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REGULAR PAPER – GENETICS/DEVELOPMENTAL BIOLOGY

Cold-upregulated glycosyltransferase gene 1 (*OsCUGT1*) plays important roles in rice height and spikelet fertility

Lanxin Zhao[1] · Hui Liu[1] · Kangli Peng[1] · Xiaozhen Huang[1,2]

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Abstract

Glycosyltransferases (GTs) regulate many physiological processes and stress responses in plants. However, little is known about the function of GT in rice development. In this study, molecular analyses revealed that the expression of a rice GT gene (Cold-Upregulated Glycosyltransferase Gene 1, *CUGT1*) is developmentally controlled and stress-induced. *OsCUGT1* was knocked out by using the clustered regularly interspaced short palindromic repeats (CRISPR) system to obtain the mutant *oscugt1*, which showed a severe dwarf and sterility phenotype. Further cytological analyses indicated that the dwarfism seen in the *oscugt1* mutant might be caused by fewer and smaller cells. Histological pollen analysis suggests that the spikelet sterility in *oscugt1* mutants may be caused by abnormal microsporogenesis. Moreover, multiple transgenic plants with knockdown of *OsCUGT1* expression through RNA interference were obtained, which also showed obvious defects in plant height and fertility. RNA sequencing revealed that multiple biological processes associated with phenylpropanoid biosynthesis, cytokinin metabolism and pollen development are affected in the *oscugt1* mutant. Overall, these results suggest that rice *OsCUGT1* plays an essential role in rice development.

Keywords Glycosyltransferases · Plant height · Rice · Secondary metabolism · Spikelet fertility

Introduction

The development of stems and inflorescences in crop plants is fundamentally important to their growth and productivity (Wang et al. 2018). In rice (*Oryza sativa* L.), plant height and spikelet fertility rely on a sophisticated regulatory network in which thousands of genes work in a coordinated and integrated fashion during development (Liu et al. 2018; Wang et al. 2018; Zeng et al. 2017). In the past few decades, genetic and genomic studies in model plant species have uncovered many of the molecular mechanisms underlying the control of plant height, which is primarily determined by several endogenous phytohormones, including gibberellins, brassinosteroids, cytokinins, auxin and strigolactones (Liu et al. 2018). It has been reported that functional alleles in the gibberellin biosynthesis gene *semidwarf 1* (*sd1*) result in dwarfism to different degrees (Spielmeyer et al. 2002; Ueguchi-Tanaka et al. 2005). In addition, cytokinins also exert essential roles in the regulation of plant height because they can control many agriculturally important processes, including cell division, stem elongation and other developmental processes (Heyl and Schmulling 2003). Decapitation of cytokinin catabolism or the disturbance of cytokinin signaling dramatically decreases plant yield and height (Xiao et al. 2020). For example, reduced expression of *CYTOKININ OXIDASE/DEHYDROGENASE 2* (*OsCKX2*), an enzyme that functions in cytokinin oxidative cleavage and homeostasis, enhanced grain yield (Ashikari et al. 2005). Overexpression of *OsCKX2* resulted in developmental defects due to cytokinin level reduction (Yan et al. 2020). In addition, it has been reported that overexpression of a *B-TYPE CYTOKININ RESPONSE REGULATOR* (*OsORR2*) also reduces rice height (Shi et al. 2020).

Spikelet fertility is also vital for crop production. The genetic basis of floral organ initiation and development has been extensively studied in Arabidopsis (*Arabidopsis*

✉ Xiaozhen Huang
xzhuang@gzu.edu.cn

[1] Key Laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), College of Life Sciences/ Institute of Agro-Bioengineering, Guizhou University, Guiyang 550025, China

[2] College of Tea Sciences, Guizhou University, Guiyang 550025, China

thaliana) and rice (Wang and Li 2008). Plant reproductive success is influenced by various environmental and developmental factors (Nubankoh et al. 2020). Numerous physical factors, important molecular components, and complex regulatory interactions contribute to spikelet fertility by affecting pollen formation and viability, anther dehiscence, and flower opening (Hu et al. 2021; Jagadish et al. 2007; Qi and Wu 2022; Zhou et al. 2017). Accumulating genetic evidence underscores the importance of multiple transcription factors, lipid metabolism- and sugar metabolism-related enzymes in spikelet fertility. For example, *TAPETUM DEGENERATION RETARDATION* (*OsTDR*) encodes a putative basic helix–loop–helix transcription factor that functions in tapetum degradation and anther development (Li et al. 2006). *SUCROSE PHOSPHATE SYNTHASE* (*OsSPS1*) is essential in pollen germination by modulating sucrose synthesis (Hirose et al. 2014). Despite extensive efforts to elucidate the molecular regulation of plant height and spikelet fertility in rice, much still remains unknown.

Glycosyltransferases (GTs) are the most diverse group of enzymes present in all eukaryotic and prokaryotic cells (Cao et al. 2008; Hu and Walker 2002). They can transfer glycosyl groups from the activated donor (usually a UDP-glycosyl group) to the hydroxyl group of numerous substrates, such as lipids, proteins, hormones and secondary metabolites (Hou et al. 2004). The glycosylation catalyzed by GTs functions as an important modulator in regulating hormone homeostasis, stem growth, pollen and seed maturation, defense responses and abiotic stress tolerance (Bowles 2002; Gachon et al. 2005; Rehman et al. 2022; Wang et al. 2011, 2022; Yang et al. 2022). For example, *OsGT1* genes have been reported to be essential for the production of viable pollen grains in rice (Moon et al. 2013). Moreover, a series of recent studies have shown that GTs can determine phenylpropanoid pathway flux, and therefore affect plant development and abiotic tolerance (Dare et al. 2017; Dong et al. 2020; Peng et al. 2017). In apple plants, dysfunction of a phloretin-specific glycosyltransferase *UGT88F1* led to a severely dwarfed phenotype by modulating the phenylpropanoid pathway. In rice, *OsUGT707A2* regulates flavone accumulation and UV tolerance (Peng et al. 2017).

Although much evidence has suggested that GTs participate in many physiological processes, growth/development and stress responses in plants, little is known about the molecular function of *OsCUGT1* (MSU: LOC_Os01g43380). Previously, *OsCUGT1* was isolated as a cold-induced glycosyltransferase based on the cold treatment transcriptome library of Guizhou landrace rice 'Ping Tang Wild-type' (PTWT, *Oryza sativa* ssp. *japonica*) (Cai et al. 2021). One of OsCUGT1's homologous gene At5g04480 in Arabidopsis was reported to participate in pollen tube development (Hoedemaekers et al. 2015). However, the biological function of *OsCUGT1* in rice is still unknown. In this study, we report that *OsCUGT1* plays a prominent role in regulating stem height and spikelet fertility in rice. *OsCUGT1* is generally expressed in rice and most highly expressed in sheaths and panicles. *oscugt1* mutants obtained by means of clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing had a short plant stature and defects in microspore development. Moreover, the *OsCUGT1* RNAi transgenic lines exhibited a weaker but similar morphological phenotype to the *oscugt1* mutants. A combination of cytological observations and comparative transcriptome analysis suggested that *OsCUGT1* regulates rice growth and development by modulating essential genes involved in phenylpropanoid biosynthesis, cytokinin metabolism and pollen development. These findings may provide a more comprehensive understanding of GT functions in rice.

Materials and methods

Plant materials and growth conditions

The rice *japonica* cultivars 'Zhonghua11 (ZH11)' and 'PTWT', a black glutinous rice landrace in Guizhou, were used in this study. ZH11 and mutant plants were grown in a greenhouse at 30 °C (16-h light) and 22 °C (8-h dark) or in a paddy field at Guizhou University in Guiyang, China, from March to November of each year.

