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ORIGINAL ARTICLE

Genetic Mapping and Transcriptomic Analysis Revealed the Molecular Mechanism Underlying Leaf-Rolling and a Candidate Protein Phosphatase Gene for the *Rolled Leaf-Dominant* (*RL-D*) Mutant in Rice

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Abstract

In rice, moderate leaf rolling improves photosynthesis and crop yield. However, the molecular mechanisms underlying this important agronomic trait remain incompletely understood. Here, we investigated a dominant rolled leaf mutant (*RL-D*) developed from Nipponbare rice (WT). From the six-leaf stage, the leaves of the mutant rolled inward, and abnormal sclerenchyma tissues developed on the abaxial side of the leaf midribs. Additionally, leaf length, plant height, grain weight, and chlorophyll content were significantly greater in the mutant as compared to the WT. Genetic mapping analysis suggested that the leaf-rolling trait in the *RL-D* mutant was controlled by a single dominant gene, which was located in a 743-kb region on rice chromosome 3. Re-sequencing analysis showed that one gene in the mapped region encoding a protein phosphatase, *Os03g0395100* (herein designated *OsPP2C*), had base mutations in the first exon. These mutations may have produced a truncated form of the OsPP2C protein in *RL-D*. Further transcriptomic analysis revealed that several biological processes, especially secondary cell wall formation and protein phosphorylation, were overrepresented among the differentially expressed genes (DEGs) between the mutant and the wild type. qRT-PCR verification also demonstrated that specific genes associated with leaf polarity and secondary cell wall formation were differentially expressed in the mutant. This study presents a novel dominant rolled-leaf germplasm that may help to improve rice leaf morphology in the future. The results also suggested that the *RL-D* phenotype might result from abnormal sclerenchyma tissue development, possibly regulated by *OsPP2C* via the dephosphorylation pathway. This may present a novel mechanism underlying leaf-rolling in rice.

Keywords Rice · Rolled leaf-dominant · Gene mapping · Transcriptomic analysis · Protein phosphatase

Key Message

- This study investigated the dominant rolled-leaf (*RL-D*) rice mutant and presented a novel dominant rolled-leaf germplasm that may help to improve rice leaf morphology. The results also suggested that the *RL-D* phenotype might result from abnormal sclerenchyma tissues, possibly regulated by a protein phosphatase encoding gene *OsPP2C* via the dephosphorylation pathway.

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Introduction

Rice is a staple food feeding over half of the earth's population (Muthayya et al. 2014). Changes in leaf morphology, especially moderate leaf rolling, improve photosynthesis and crop yield; thus, leaf rolling is usually considered a crucial agronomic trait in high-yield rice breeding (Xu et al. 2018).

During leaf maturation, the development of adaxial-abaxial polarity plays an important role (Bowman et al. 2002). Leaf adaxial development is mainly controlled by genes in the *PHAN* or *homeodomain-leucine zipper III* (*HD-ZIPIII*) families (Waites et al. 1998; Itoh et al. 2008), while leaf abaxial development is mainly controlled by genes in the *YABBY* or *KANADI* families (Dai et al. 2007; Liu et al. 2007a; Toriba et al. 2007; Eshed et al. 2004; Kerstetter et al. 2001; Zhang et al. 2009). Many of the leaf-rolling genes identified in rice to date regulate the development of leaf adaxial-abaxial polarity, including *ADL1* (Hibara et al. 2009), *ACL1* (Li et al. 2010; Fang et al. 2021), and *OsAGO7* (Shi et al. 2007).

