in Table S1. After

confirmation by DNA sequencing, the amplified sequence was cloned into the binary vector pCUbi1390 for *PEX1*overexpression in rice. The final constructs were electroporated into Agrobacterium tumefaciens strain EHA105.

For promoter analysis, a1572 bp DNA fragment immediately upstream of OsPEX1 start codon was amplified using primers LRXPF and LRXPF (Table S1) and inserted into the *EcoRI* and *Nco*I sites of the pCAMBIA1305.1 vector. The resulting plasmid was introduced into wild-type plant by Agrobacterium tumefaciens-mediated transformation. Transgenic plants were selected on Hygromycin medium, and T_2 transgenic plants were used to analyse GUS activity.

Quantitative RT-PCR

Total RNA was extracted from frozen samples with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The 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. Investigated genes and their specific primer sets are shown in Supplemental Table S1. The relative expression level of a target gene was normalized to that of rice *25sRNA*.

Histochemical analysis

The mature caryopses are soaked in FAA solution (50% ethanol, 5% glacial acetic acid and 5% formaldehyde) overnight at 4°C for cell fixation and dehydrated in a graded alcohol series. Then the samples were sectioned transversely into 2-mm slices using a razor blade and embedded in paraffin. Semi-thin sections were cut using a glass knife in a Leica ultra-microtome, stained with 0.05% (w/v) Fast Green, and 0.1% (w/v) Safranin. The histochemical slices were observed under a light microscope. By using Image J software, the cell size was calculated.

Results

pex1 produces normal size of glumes but smaller caryopsis

In previous study, we characterized a rice semi-dominant dwarf mutant *pex1* (Ke et al., 2019), which was obtained from a transposon-tagged population in the *japonica* rice variety Zhonghua 11(Liu et al., 2007). Interestingly, this mutant has almost the same size of glumes as the wild-type (WT) plants, but with obviously smaller caryopsis (Fig. 1a). Co-segregation analysis indicated that the dwarfism of *pex1* was companied by smaller caryopsis (data no shown). Compared with the wild type, caryopses width and the length of the heterozygous *pex1* mutant (*pex1/+*) were reduced by 18.5% and 4.0%, respectively, while the homozygous *pex1* mutant (*pex1/pex1*) had a 25.3% reduction in caryopses width and a 7.2% reduction in caryopses length (Fig. 1b). As a result, the 1000-grain weight of *pex1/+* and *pex1/pex1* were reduced by 25.0% and 33.8%, respectively (Fig. 1b).

Developmental characteristics of *pex1* caryopsis

Morphological analysis was carried out for the grain and caryopses of wild type and *pex1* mutant grown in a paddy field. The anthesis date of each floret was marked on the surface of the lemma using a marker, and samples were collected. The glumes of *pex1* plants were almost indistinguishable from wild type (WT) plants (Fig. 2a). However, the *pex1* mutant produced significantly smaller caryopses from 7 days after pollination (DAP) compared with the wild type (Fig. 2 b, c). The major extension of the rice caryopsis along the long axis occurred from 1 to 7 DAP, while major expansion along the transversal axis occurred between 1 and 15 DAP (Fig. 2 b, c). The results suggested that the growth pattern for caryopsis width differed from that of caryopsis length in rice.

OsPEX1 affects caryopsis size by influencing cell expansion

To test whether cell size is affected in those caryopses, aleurone cells of the caryopses from the WT and *pex1* plants were examined and indeed, smaller size aleurone cells were observed in the caryopses of the *pex1* plants (Fig. 3a-c). In order to examine the phenotype by molecular analyses, expression of several representative genes involved in cell expansion was investigated by quantitative RT-PCR (Fig. 3d). The cell expansion-related gene such as *OsEXP6* (LOC_Os03g21820) and xyloglucan endo-transglycosylase/hydrolase (XTH) genes including *XTH10* (LOC_Os06g48200) and *XTH17* (LOC_Os08g13920) were significantly downregulated in the caryopses of *pex1* mutants compared with the WT control. These results suggested the *pex1* mutation affected caryopsis size by decreased cell size.