Plasmid construction and rice transformation

High-fidelity DNA polymerase (Genestar) was used for PCR amplification; final plasmids were also verified by Sanger sequencing. *OsCUGT1* knockout mutants were obtained via CRISPR/CRISPR-associated protein 9 (Cas9)-mediated genome editing. The guide RNAs targeting the first exon of *OsCUGT1* were cloned into the rice CRISPR/Cas9 vector pHUE411-2gR with a U3 promoter (Xing et al. 2014). pTCK303 (Wang et al. 2004) was used as the binary vector for hpRNA-producing construction. A 360 bp fragment of *OsCUGT1* was inserted into the sense/antisense orientation of pTCK303. All resulting recombinant plasmids were then introduced into ZH11 by means of a modified Agrobacterium (*Agrobacterium tumefaciens* EHA105)-mediated transformation method. The coding sequences of *OsCUGT1* were PCR amplified from PTWT rice plants and cloned into the binary vector pCAMBIA1300-UBI (Bio-Transduction) to obtain *OsCUGT1* overexpression lines.

RNA extraction and quantitative real-time PCR (qRT−PCR)

Total RNA was isolated from rice plants at the mature stage using an RNA Extraction Kit (Omeaga). qRT−PCR was

performed using a 2 × SYBR Green Power PCR Master Mix kit (Genestar) as previously described (Huang et al. 2022). Values are the means ± standard deviations (SD) of three repeats, with Student's *t* test used for statistical analysis (Miura et al. 2007). All primers used in this study are listed in Table S1.

Microscopy observations

Paraffin sections were prepared as previously described (Huang et al. 2022). Internodes and spikelets from ZH11 and *oscugt1* plants were collected and fixed in 75% (v/v) or 50% (v/v) formaldehyde-acetic-acid (FAA) ethanol fixative solution. The treated samples were dehydrated, embedded and double-stained with safranin O and fast green or toluidine blue (Service-bio), then finally observed, imaged and measured with the Case-Viewer digital microscopy application (3DHISTECH). Fresh pollen grains were examined using scanning electron microscopy (SEM) (Huang et al. 2022). Pollen grains were collected, fixed and washed with 2.5% (w/v) glutaraldehyde (Ser-vice-bio), 0.1 M sodium phosphate buffer (pH 7.4) and 1% (w/v) osmic acid sequentially. Then, the treated samples were dehydrated using a graded series of ethanol, dried and coated with gold films before SEM observation. (HITACHI, SU8100).

Transcription analysis

Total RNA was isolated from the leaves and spikelets of ZH11 and *OsCUGT1* rice plants at the mature stage. Both sets of experiments were performed by the Metware, Jiaxing. mRNA libraries for transcriptome deep sequencing (RNA-seq) were sequenced on the Illumina platform. Sequencing data were collected from three independent experiments for each sample. Differentially expressed genes (DEGs) between *OsCUGT1* and WT plants were determined with the R package DEseq2 (Love et al. 2014). The Benjamini–Hochberg multiple comparison adjustment ($p < 0.05$) was used to control false discovery rate (FDR) (Benjamini et al. 2001). Genes with FDR lower than 0.05 and |log2Fold Change|> = 1 were identified as DEGs.

Results

Molecular expression patterns of *OsCUGT1*

Previously, *OsCUGT1* was isolated as a cold-induced glycosyltransferase based on the cold treatment transcriptome library of Guizhou landrace rice PTWT (Cai et al. 2021). According to the CAZy (Carbohydrate-Active EnZymes) database (http://www.cazy.org/GlycosylTransferases.html), OsCUGT1 belongs to the glycosyl transferase family 1 which is known to be involved in the modification of certain secondary metabolites and hormones (Cao et al. 2008). Glycosyl transferase family 1 consisting of 35 members in rice, which can be further clustered into six groups (Fig. S1). OsCUGT1 and its rice homolog LOC_Os10g39900 belongs to the Group II of family 1 (Fig. S1). Multiple sequence alignment of OsCUGT1, LOC_Os10g39900 and their Arabidopsis homolog (At4g01210 and At5g04480) revealed that these proteins all contain glycosyl transferase group 1 domains (PF00534) and display relatively high-level sequence conservation in the C-terminal domains (Fig. 1a). BLAST analysis showed that At4g01210 and At5g04480 in Arabidopsis and LOC_Os10g39900 in rice share approximately 39%, 28% and 26% amino acid sequence identity with OsCUGT1, respectively, which suggests functional similarity among these proteins. These results indicated that *OsCUGT1* may act on plant development like At5g04480. In addition, divergence in the spatial expression patterns of *OsCUGT1* was examined to explore the possible biological function. qRT−PCR analyses indicated that *OsCUGT1* is ubiquitously expressed in rice, with relatively low expression in roots, leaves, seedlings and mature panicles, and the highest expression in sheaths and young panicles (Fig. 1b). This finding suggested that *OsCUGT1* may function mainly in rice sheaths and panicles. In addition, promoter analysis showed that *OsCUGT1* had several cis-acting elements, including ABA-responsive elements (ABRE), drought-responsive elements (DRE) and anoxic-responsive elements (ARE). qRT−PCR confirmed that *OsCUGT1* was indeed upregulated by multiple external stress stimuli. For example, the expression of *OsCUGT1* was upregulated nearly 48-fold and sixfold by NaCl and mannitol treatment, respectively. Moreover, *OsCUGT1* was also upregulated by many hormone treatments, especially by cytokinin, which increased the expression of *OsCUGT1* 22-fold (Fig. 1c). These results indicate that *OsCUGT1* may play roles in regulating plant growth and their adaptation to environmental conditions.

oscugt1 mutants exhibit a dwarf phenotype

To investigate the biological function of *OsCUGT1*, we used a genetic approach to understand the role of *OsCUGT1* in the rice cultivar ZH11 via CRISPR/Cas9-mediated genome editing. Nineteen (90.5%) plants were successfully edited from 21 T_0 transgenic plants. Many independent lines carrying different types of homozygous mutations in the *OsCUGT1* gene were obtained (Fig. 2a). For example, the *oscugt1-1* allele harbored a 1 bp A insertion, while *oscugt1-2* carried a 1 bp T deletion 317 bp downstream of the ATG in *OsCUGT1* (Fig. 2a). All homozygous mutants exhibited a dwarf phenotype compared to their WT (Fig. 2b). Statistically, the plant height of *oscugt1* mutants was approximately three-fifths of that