Several other genes have been shown to participate in the regulation of leaf rolling in rice, including genes associated with bulliform cells, such as *REL1* (Chen et al. 2015), *SRL1* (Xiang et al. 2012; Li et al. 2017), *RL14* (Fang et al. 2012), *Roc5* (Zou et al. 2011), *NRL1* (Hu et al. 2010), *OsLBD3-7* (Li et al. 2016a, b), *REL2* (Yang et al. 2016), *Hal1* (Matsumoto et al. 2018), and *PSL1* (Zhang et al. 2021); genes associated with the development of sclerenchyma tissue around leaf vascular bundles, such as *SLL1* (Zhang et al. 2009), *SRL2* (Liu et al. 2016), and *NRL2* (Zhao et al. 2016); and genes associated with cuticle development, such as *CFL1* (Wu et al. 2011) and *OsCHR4* (Guo et al. 2019). Other factors, such as the micro RNAs *miR165/166* (Juarez et al. 2004; Mallory et al. 2004) and *TAS3* (Fahlgren et al. 2006; Hunter et al. 2006), may indirectly control leaf morphology in plants by regulating downstream genes related to the development of leaf polarity. Some genes involved in the regulation of the auxin (IAA) response, such as *miR160* (Mallory et al. 2005; Liu et al. 2007b), or in IAA biosynthesis, such as *OsNAL7* (Fujino et al. 2008) and *OsFMO*$_{(t)}$ (Yi et al. 2013), may also participate in rolled leaf formation in rice, possibly by regulating the development of leaf polarity or vascular bundles.

Nevertheless, the molecular mechanisms underlying the leaf-rolling phenotype are still incompletely characterized in rice. Furthermore, in comparison with recessive mutants, dominant leaf-rolling mutants are advantageous for rice breeding because the hybrid progeny are easily identifiable by the rolled-leaf phenotype. However, to date, most identified rolled-leaf mutants are recessive; only a few dominant mutants have been identified in rice, including *REL1* (Chen et al. 2015; Liang et al. 2018), *CFL1* (Wu et al. 2011), *R05* (Shi et al. 2007), and *ACL-D* (Xu et al. 2014), and the mechanisms controlling dominant leaf-rolling in rice remain unclear. In order to effectively utilize the dominant rolled-leaf phenotype in rice breeding, it is necessary to identify additional dominant leaf-rolling functional genes and to elucidate the underlying mechanisms.

In our preliminary study, a dominant rolled-leaf mutant (*RL-D*) was obtained via the ethyl methanesulfonate (EMS) mutagenesis of Nipponbare rice (*Oryza sativa* L. ssp. *japonica*) (Chen et al. 2013). However, it was unclear how this mutant differed from previously reported dominant leaf-rolling rice mutants. The underlying cause of this mutation in the *RL-D* mutant was also unknown. In the present study, the detailed phenotyping, histological alterations, and molecular mechanisms underlying leaf rolling in *RL-D* were investigated. Genetic mapping showed that the *RL-D* locus was defined in a 743-kb region on rice chromosome 3, while re-sequencing analysis showed that the first exon of a rice gene (*Os03g0395100*) encoding protein phosphatase (herein named OsPP2C) contained base mutations. These mutations may have produced a truncated form of the OsPP2C protein in the *RL-D* mutant. Our results also suggested that several metabolic pathways, especially secondary cell wall formation and protein phosphorylation, might participate in rolled-leaf formation in *RL-D*. Our study provided a novel dominant rolled-leaf germplasm, which may be valuable for high-yield rice breeding, and presented an analysis of the mechanisms underlying the *RL-D* mutation.

Materials and Methods

Plant Materials and Experimental Design

The rice *RL-D* mutant was obtained by performing EMS on suspension-cultured cells of Nipponbare rice (*Oryza sativa* L. ssp. *japonica*) (Chen et al. 2013). In the present study, the *RL-D* mutant was used for phenotypic, histological, transcriptomics, and qRT-PCR analyses, as compared to the wild-type Nipponbare rice variety (herein referred to as WT). In addition, we constructed two F_2 populations: one F_2 population (744 individuals in total), constructed by crossing *RL-D* (the male parent) with the wild type (WT), and the second F_2 population (689 individuals in total), constructed by crossing *RL-D* (the male parent) with Jingxian 89 (a widely compatible flat-leaf rice variety), were used for genetic analysis. All 164 recessive (normal flat-leaf) individuals in the second F_2 population were used for the molecular mapping of the rolled-leaf locus *RL-D*.