Overexpression of *PEX1* Leads to a *pex1*-like Phenotype

Molecular and genetic analyses revealed that the dwarf phenotype of *pex1* was caused by ectopic expression of a leucine-rich repeat extension-like gene, *OsPEX1* (Ke et al., 2019). To confirm that ectopic expression of *OsPEX1* (*LOC_Os11g43640*) is also responsible for the small caryopsis in *pex1*, we overexpressed *LOC_Os11g43640* in the wild type plant under the control of the maize (*Zea mays*) Ubiquitin promoter. Compared to WT control, all positive transformants displayed a normal size of glumes but smaller caryopsis, similar to the phenotype characteristic of the *pex1* mutants (Fig. 4). Thus, we concluded that the abnormal phenotypes of the *pex1* mutant results from ectopic expression of *OsPEX1* (*LOC_Os11g43640*).

Expression pattern of *OsPEX1*

Given that *pex1* exhibits the normal size of glumes but smaller caryopsis than WT, we compared the transcriptional level of *OsPEX1* in glumes and caryopsis between WT and *pex1* mutant. We found that in

the caryopsis *OsPEX1* gene in *pex1* mutants was transcribed approximately fourfold more abundant than in WT, whereas in the glumes *OsPEX1* was not differentially expressed between the WT and *pex1* mutants (Fig. 5a). The findings indicate that *OsPEX1* negatively modulates caryopsis size in rice. To elucidate the role of *OsPEX1* in caryopsis development, we further investigate the expression pattern of *OsPEX1*during caryopsis development. As shown in Fig. 5b, the transcript of *OsPEX1* was rapidly increased after flowering, reaching peak (approximately seven folds) 7 DAP, and then reducing but retain higher level at 10 DAP.

Further promoter-β-glucuronidase (GUS) fusion analysis revealed that *OsPEX1* was expressed in the outer surface of the endosperm, including the aleurone layer of developing caryopses (Fig. 5c), especially the dorsal vascular bundle of developing caryopses with high levels of GUS expression (Supplemental Fig. S1). Interestingly, GUS expression was restricted to embryo at mature seed, no GUS staining was detected at the aleurone layer of mature seed.

We also conducted the expression analysis of several key genes involved in glume regulation in WT and *pex1* mutant. As expected, qRT-PCR (Quantitative reverse transcription PCR) results showed that the transcriptional level of eight genes tested in *pex1* mutant were similar to that in WT (Supplemental Fig. S2), suggesting the ectopic expression of *OsPEX1* have less effects on genes related to glume size, consistent with the *pex1* phenotype with normal glume compared to WT plants.

Phylogenetic analyses of LRXs family in rice and *Arabidopsis*

We investigated the phylogenetic relationship between rice and Arabidopsis LRXs genes by comparing the putative full-length protein sequences, which can be divided into two sub-groups, LRX sub-group and PEX one (Fig. 6a), as previous reported by Baumberger et al (Baumberger et al., 2003). All of the putative OsLRX proteins contain the conserved leucine-rich repeat (LRR) motifs for protein-protein interactions and extensin domain probably involved in cross-linking to cell wall components (Fig. 6B). A cysteine-rich domain (CRD) might act to stabilize the structure of the LRX protein through forming disulfide bonds with cysteine residues of the LRR domain (Herger et al., 2019; Moussu et al., 2020). None of the *OsLRXs* genes except for OsPEX2 has introns (Fig. 6b). *OsPEX1* has the longest coding sequence (CDS) in the LRXs members of rice (Fig. 6b).

Discussion

Zhu and colleagues reported that OsKinesin-13A, a microtubule depolymerase plays a role in the regulation of glume length by affecting cell elongation (Deng et al., 2015). The authors showed that the mutant of *OsKinesin-13A*, *sar1*, displayed length reduction in the glume. However, WT and *sar1* caryopses matured under glume-cutting conditions were similar in length. The observations indicated that the grain phenotype was caused by reduction in glume length, which indirectly restricted caryopsis size. Although some studies of the relationship between glume and caryopsis development were discussed based on

notched grains in rice (Takeda et al., 1981; Xiong et al., 1986), the observations by Deng and colleagues (Deng et al., 2015) suggest that the size and length of floral glume and caryopsis can be regulated independently.

The length reduction in *sar1* caryopses was not a direct consequence of mutation in *OsKinesin-13A*, but resulted from the space restrictions due to shortened glumes (Deng et al., 2015), indicating that *OsKinesin-13A* controls glume size but not caryopsis size. By contrast, mutation in *OsPEX1* directly affected caryopsis size but had little effect on glume size. These results indicate that *OsPEX1* is directly involved in regulating caryopsis size. Thus, our findings strongly support the model that caryopsis development can be separated from maternally controlled glume development.