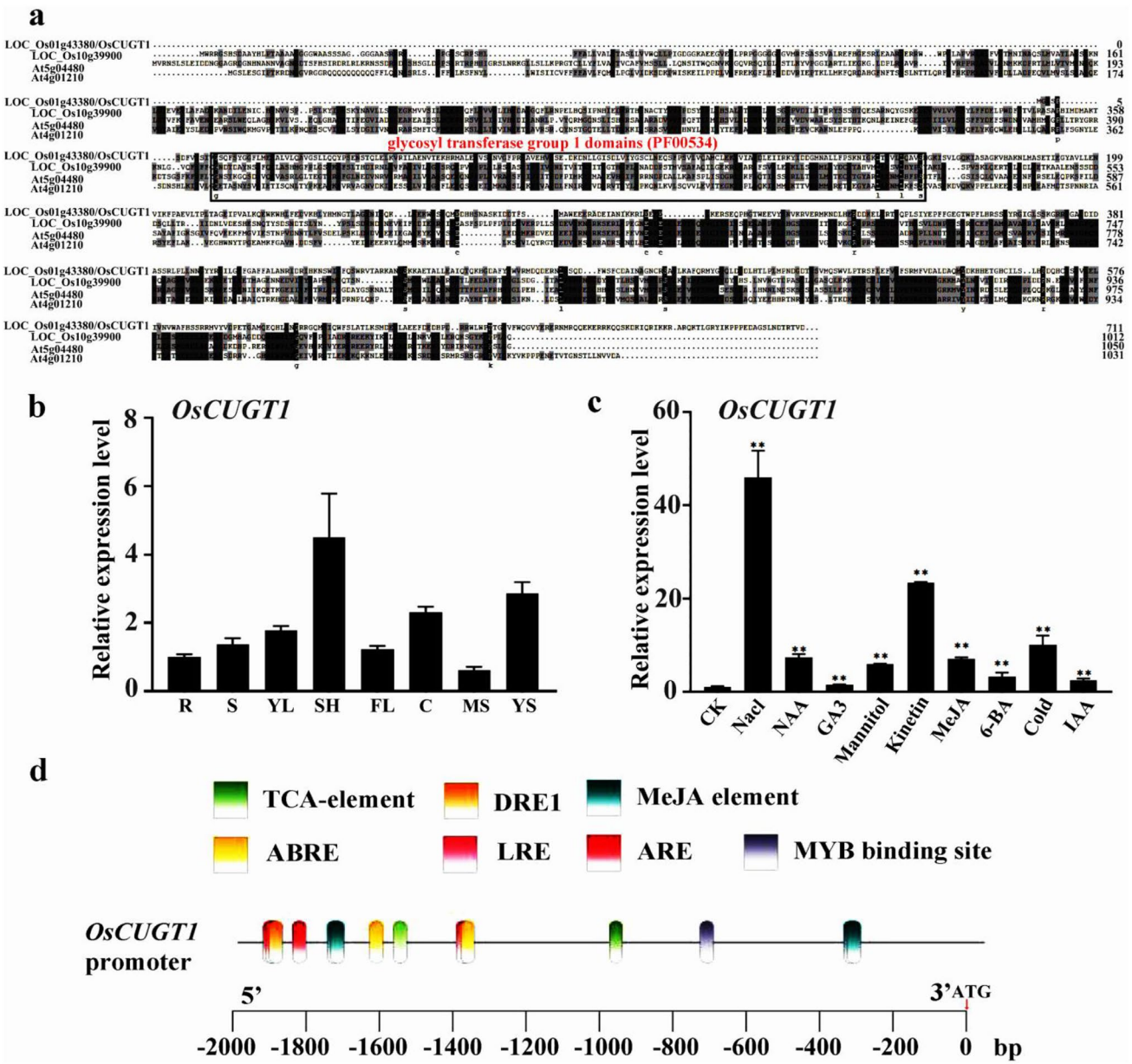

Fig. 1 Characterization of the *OsCUGT1* sequence and expression pattern. **a** Multiple sequence alignment of OsCUGT1 and its homologs from rice and Arabidopsis. These proteins all contain glycosyl transferase group 1 domains (PF00534). **b** The spatial expression patterns of *OsCUGT1* were determined by qRT−PCR. Total RNA was extracted from various rice tissues. Abbreviations: R, root; S, seedling; YL, young leaves; SH, sheaths; FL, flag leave; C, culm; MP, mature panicle; YP, young panicle. **c** The expression level of *OsCUGT1* was upregulated by multiple external stress stimuli and hormone treatments. Values are presented as means ± SD ($n = 3$); $^{**}P < 0.01$ by Student's *t*-test. **d** Analysis of *cis*-acting elements in the promoter of *OsCUGT1*. TCA-element, salicylic acid responsive elements; *ABRE* ABA-responsive elements, *DRE* drought-responsive elements, *LRE* light-responsive elements, *ARE* anoxic-responsive elements

of WT at the maturity stage (Fig. 2c). The rice internodes from the first to the fifth main tiller were dramatically shorter in oscugt1 mutants (Fig. 2d). These results suggested that *OsCUGT1* plays an important role in rice height.

Cell division and expansion of the stem are affected in the *oscugt1* mutant

We characterized the morphology of epidermal cells in the first internode in the WT and the *oscugt1* mutants through

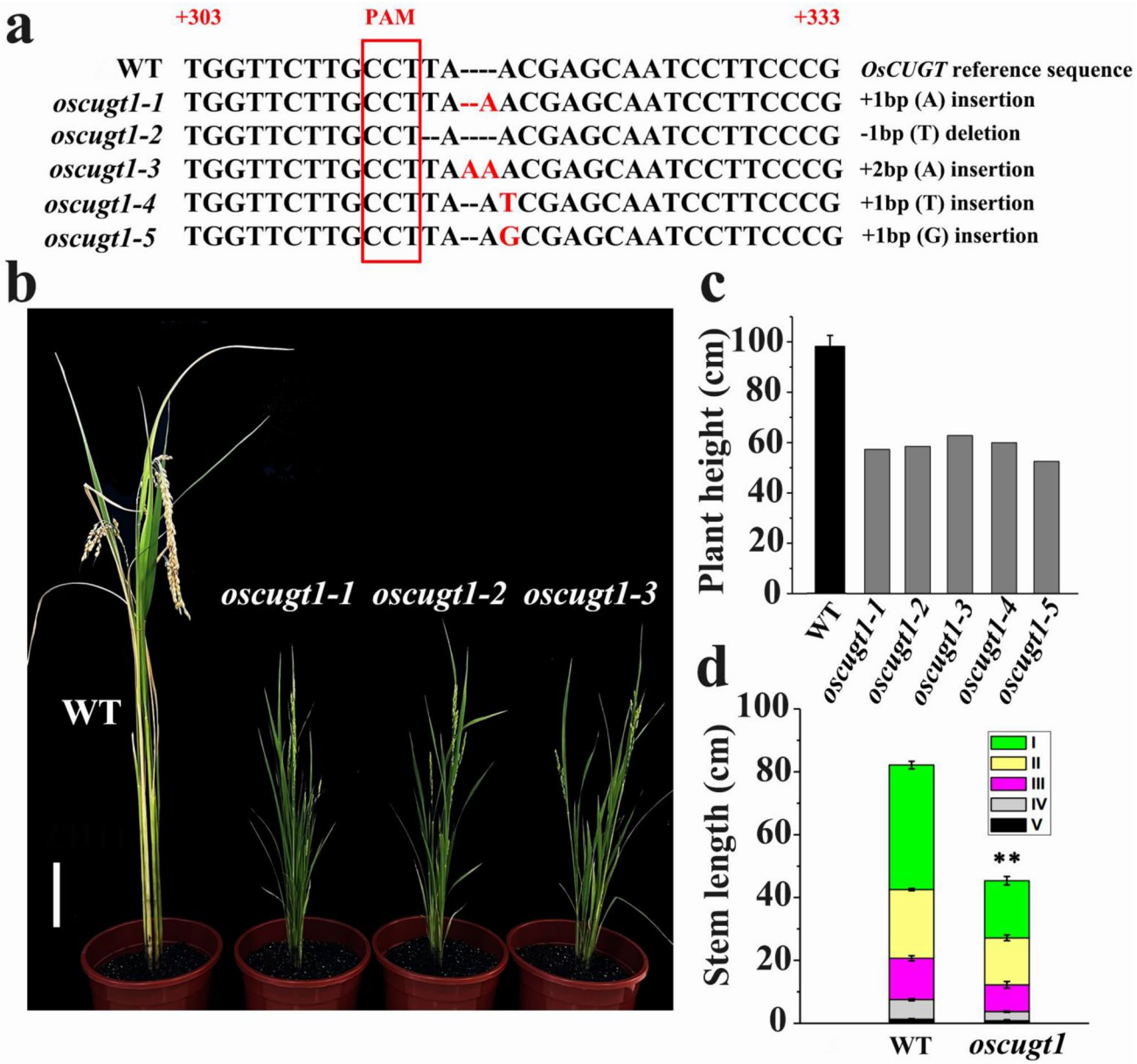

Fig. 2 Isolation and characterization of mutants in *OsCUGT1*. **a** Alignment of genomic sequences from the wild type (WT) in several independent *oscugt1* mutants. **b** Dwarf phenotype of the *oscugt1* mutant during the mature period. Bar = 10 cm. **c** Plant height of WT and five independent *oscugt1* mutant lines. **d** Internode length comparison of WT and *oscugt1*. Values are expressed as the mean ± SD ($n = 3$); $^{**}P < 0.01$ by Student's *t*-test

microscopic observations (Fig. 3). Longitudinal sections of the first internode in *oscugt1-1* and *oscugt1-2* showed shorter cell lengths compared to WT (Fig. 3a). We obtained support for this observation by quantifying the epidermal cell length in *oscugt1* mutants with the imaging software Case-Viewer, which revealed a dramatic reduction of nearly 40% in cell length compared to WT (Fig. 3b). We also estimated the cell number of the WT and mutants by measuring their first internode length and dividing it by the average cell length. Analysis showed that the length of the first internode in *oscugt1-1* and *oscugt1-2* was approximately 50% and 42% of that in the WT, respectively (Fig. 3c). Therefore, the cell number in *oscugt1-1* and *oscugt1-2* decreased by approximately 20 and 24%, respectively, compared to that in the WT (Fig. 3d). These results indicated that the dwarf stature of *oscugt1* is caused by the reduction in both cell number and cell size. Moreover, the expression of two genes that are mainly related to cell elongation (*OsXTH8*, *Xyloglucan endotransglucosylases/hydrolases 5*) (Fig. 3e) and cell division (*OsRAN2*, *Ras-related nuclear protein GTPase 2*)