Phenotypic Investigation

At maturity, 30 *RL-D* or WT plants were randomly selected for the investigation of agronomic traits, and the rolled-leaf phenotypes were recorded for all plants from each F_2 population. The top two leaves from each plant were used to calculate the leaf-rolling index (LRI) as previously described (Wang et al. 2017). Briefly, leaf width was measured in both a natural state (Ln) and an unfolded state (Lw), and then, LRI was calculated as $(Lw - Ln)/Lw \times 100\%$. The flag leaves at the maximum booting stage were used for the determination of chlorophyll content. Briefly, leaves from three randomly selected rice plants were ground to powder in liquid nitrogen, and chlorophyll was extracted by incubating 0.1 g of leaf powder in 95% ethanol for 12 h at room temperature in the dark. After incubation, the mixture was centrifuged (12,000 rpm, 10 min), and the chlorophyll absorbance of the supernatant was measured using a BioTek Epoch spectrophotometer at 649 nm and 665 nm. Total chlorophyll content (a + b) was calculated following Li et al. (2019). Three independent biological replicates of all experiments were performed.

Histological Observations of Leaves

Free-hand and semi-/ultra-thin transverse leaf sections were used for histological observation. To produce free-hand

sections, the top leaf was harvested from a variety of WT and *RL-D* rice plants at different stages of growth. Each leaf was cut into slices (each about 100 μm thick) in a Petri dish with a small amount of ddH_2O using a sharp blade, and the sections were observed under a normal light microscope. To produce semi-/ultra-thin sections, flag leaves at the boot stage were fixed in a solution of 4% paraformaldehyde-0.25% glutaraldehyde containing 0.1 mmol/L sodium phosphate (pH 7.0) and 0.1% Tween-20 for 24 h, and then transferred to a 1% osmic acid solution for 1.5 h. After fixation, the samples were dehydrated in a series of increasing concentrations of ethanol, infiltrated in acetone, and embedded in Eponate 12TM resin. The embedded samples were then polymerized for 24 h at 65 °C. Semi-thin sections (2 μm thick) and ultra-thin sections (80–90 nm thick) were prepared using a Leica UCT ultramicrotome. Semi-thin sections were stained with 0.1% toluidine blue solution, and then examined and photographed using a Zeiss Axio Observer D1 microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate, and then examined and photographed using a Zeiss EVO MA 15 microscope.

Mapping of the *RL-D* Locus

The polymorphisms between *RL-D* (the dominant rolled-leaf mutant, male parent) and Jingxian 89 (female parent) were screened using simple sequence repeat (SSR) markers selected from the Gramene database (http://www.gramene.org/). The polymorphic markers were used for co-segregation analysis with 15 recessive (normal leaf) plants selected from the mapping population of the F_2 generation. The identified roughly linked markers were used for an additional segregation analysis with all 164 recessive (normal leaf) individuals in the F_2 generation. We used BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify InDel variations between the reference genomic sequences of *indica* and *japonica* rice (downloaded from the nucleic acid database GenBank; https://www.ncbi.nlm.nih.gov/genbank/) in the genomic region anchored by the rough-mapped markers. New InDel markers, based on these sequence variations, were designed to fine map the *RL-D* locus.

Total genomic DNA was extracted from rice leaves for molecular mapping analysis using the CTAB method (Abdel-Latif and Osman 2017), and then used as templates for PCR amplification. To amplify SSR or insertion/deletion (InDel) markers, PCR amplifications were performed in 15.0 μL volumes, each containing 1.0 μL of DNA template, 1.5 μL of 10 × reaction buffer, 0.3 μL of 10 μM dNTPs mixture, 0.3 μL each of 10 μM forward and reverse primers, 0.15 μL (1.0 U) of *Taq* DNA polymerase, and 11.45 μL of ddH_2O, using a MJ Research PTC-100 PCR instrument. The PCR cycling conditions were as follows: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a final extension of 72 °C for 10 min. The amplified products were subjected to 10% non-denaturing polyacrylamide gel electrophoresis (PAGE) and were visualized using the silver staining method (Xu et al. 2002).