The *OsPEX1* gene is a member of *LRX* (*LRR/EXTENSIN*) gene family. The LRX proteins are characterized by a domain with leucine-rich repeats in addition to the extension domain. LRRs are frequently implicated in protein-protein interactions and signal transduction during development or in pathogen recognition and defense (Song et al., 1995; Torii et al., 1996; Jinn et al., 2000). Furthermore, one proposed function of extensins is to lock-in cell shape upon cell expansion (Carpita and Gibeaut, 1993; Hall and Cannon, 2002). Together, the properties of LRR and extensins, LRXs could potentially be involved in the regulation of cell wall expansion in response to signals. Such function in cell morphogenesis is highlighted by the finding that the Arabidopsis *lrx1* mutant developed aberrant root hairs (Baumberger et al., 2001). The idea is also supported by the observation in this study that *pex1* had smaller aleurone cells than WT (Fig. 3), consistent with a role of OsPEX1 in cell wall expansion. Together with the observations that overexpression of *OsPEX1* resulted in reduction of smaller caryopsis, our results support the existence of a negative regulatory role of *OsPEX1* on caryopsis development.

According to expression pattern, *LRX* genes of higher plants can be classified as vegetatively expressed or predominantly expressed in reproductive tissue, two categories that almost completely overlap with the phylogenetic clades (Baumberger et al., 2003; Herger et al., 2019). Interestingly, in spite of the fact that OsPEX1 belong to PEX (pollen expression LRX) subfamily, it is highly expressed in root and stem as well as reproductive tissue such as developing caryopsis, but is expressed at much lower level in leaf and glume, consist with the pleiotropic phenotypes of the *pex1* plants with dwarfism (Ke et al., 2019)and small caryopsis (Fig. 1), but have less effect on leaves (Ke et al., 2019) and glume developments (Fig. 1).

Our results indicate that *OsPEX1* plays an important role in caryopsis development. However, the regulatory mechanisms of caryopsis development by *OsPEX1* remain to be elucidated. It has been shown that the LRR domain of LRX proteins, which is highly conserved in higher plants, can directly interact with RALFs (rapid alkalinization factors) peptides hormone and CrRLK1L (*Catharanthus roseus* receptor-like kinase1-like) transmembrane receptors, and hence LRXs have a regulatory or signaling function during cell growth in *Arabidopsis* (Herger et al., 2019). Rice *RALF* and *CrRLK1L* gene family contain 46 and 16 members, respectively (http://rice.plantbiology.msu.edu/). Future research is need to investigate which RALF or CrRLK1L members influence the functions of OsPEX1, and whether they can physically interaction with OsPEX1 protein.

Conclusion

In the present study, we propose that *OsPEX1* negatively regulates caryopsis size through influencing cell expansion in rice. In addition, our results suggest caryopsis development can be separated from maternally controlled glume development.

Abbreviations

Brassinosteroid (BR); CrRLK1L (*Catharanthus roseus* receptor-like kinase1-like); Cysteine-rich domain (CRD); Days after pollination (DAP); β-glucuronidase (GUS) GWs (grain width); Heterotrimeric G protein alpha subunit (*RGA1*); Leucine-rich repeat (LRR); Leucine-rich repeat extensins (LRXs); Mitogen-activated protein kinase (MAPK); PEX (pollen expression LRX); Quantitative reverse transcription PCR (qRT-PCR); Rapid alkalinization factors (RALFs).

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

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Authors' contributions

S.L., S.K., G.T., D.H. and M.W. performed experiments and conducted fieldwork. Y.Z. worked on the transgenic lines. G.Q. designed the experiments and analyzed the data; X.Q.Z. conceived the project and wrote the article with contributions of all the authors.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