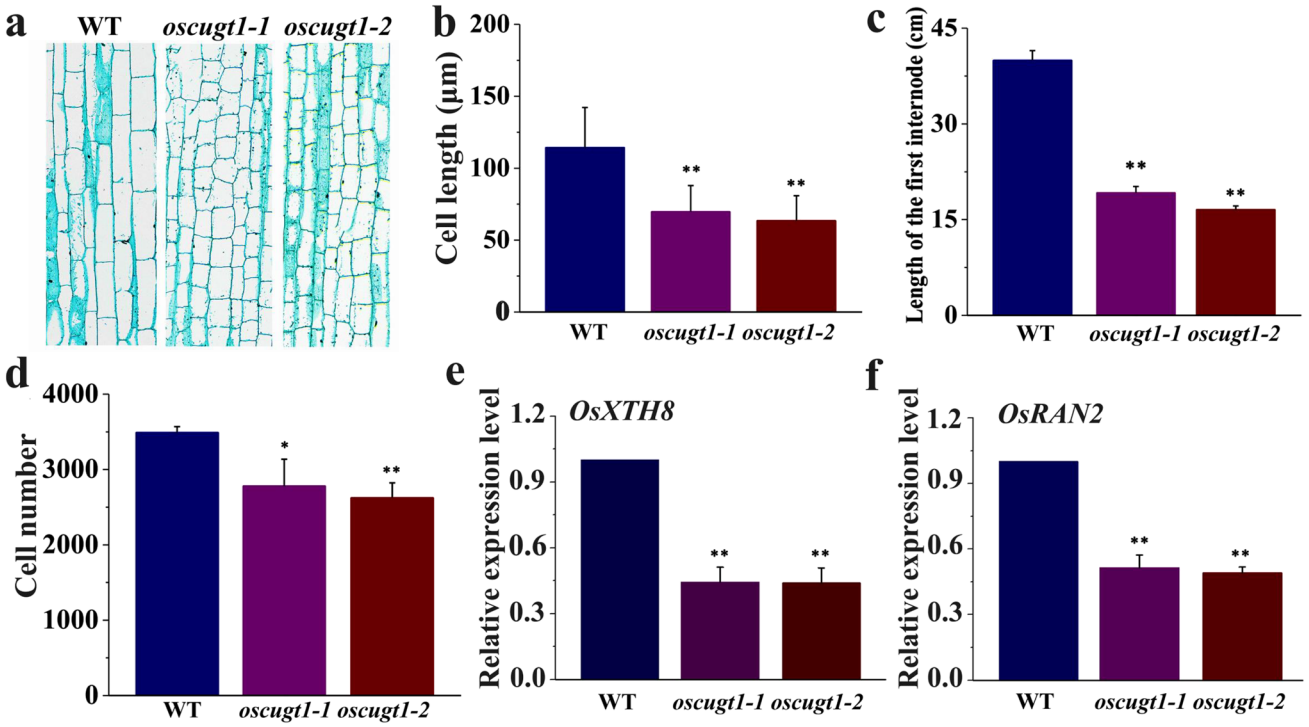

Fig. 3 Histological characterization of stems and roots from wild-type (WT) and the *oscugt1* mutant. **a** Comparison of the first internode's longitudinal section between WT and *oscugt1*. **b** Comparison of the first internode's mean cell length between WT and *oscugt1*. Values are presented as means ± SD ($n = 100$ cells). **c** Comparison of the first internode's mean length between WT and *oscugt1*. Values are presented as means ± SD ($n = 3$). **d** Comparison of the first internode's mean cell number between WT and *oscugt1*. The cell number were calculated by dividing the length of the first internode by the average cell length. Values are presented as means ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test. **e** Relative expression levels of *OsXTH8* as determined by qRT−PCR. **f** Relative expression levels of *OsRAN2* as determined by qRT−PCR. Both of *OsXTH8* and *OsRAN2* with lower expression in *oscugt1* compared to WT, $^{**}P < 0.01$ by Student's *t*-test

(Fig. 3f) downregulated in the *oscugt1* mutant, suggesting that both cell expansion and cell division might be affected in the *oscugt1* mutant.

The *oscugt1* mutant is defective in microspore development

We noticed a shorter panicle and abnormal seed production in the *oscugt1* mutant (Fig. 4a). Panicle seed-setting rates were 0% in the *oscugt1* mutant, in sharp contrast to 94% in the WT plants (Fig. 4b). To investigate the possible causes of sterility in the *oscugt1* mutant, we performed crosses between homozygotes (*oscugt1-5*) and the wild type. When *oscugt1-5* was used as a pollen receiver (female), their seed setting rates reached 41%, while the seed settings rates of crosses between WT reached about 60%. However, *oscugt1-5* cannot serve as a pollen donor due to the lack of mature pollen. Therefore, the above results indicated that dysfunction of *OsCUGT1* causes defects in the male gamete. Then, we conducted detailed histological and cytological observations of male reproductive tissues. Compared to the WT, the length of anthers in the *oscugt1* mutant was approximately two-thirds that of the WT (Fig. 4c). The whole stamens from the *oscugt1* mutant showed clear morphological abnormalities, as they were much smaller and a lighter shade of yellow compared to WT (Fig. 4d-g). We determined from pollen grains with Lugol solution (KI-I2) staining that the pollen number and viability in *oscugt1* were markedly decreased (Fig. 4h, i). In addition, SEM confirmed that the aborted pollen grains in *oscugt1* still had an exine layer and aperture (Fig. 4j, k), although they exhibited an abnormal ornamentation with larger bacula structures (Fig. 4i-m).