Genetic maps were constructed using Mapmaker3.0 with a minimum likelihood of odd (LOD) threshold of 3.0 (Lander et al. 1987), and the Kosambi function was used to convert the recombinant rates (%) obtained from the molecular marker analysis into genetic distances (centiMorgan, cM). All coding sequences (CDSs) in the fine-mapped genomic DNA region were amplified by PCR and re-sequenced to identify sequence mutations between the *RL-D* mutant and the WT. Base positions in the mapped region and the locations of the base mutations in the tentative candidate genes were determined based on chromosome 3 of the Nipponbare genome, which is the reference genome for *japonica* rice varieties. This genome was obtained from the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu); we used BLAST against the RGAP database to perform sequence alignment and gene annotation.

Transcriptomic Analysis

We used RNA-seq to identify genes that were differently expressed during early leaf-rolling. As the leaf-rolling phenotype was first observed at the six-leaf stage in the *RL-D* mutant, we sampled the top (half-expanded) leaves of *RL-D* and WT Nipponbare plants at the early six-leaf stage. To ensure representative sampling, leaves from three different seedlings were mixed to generate a biological replicate, and three biological replicates were constructed per genotype (18 seedlings in total). Total RNA was extracted from each replicate using TRIzol reagent (Invitrogen, USA), following the manufacturer's instructions, and then submitted to Novogene (http://www.novogene.com) for transcriptome sequencing. In brief, after RNA qualification, mRNA was enriched with Oligo(dT)-coupled magnetic beads, and then used as template for double-stranded cDNA (dscDNA) synthesis. Subsequently, the purified dscDNA samples were used to construct sequencing libraries and paired-end high-throughput sequencing was performed using a HiSeq 4000 sequencer (Illumina), with a read length of 150 bp (see Table S1 for RNA sequence statistics). After data filtration, the clean reads were compared to the reference genome (Rice Genome Annotation Project for Nipponbare, release 7; http://rice.plantbiology.msu.edu/) using TopHat2 (Kim et al. 2013). Gene expression levels were measured as using the expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) method (Trapnell et al. 2010). DESeq (Anders and Huber 2010, 2013) was used to calculate *P*–values, and these *P*–values were adjusted using the Benjamini–Hochberg correction method. We identified differentially expressed genes (DEGs)

between the mutant and the WT as those genes where $|\log_2$ fold change$| \geq 1$ and P–value < 0.05. We used GOseq (Young et al. 2010) to determine Gene Ontology (GO) enrichment, and KOBAS 2.0 (Mao et al. 2005) to determine pathway enrichment against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/). Terms were considered significantly enriched in the DEGs when the P–value was < 0.05. Clean RNA-seq data were uploaded to the SRA database (http://www.ncbi.nlm.nih.gov/sra; accession no. PRJNA613143).

Quantitative Real-time RT-PCR (qRT-PCR)

Total RNA from rice leaves at different growth stages was extracted for gene expression analysis using TRIzol reagent (Invitrogen), and then treated with RNase-free DNase I (NEB), following the manufacturer's instructions. Single-stranded cDNA was synthesized from 1.0 µg of total RNA using HiScript II Q Select RT SuperMix reverse transcriptase kits (Vazyme), and then used as templates for qRT-PCR. qRT-PCR amplifications were performed with specific primers (Table S2) using a Bio-Rad CFX96 and the 2 × SsoFast EvaGreen Supermix (Bio-Rad, USA), following the manufacturer's instructions. The PCR cycling conditions were as follows: 95 °C for 3 min, followed by 38 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s. The expression levels of the target genes were normalized against the expression of *O. sativa* actin (*OsActin2*, *Os10g0510000*) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All PCRs were repeated three times.