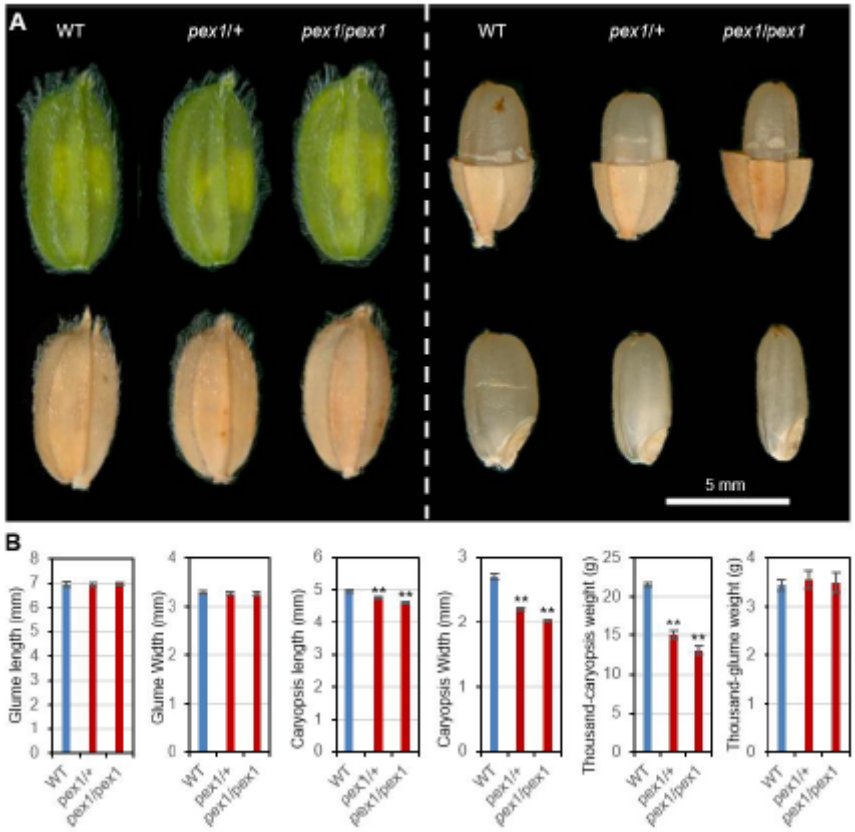

Figure 1

Phenotypic characterization of the pex1 mutant. a pex1 exhibits normal size of glumes but smaller caryopsis. b Comparisons of glume length, glume width, caryopsis length, caryopsis width and 1000-caryopsis, glume weight of WT and pex1 mutant. All data are means ± SD (n = 30). **P < 0.01, determined by Student's t-tests.