To further investigate the exact role of *OsCUGT1* in microsporogenesis, we characterized the progression of anther development in WT and *oscugt1-1* over eight developmental stages (Fig. 5). We detected no obvious differences in either the early pre-meiosis stage or the young microspore stage between WT and *oscugt1-1*, as both genotypes went through normal meiosis and released free microspores from their tetrads (Fig. 5a–d, i–l). Micro-spores from WT were plump and spherical, as expected for healthy microspores (Fig. 5e), whereas *oscugt1-1* microspores were adhesive, shriveled and deformed (Fig. 5m). The differences between

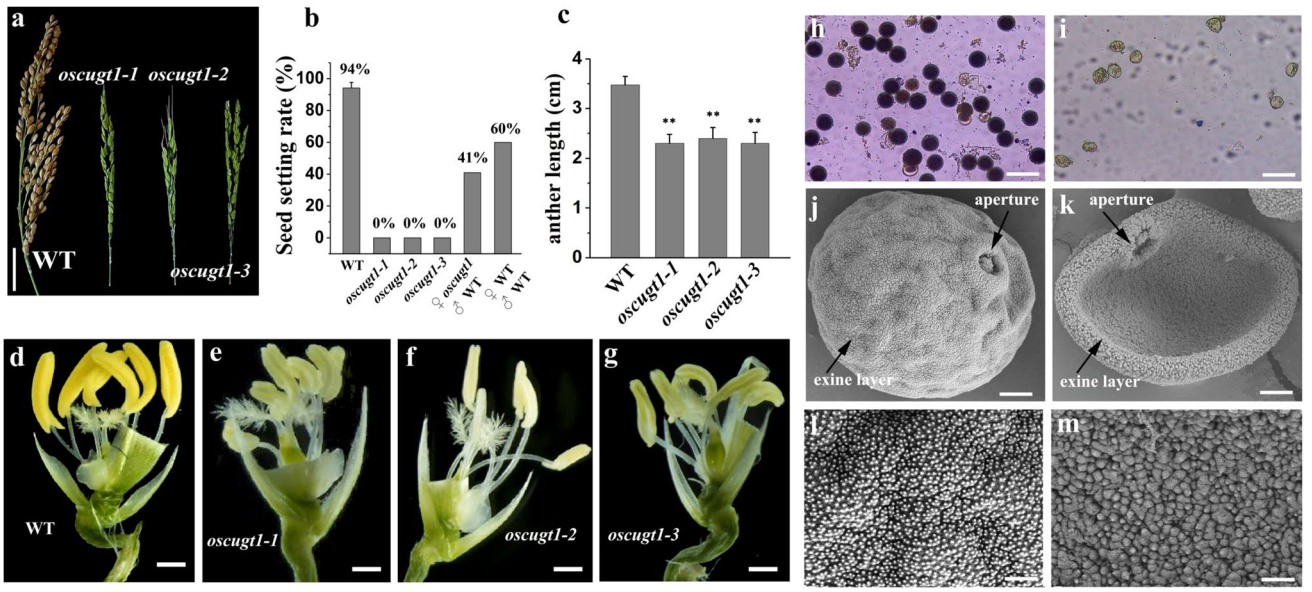

Fig. 4 Floret phenotypes in wild-type (WT) and the *oscugt1* mutant. **a** Representative panicles of WT and *oscugt1* mutants. Bar = 4 cm. **b** Average seed-setting rates of WT and *oscugt1* mutants. **c** Average anther length of WT and *oscugt1* mutants. Values are presented as means ± SD ($n = 6$). Data were analyzed using Student's *t*-test; $^{**}P < 0.01$. Floret phenotypes of WT (**d**) and *oscugt1* mutants (**e**–**g**). Bar = 2 mm. Pollen stained with Lugol solution in WT (**h**) and *oscugt1* mutants (**i**). Normal pollen appears dark. Bar = 50 μm. **j**–**m** Scanning electron micrographs of pollen grains. General view of WT pollen (**j**) and *oscugt1* pollen (**k**) with exine and aperture ornamentation. Enlarged view of WT exine ornamentation with normal bacula structures (**l**) and *oscugt1* exine ornamentation with larger bacula structures (**m**). Bar = 1 μm (**j** and **k**), Bar = 0.1 μm (**l** and **m**)

the two genotypes became obvious at the vacuolated pollen stage, during which WT pollen transformed into large vacuolated microspores with degraded tapetal layer cells (Fig. 5f, g). However, most of the central vacuole in *oscugt1-1* appeared deformed and shrunken (Fig. 5n, o). At the mature pollen stage, each anther lobe in WT was full with viable pollen grains that accumulated plentiful starch granules and nutrients on their surface (Fig. 5h). In contrast, the structures of the four anther lobes were highly abnormal in the *oscugt1-1* mutant, as nearly all lobes failed to produce viable and plump pollen grains, instead presenting very shrunken and deformed pollen grains (Fig. 5p). Collectively, these data indicated that the male sterility phenotype observed in the *oscugt1* mutant might be due to defective microspore development.

Knockdown of *OsCUGT1* by RNAi results in a reduction in rice height and fertility

To further elucidate *OsCUGT1*'s functions in the regulation of plant height and spikelet fertility, the *OsCUGT1*-RNAi vector was constructed (Fig. 6a) and used to transform the rice ZH11. Based on hygromycin-resistance selection, twenty-five T_0 RNAi plants were obtained. Semiquantitative PCR revealed that *OsCUGT1* expression was knocked down in several RNAi lines (Fig. 6b). The height and seed setting rate of these plants decreased to varying degrees compared with those of the WT (Fig. 6c-f). In addition, two RNAi lines from the twenty-five T_0 plants exhibited complete sterility, similar to *oscugt1* (Fig. 6f). These results suggested that the RNAi lines exhibited a weaker morphological phenotype than the knockout *oscugt1* mutants and demonstrated that *OsCUGT1* plays a crucial role in the regulation of plant development.

RNA-seq analysis of the *oscugt1* mutant

To unravel the underlying reason for the shorter stature and abnormal pollen grains in the *oscugt1* mutant, we performed RNA-seq using the leaves (L) and spikelets (SP) of WT and *oscugt1* mutant plants. From two comparison groups (WT-L vs. GT-L; WT-SP vs. GT-SP), 2108 and 4224 differentially expressed genes (DEGs) were identified, respectively (Fig. 7a). Only 375 genes were shared between groups "L" and "SP" (Fig. 7b), indicating that gene expression patterns and the regulatory pathway in leaves vs. spikelets are quite different. Pearson correlation analysis revealed that there was little variation among experimental replicates (Fig. 7c). Therefore, these RNA-seq data have high reliability and could be used for further analysis.

Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification of the DEGs from the two comparison groups showed that a wide spectrum of biological processes and metabolic