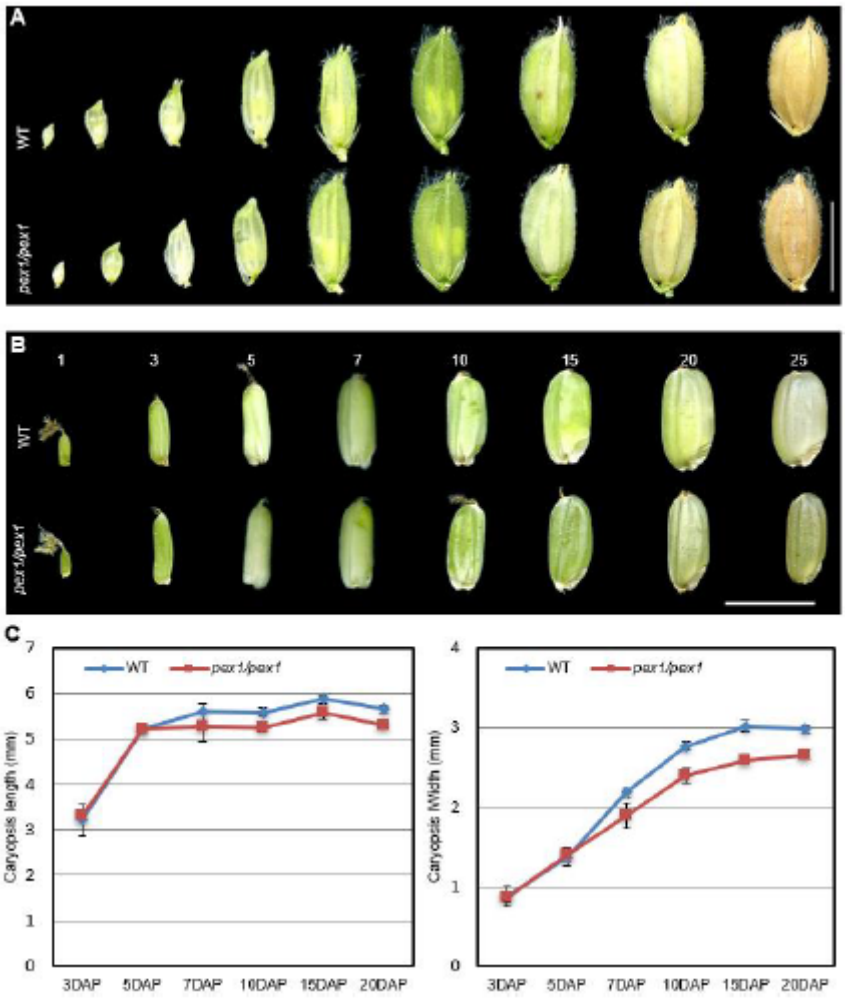

Figure 2

Dynamic characteristics of the pex1caryopsis development. Rice grain (a) and caryopsis (b) development from 0 to 25 days after pollination (DAP). Numbers above denote the DAP. The rapid elongation of the caryopsis occurred in the first 5 DAP; The major period of expansion occurred in the first 15 DAP. c comparison of caryopsis length and caryopsis width of WT and pex1 mutant. Scale bar = 5mm

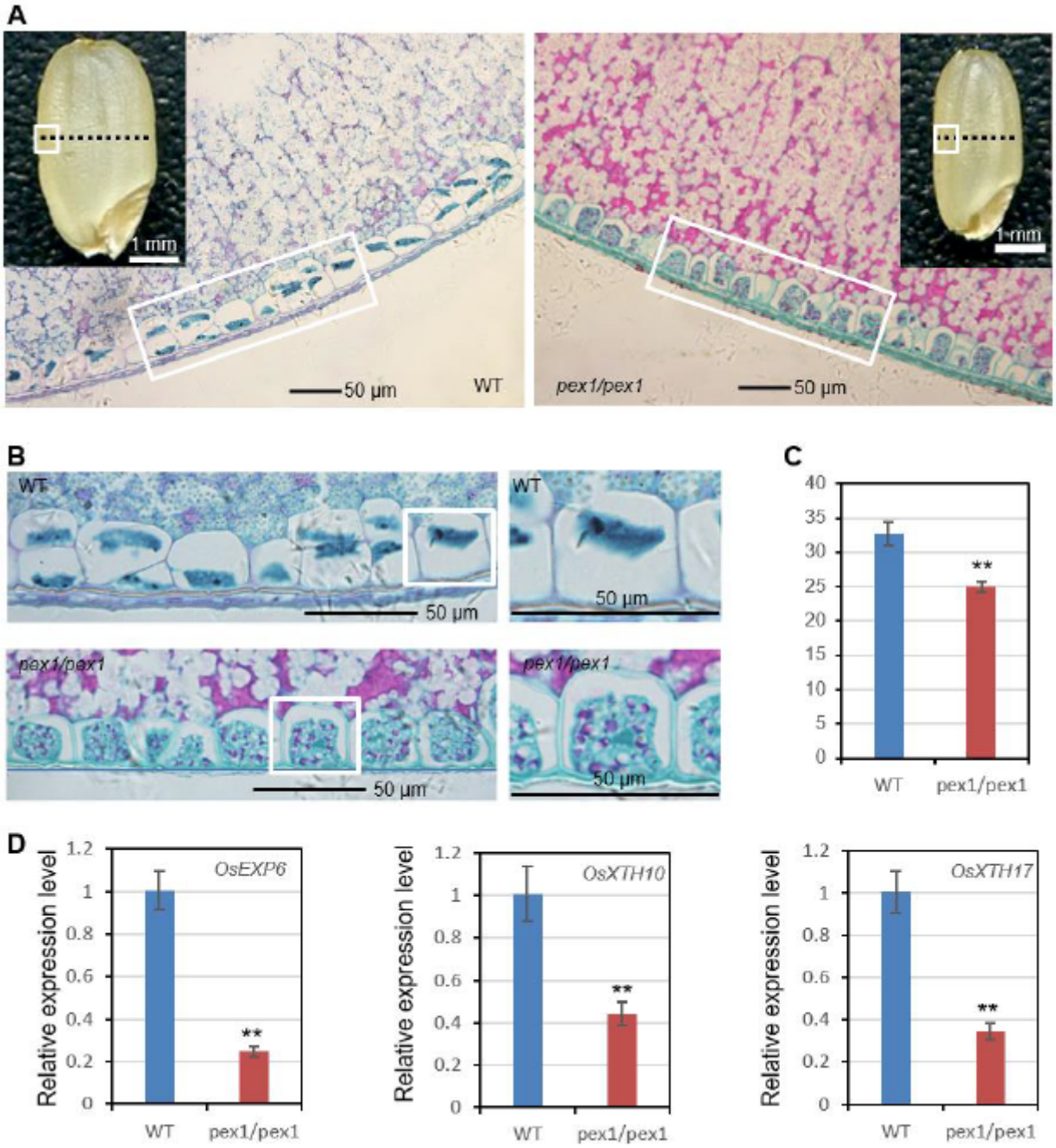

Figure 3

The pex1 mutation affected caryopsis size by decreased cell size. a Cross sections of mature caryopses at the ventral positions of WT and pex1 endosperms. Dotted lines indicate sites of cross sections in the WT and pex1caryopses. b Magnified images of the boxed areas in (a). c Comparison of average length of each cell in the aleurone layer. All data are means ± SD. (n = 10). **P < 0.01, determined by Student's t-tests. d, Transcript levels of cell expansion-related genes in caryopsis of pex1/pex1 at 7 DAF, relative to WT. The rice 25sRNA gene was used as an internal control. Data are means ± SD. (n = 3), **P < 0.01, determined by Student's t-tests.

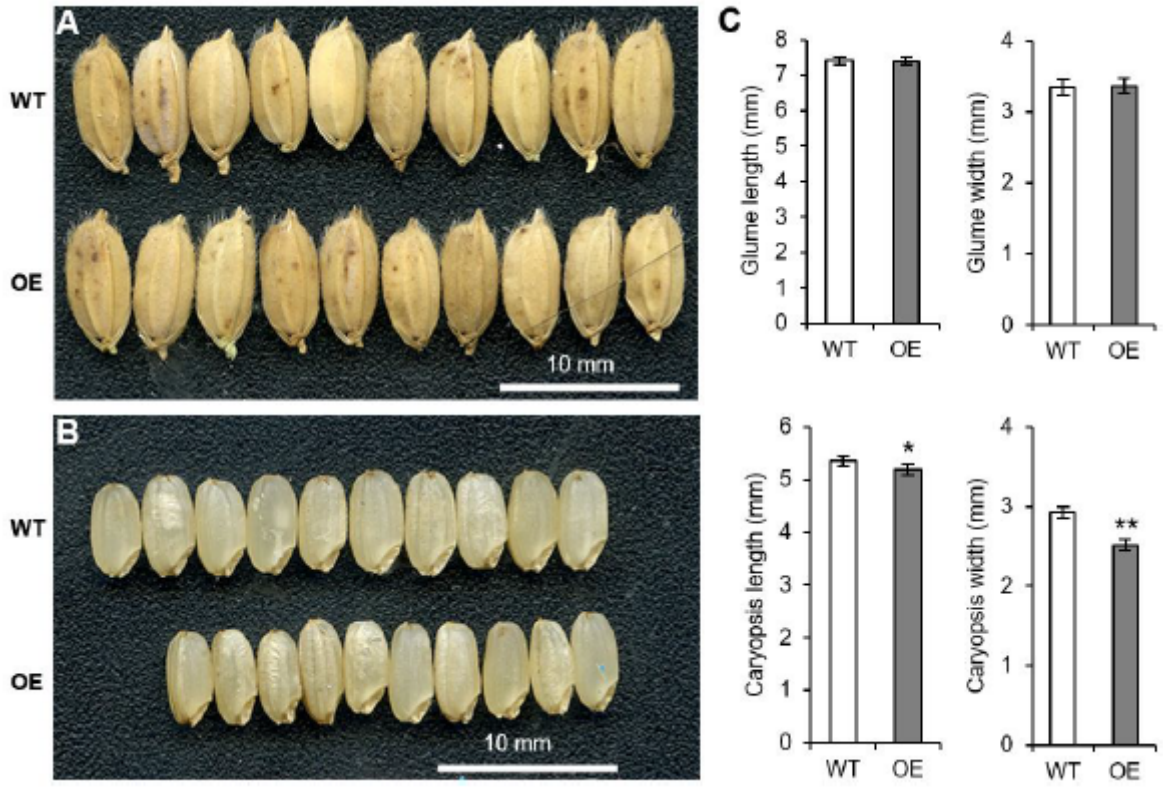

Figure 4

Overexpression of OsPEX1 phenocopies the pex1 mutant phenotype. The grain (a) and caryopsis (b) morphologies of the wild-type (WT) and transgenic plants overexpressing OsPEX1. OE indicated transgenic plants overexpressing rice PEX1. c Characterization of glume length, glume width, caryopsis length and caryopsis width in the wild type and the transgenic plants (OE) overexpressing rice OsPEX1. Values are means ± SD. In each graph, statistically significant differences are indicated by the asterisks (*P<0.05, **P<0.01).