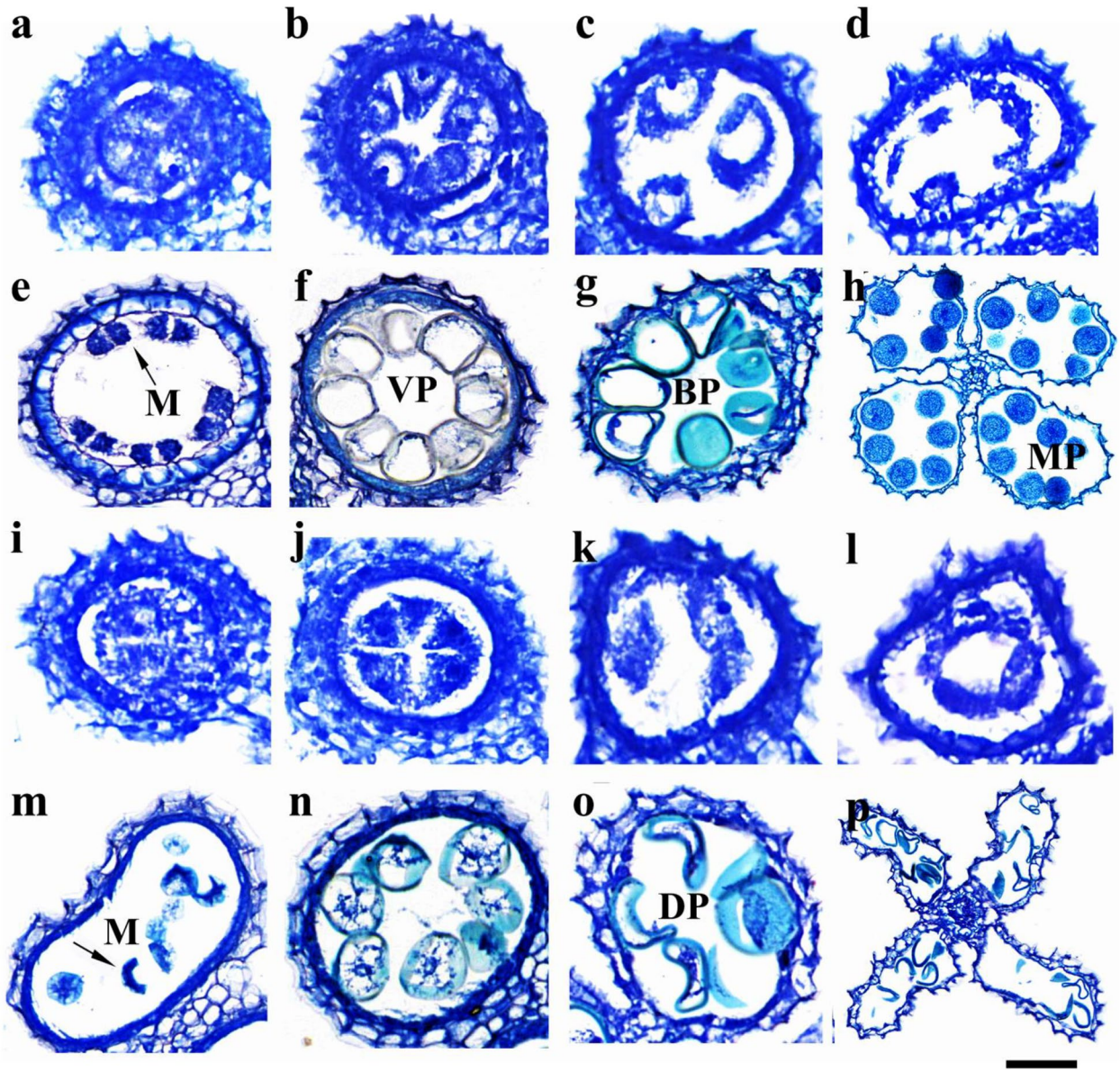

Fig. 5 Histological analysis of wild-type (WT) and *oscugt1* mutant microspores at eight developmental stages. Transverse sections of wild-type (WT) and *oscugt1* single anther locules at the meiosis stage (**a**–**c**, **i**–**k**), the tetrad stage (**d** and **l**), the young microspore stage (e and m), the vacuolated pollen stage (**f** and **m**) and the pollen mitosis stage (**g** and **o**). Cross-sections of WT and *oscugt1* mutant whole anthers with four locules at the mature pollen stage (**h** and **p**). BP, bicellular; DP, degraded microspore; M, microspore; MP, mature pollen, VP, vacuolated pollen. Bars, 20 μm (**a**–**g**, **j**–**o**); 40 μm (**h**, **p**)

pathways were enriched (Fig. S2-S5). Further analysis revealed that many DEGs in the comparison group "WT-L vs. GT-L" are related to the biosynthesis of secondary metabolites (Fig. S3). Since phenylpropanoids are termed 'secondary metabolites', we noticed that a large number of phenylpropanoid pathway genes, such as several *PHENYLALANINE AMMONIA LYASE* (*PAL*) genes, including *OsPAL1*, *OsPAL2*, *OsPAL3*, *OsPAL4*, *OsPAL6*, *OsPAL7* and *CINNAMYL ALCOHOL DEHYDROGENASE* (*OsCAD2*), *OsCAD3*, *CAFFEIC ACID 3-O-METHYLTRANSFERASE* (*OsCOMT*), 4-*COUMARATE:COENZYME A LIGASE 1* (*Os4CL1*), *Os4CL2*, *Os4CL3*, *Os4CL5*, and *FLAVANONE 3'-HYDROXYLASE* (*OsF3'H*), which are involved in phenylpropanoid compound biosynthesis in rice, were all downregulated in the *oscugt1* mutant relative to WT (Fig. 7d, e). These results suggested that the loss of *OsCUGT1* function disturbs the transcript levels of genes encoding phenylpropanoid biosynthetic enzymes, thereby impairing the accumulation of related metabolites that are important modulators of plant growth and development.

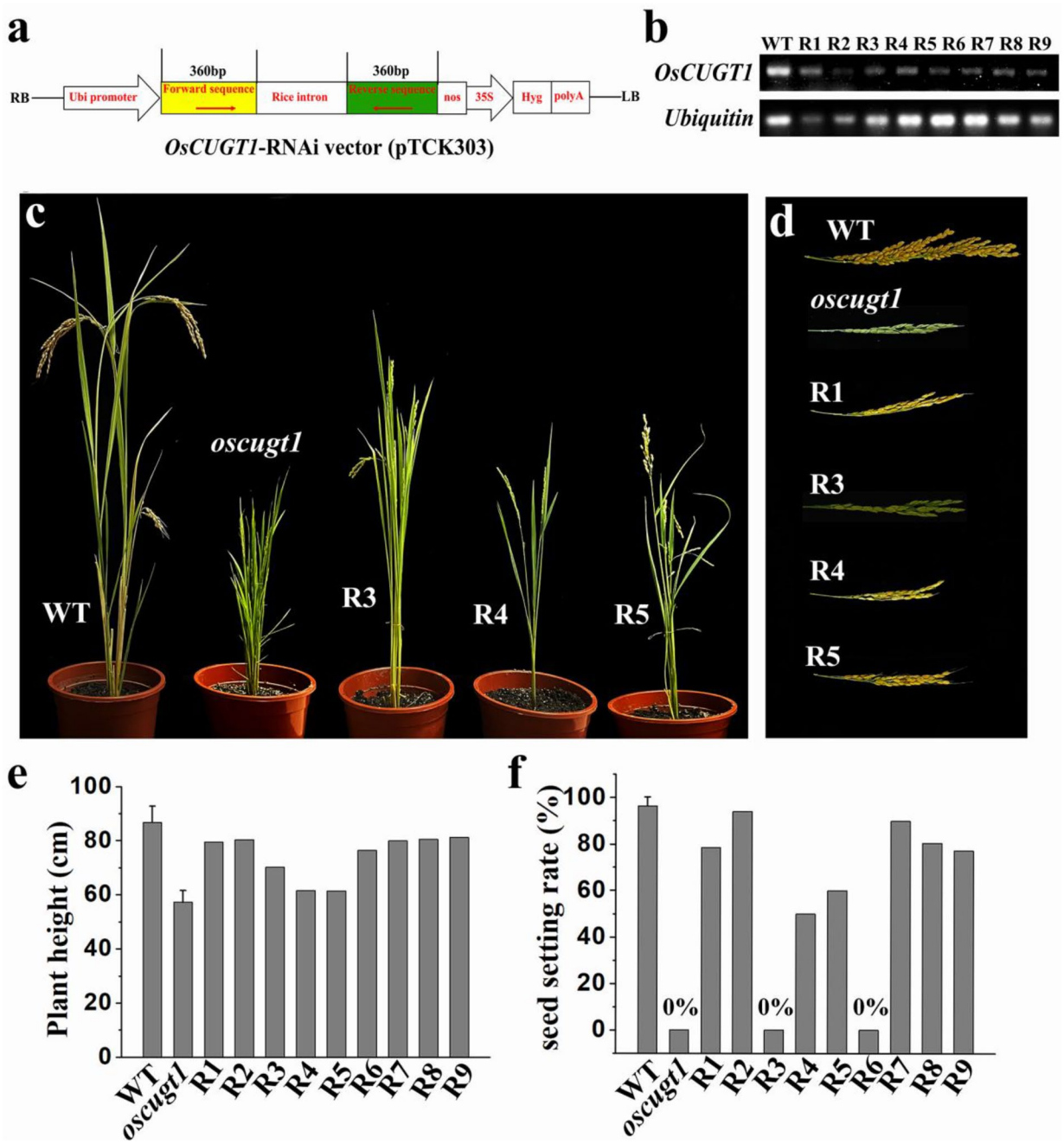

Fig. 6 The generation of *OsCUGT1* RNAi transgenic plants through the RNA interference system. **a** pTCK303 was used as the binary vector for hpRNA-producing construction. A 360 bp fragment of *OsCUGT1* was inserted into the sense/antisense orientation of pTCK303. **b** Relative expression levels of *OsCUGT1* in the RNAi transgenic plants, as determined by semi-quantitative PCR. **c** Dwarf phenotype of the RNAi transgenic plants during the mature period. **d** Representative panicles of WT and RNAi transgenic plants. **e** Plant height of WT and nine independent RNAi transgenic lines. **f** Seed setting rate of WT and nine independent RNAi transgenic lines

Additionally, we found that many DEGs from the "WT-L vs. GT-L" group, which participate in cytokinin metabolism, were affected in the *oscugt1* mutant. Genes involved in cytokinin biosynthesis, such as *LONELY GUY OsLOG1* and *OsLOG10*, which encode cytokinin-activating enzymes (Kurakawa et al. 2007), were both repressed in *oscugt1* (Fig. 7f). By contrast, genes functioning in cytokinin degradation/inactivation, such as cytokinin-O-glucosyltransferases (CGTs) and cytokinin oxidase/dehydrogenase (CKX) family genes, were highly expressed in the *oscugt1* mutant compared to the WT (Fig. 7g, h).