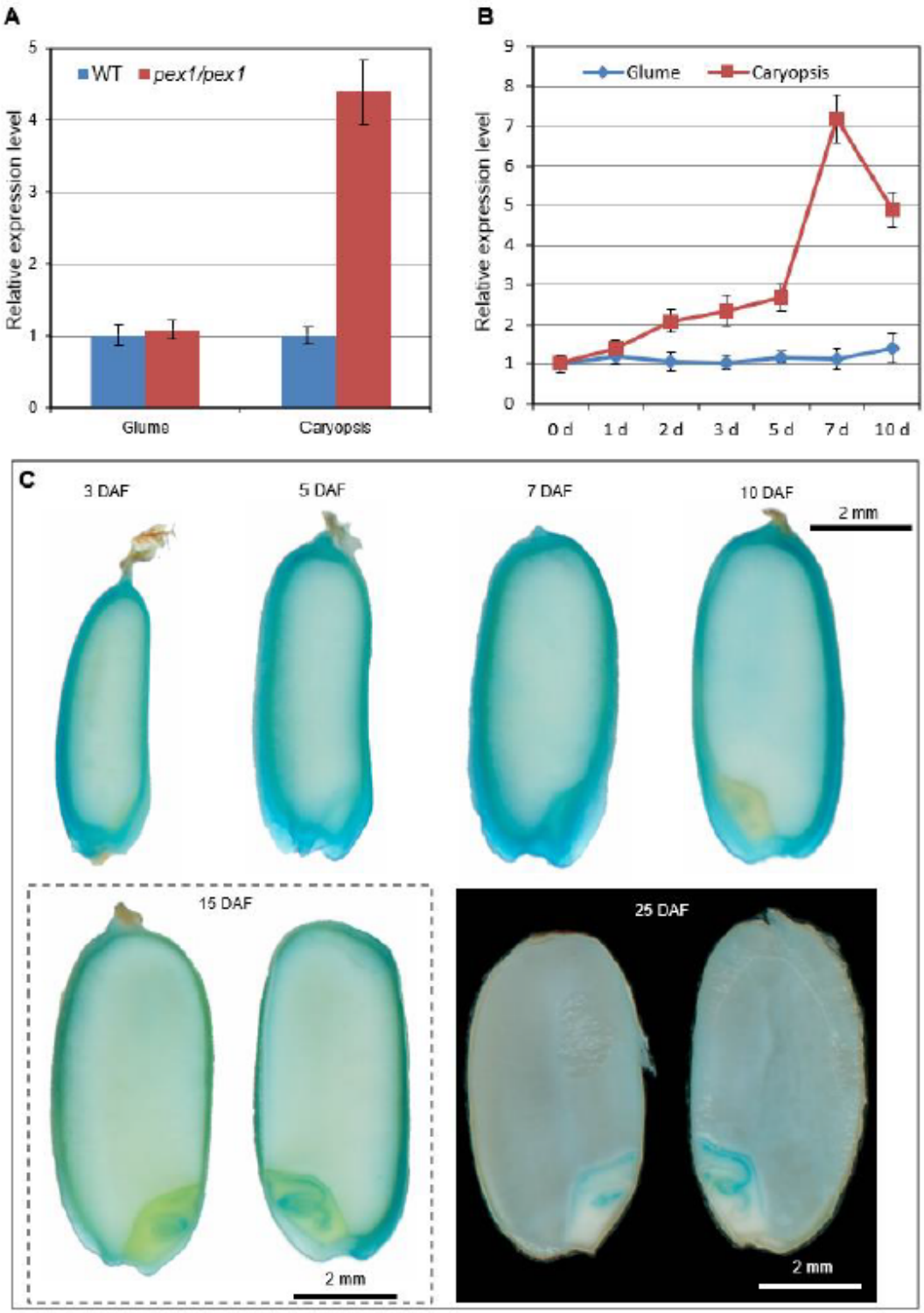

Figure 5

Expression pattern of OsPEX1. a Comparisons of the transcriptional level of OsPEX1 in glumes and caryopsis between WT and pex1 mutant plants. b Change over time in the OsPEX1 transcription levels of rice caryopsis from 0 to 10 day after flowering. c OsPEX1p::Gus expression pattern in developing caryopses.

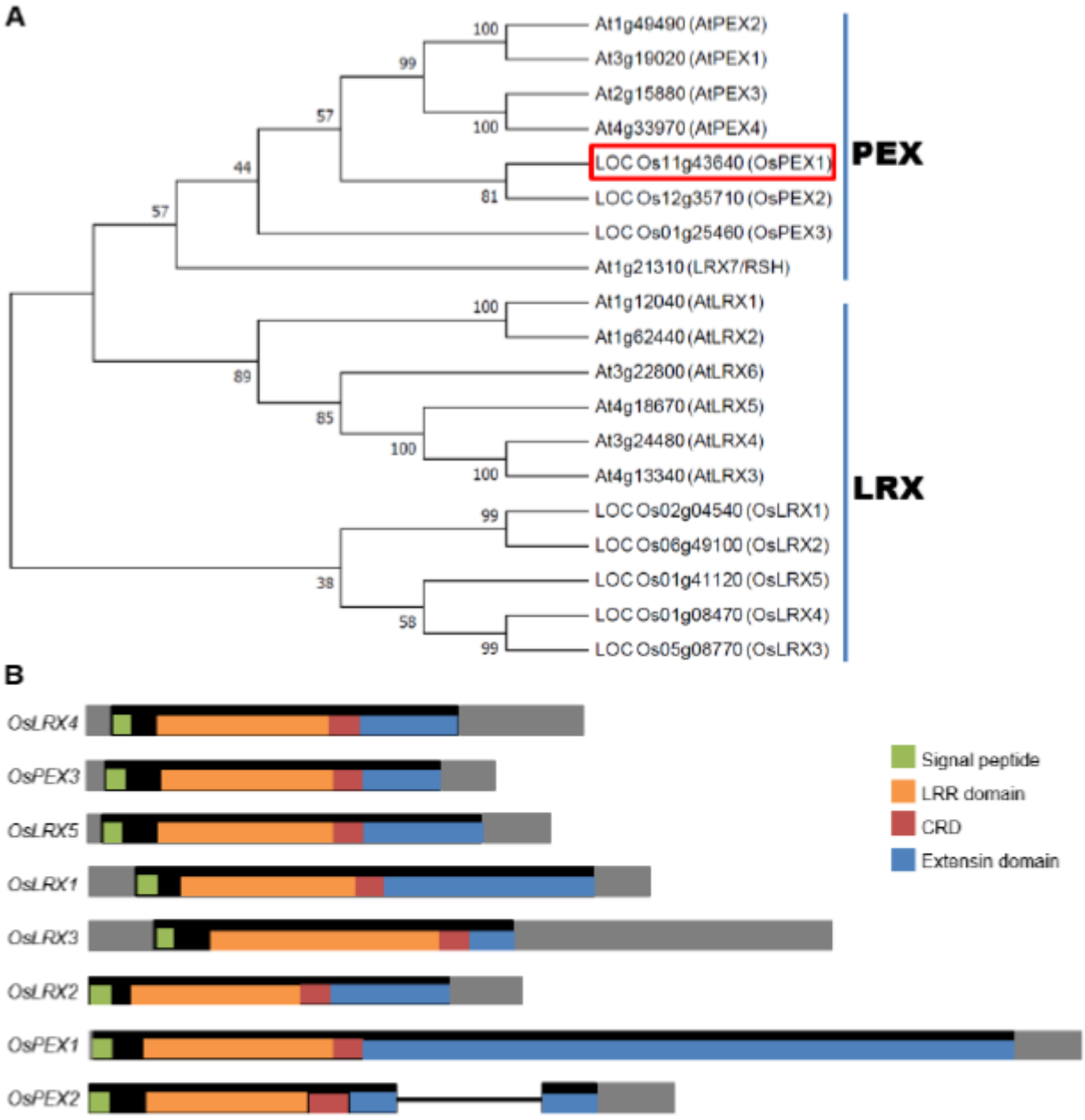

Figure 6

Identification and analysis of putative rice LRX genes. a Phylogenic analysis of OsLRXs. The phylogenetic trees were constructed using Mega7.0 program with neighbor-joining method; bootstrap analysis was performed with 1000 replicates and excluding positions with gaps. Based on the analysis, OsLRXs were divided into two sub-groups. b Gene structures and conserved domains of the OsLRXs. Thin lines represent introns, dark bars refer to putative protein-coding sequence (CDS), the untranslated regions are shown in gray rectangles. LRX proteins contain a signal peptide for protein export, an LRR domain, a cysteine-rich linker domain (CRD) and an extensin domain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SI202722FiguresTables.pdf