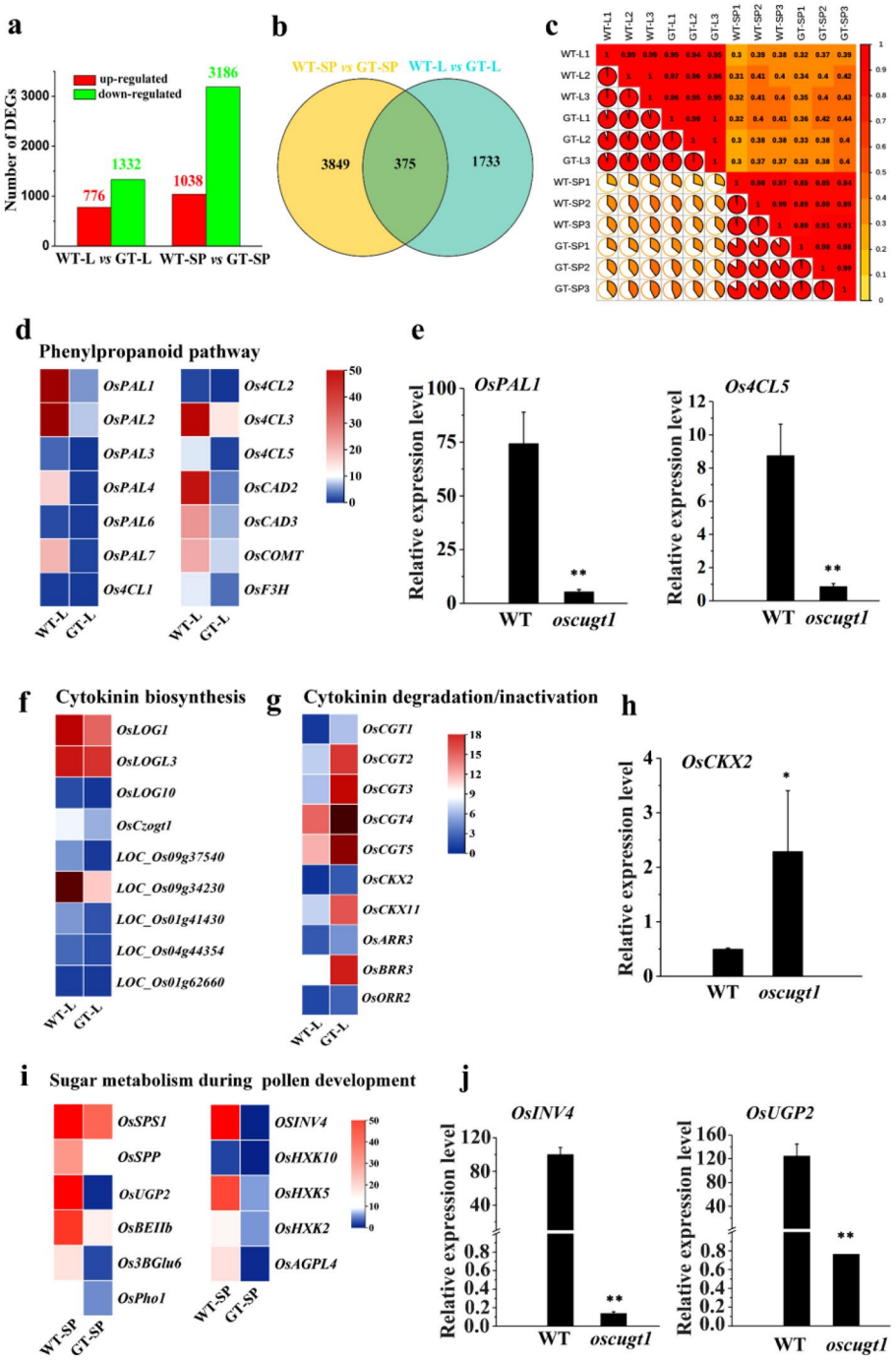

Fig. 7 Transcriptome profiling of wild-type (WT) and *oscugt1* plants. Transcriptome profiling of wild-type (WT) and *oscugt1* plants. **a** Overview of differentially expressed genes (DEGs) in the leaves (L) and spikelets (SP) of WT and *oscugt1* mutant plants (GT). **b** The DEGs were picked out from two comparison groups (WT-L vs. GT-L; WT-SP vs.GT-SP) to draw the Venn diagram. **c** Pearson correlation coefficients of RNA-seq data from all samples. **d** DEGs in phenylpropanoid pathway. The color in each cell indicates the value of the gene expression levels as fragments per kilobase of transcript per million mapped reads (FPKM). (**e**) Differential expression of *OsPAL1* and *Os4CL5* was analyzed based on the transcriptome data. **f** DEGs in cytokinin biosynthesis pathway. **g** DEGs in cytokinin degradation/inactivation pathway. The color in each cell indicates the value of FPKM. **h** Differential expression of *OsCKX2* was analyzed based on the transcriptome data. **i** DEGs in sugar metabolism during pollen development. **j** Differential expression of *OsUGP2* and *OsINV4* was analyzed based on the transcriptome data. Values are presented as means ± SD ($n = 3$). Data were analyzed using Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$

These results suggested that the loss of *OsCUGT1* may affect the cytokinin levels in plant leaf cells.

Furthermore, DEG analysis was conducted in the comparison groups "WT-SP vs. GT-SP". KEGG analysis revealed that the top three enriched pathways were metabolic pathways, biosynthesis of secondary metabolites and starch and sucrose metabolism (Fig. S5). Defective sugar metabolism during anther and pollen development is known to contribute to male sterility (Liu et al. 2021). Consistently, we found that a large number of genes involved in sugar and starch synthesis during microsporogenesis were significantly downregulated in the *oscugt1* mutants (Fig. 7i, j). For example, *SUCROSE PHOSPHATE SYNTHASE* (*OsSPS1*), *UDP-GLUCOSE PYROPHOSPHORYLASE GENE* (*OsUGP2*), *ANTHER-SPECIFIC CELL WALL INVERTASE GENE* (*OsINV4*), and *LARGE SUBUNIT OF ADP-GLUCOSE PYROPHOSPHORYLASE* (*OsAGPL4*) were all significantly less expressed in *oscugt1*. In addition, the transcripts of several anther development-specific genes, including *MALE STERILITY 1* (*OsMS1*), *OsMS2*, *MONOSACCHARIDE*

TRANSPORTER GENE (*OsMST5*), *RICE ANTHER-SPECIFIC GENE* (*OsRTS*), and *TAPETUM DEGENERATION RETARDATION* (*OsTDR*), were all nearly abolished in the *oscugt1* mutant (Fig. S6).

Together, these results indicated that loss of *OsCUGT1* affects the expression of genes related to multiple biological processes, especially in the phenylpropanoid biosynthetic pathway, cytokinin homeostasis and pollen development.

Discussion

GTs are encoded by large multigene families, which sometimes comprise hundreds of genes in plants (Cao et al. 2008). Many identified GTs participate in essential processes, including pathogen defense (Ke et al. 2019; Zhang et al. 2022), stress adaptation (Li et al. 2017; Wang et al. 2022) and plant growth and development (Moon et al. 2013; Zhang et al. 2014, 2022). Although a large number of GTs have been functionally characterized in rice, the biological roles of *OsCUGT1* remain unknown. In this study, we characterized *OsCUGT1* and revealed its vital roles in rice height and spikelet fertility. Our results confirm the conserved function of *OsCUGT1* in maintaining the balance of cellular phenylpropanoid metabolite levels and highlight its potential functions in cytokinin homeostasis and pollen development, especially in pollen starch and sugar metabolism.

It has been reported that many GTs exhibit stress induction and are involved in plant stress tolerance via different pathways (Li et al. 2017, 2020; Wang et al. 2022). Previously, *OsCUGT1* was isolated as a cold-induced glycosyltransferase based on the cold treatment transcriptome library of Guizhou landrace rice PTWT. The analysis of transcript expression patterns in this study indicated that *OsCUGT1* was indeed induced by multiple external stimuli (Fig. 1c), pointing to a possible role in environmental adaptation. qRT−PCR analyses suggested that *OsCUGT1* may function mainly in rice sheaths and panicles (Fig. 1b). In agreement, the *oscugt1* knockout mutant exhibited distinct abnormalities, including dwarfism and sterility. The height of the main tillers in the *oscugt1* mutant reached only three-fifths that of the WT (Fig. 2b). RNA-seq revealed that a large number of genes contributing to secondary metabolite biosynthesis, especially the phenylpropanoid pathway, showed significant changes in the leaves and spikelets of the *oscugt1* mutant (Fig. 7). In the comparison group "WT-L vs. GT-L", the transcript of *OsPAL1*, a key enzyme that acts as an entry point to the phenylpropanoid pathway (Howles et al. 1996), was downregulated in the leaves of *oscugt1*. The significant reductions in gene expression for several important genes encoding phenylpropanoid biosynthetic enzymes, including *OsPALs*, *Os4CL*, *OsCOMT* and *OsF3'H* in *oscugt1*, would therefore lead to a decreased supply of precursors for the phenylpropanoid pathway and may reduce the accumulation of downstream metabolic compounds and in turn influence rice development. It is not surprising to suggest such a hypothesis, as many reports have shown that glycosylation mediated by GTs exerts an important role in regulating phenylpropanoid metabolic flux redirection and governing shoot morphology, grain size and abiotic stress tolerance (Dong et al. 2020; Li et al. 2017; Yin et al. 2014).

It is noteworthy that inside the stem of *oscugt1*, both cell number and cell length were reduced (Fig. 3). qRT−PCR confirmed that two important genes that function in cell division and expansion were downregulated, suggesting that both of them were affected in the *oscugt1* mutant. Hormones, especially auxin and cytokinin, can regulate many aspects of growth and development, including cell elongation and division (Di Mambro et al. 2017; Street et al. 2016). A number of studies have also demonstrated the effects of phenylpropanoid metabolic compounds on auxin and CK metabolism in Arabidopsis and rice, resulting in growth pattern changes in the plant (Brown et al. 2001; Dong et al. 2020; Kurepa et al. 2018; Wang et al. 2011; Yin et al. 2014). For example, disrupted activity of two Arabidopsis UGTs (UGT78D1 and UGT78D2) would contribute to the accumulation of high levels of a kaempferol glycoside and lead to dwarfed plants by perturbing polar auxin transport (Yin et al. 2014). In addition, nucleotide variations in *GSA1* reduce grain size by disturbing cell proliferation and expansion, which is the result of flavonoid-mediated imbalance of auxin homeostasis (Dong et al. 2020). Logically, the loss of *OsCUGT1* would be expected to affect auxin-related gene expression; however, RNA-seq results showed that the transcripts of some representative genes that function in auxin polar transport, synthesis and response were insignificantly changed in the *oscugt1* mutant (Fig. S7). These results indicated that *OsCUGT1* regulated rice height by modulating cell division and expansion, which may not be achieved by affecting the auxin regulatory pathway.

Intriguingly, RNA-seq revealed that the relative expression levels of a number of cytokinin metabolism-related genes were notably affected in the *oscugt1* mutant (Fig. 7). Several cytokinin biosynthesis-related genes, such as *OsLOG1*, *OsLOG10* and *LOC_Os04g44354*, which function in the final step of bioactive cytokinin synthesis in rice (Kurakawa et al. 2007), were repressed in *oscugt1*. In contrast, genes that negatively regulate cytokinin accumulation and signaling (Ashikari et al. 2005), such as the *OsCKX2*, *OsCKX11*, *OsCGT* genes and cytokinin response regulator *OsORR2*, were all highly expressed in the *oscugt1* mutant compared to the WT. *OsCKX2* can modulate rice tillering, spikelet number and organ size by regulating cytokinin metabolism (Yeh et al. 2015). Reduced *OsCKX2* expression increases tiller number and yield in transgenic rice. CGT genes, such as Arabidopsis

UGT76C2, the endogenous cytokinin level and plant growth and development are affected in its overexpression lines (Wang et al. 2011). *OsORR2* gene, a B-type cytokinin response regulator, the cytokinin signaling and rice height are affected in its overexpression lines (Shi et al. 2020). The higher expression levels of these putative cytokinin-inactivating genes could impair cytokinin homeostasis and contribute to cytokinin signaling disturbance, which would partially account for the dwarf phenotype and abnormal inflorescences of the *oscugt1* mutant. Many studies have reported that the glycosylation modifications of cytokinins are mostly catalyzed by family 1 glycosyltransferases (Hou et al. 2004; Li et al. 2020). The update of the CAZy database suggested that *OsCUGT1* belongs to glycosyl transferase family 1 (http://www.cazy.org/GlycosylTransferases.html). In addition, *OsCUGT1* was upregulated by certain hormone treatments, especially by cytokinin (Fig. 1c). These results raised the question of whether *OsCUGT1* is a cytokinin glycosyltransferase from rice. We attempted to examine the glycosyltransferase activity of *OsCUGT1* by purifying a glutathione S-transferase (GST)-OsCUGT1 fusion protein after expression in *Escherichia coli*. Unfortunately, we failed to obtain the labeling protein for further enzymatic activity assays. To date, few cytokinin glycosyltransferase genes in rice have been reported (Li et al. 2020), possibly because of the difficulty of establishing prokaryotic protein expression and performing enzyme activity assays. Further attempts may help to solve this problem and elucidate the underlying mechanisms of how *OsCUGT1* modulates the phenylpropanoid pathway and cytokinin metabolism.

In addition to dwarfism, we also noticed that *oscugt1* was completely sterile. It shows defects at a late stage of pollen development, in which pollen initiates starch accumulation. Sugar metabolism is crucial for pollen maturation and viability (Liu et al. 2021). Many studies have shown that defects in sugar metabolism during pollen development often result in male sterility. For example, two pollen-preferential "late genes", *OsUGP2* and *OsINV4*, whose knockdown transgenic rice failed to accumulate sucrose and starch in pollen and thus contributed to sterility were observed (Mu et al. 2009; Oliver et al. 2005). RNA-seq analysis revealed that both *OsUGP2* and *OsINV4* and other important sugar metabolism-related genes were significantly repressed in *oscugt1*. Therefore, we concluded that the defective microspore phenotype in *oscugt1* may arise because of abnormal sugar metabolism. These results also demonstrated that *OsCUGT1* is necessary for pollen maturation, especially for starch and sugar accumulation in pollen grains.

In conclusion, the combination of phenotypic analysis, microscopic observations and transcriptome analyses in this study revealed that *OsCUGT1* regulates rice height and spikelet fertility by modulating essential genes involved in the phenylpropanoid pathway, cytokinin homeostasis and pollen development.

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Declarations

Conflict of interest The authors declare no conflicts of interest in the authorship and publication of this document.

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