Subcellular Localization of the Candidate Gene

Preliminary results identified an *O. sativa* protein phosphatase (OsPP2C) as the candidate gene for the *RL-D* locus. To determine whether mutations in this gene in the *RL-D* mutant altered the subcellular location of OsPP2C, we successively cloned the cauliflower mosaic virus (CaMV) *35S* promoter, the *green florescence protein* (*GFP*) fragment, and the *nopaline synthase* (*NOS*) terminator of *Agrobacterium tumefaciens* into the pUC18 plasmid to construct the transient expression vector p35S-GFP, as described previously (Yang et al. 2014). The fragments containing the entire OsPP2C coding sequence without an end codon were amplified from the WT (Nipponbare) and the *RL-D* mutant using qRT-PCR. Each fragment was then inserted into separate p35S-GFP cloning vectors, upstream of GFP, to form WT and mutant p35S-OsPP2C-GFP fusion proteins. As described previously (Chen et al. 2006), recombinant vectors were transformed into rice protoplasts prepared from the leaf sheaths of 2-week-old seedlings of Zhonghua11 (ZH11, *Oryza sativa* L. ssp. *japonica*), and transient expression was analyzed.

Results

Phenotypic, Histological, and Physiological Characteristics of the *RL-D* Mutant

Until the five-leaf stage, the leaves of the *RL-D* mutant appeared similar to those of the WT (Fig. 1a). However, beginning at the six-leaf stage and continuing to maturity,

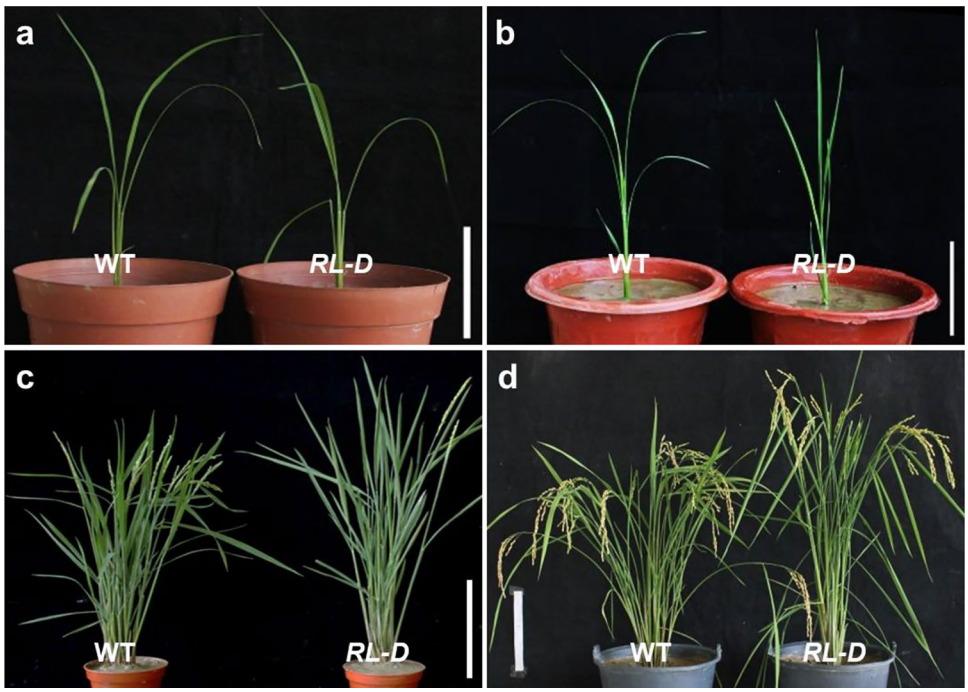


Fig. 1 Phenotype of the dominant rolled-leaf mutant rice (*RL-D*) and the wild-type Nipponbare rice (WT). **a** Five-leaf stage. **b** Six-leaf stage. **c** Booting stage. **d** Maturity. Scale bar, 10 cm

the leaves of the *RL-D* mutant exhibited an obvious inward rolling (Fig. 1b–d). At maturity, the LRIs of the first two *RL-D* leaves (~49%) were significantly greater than those of the first two WT leaves (0%; Fig. 2a; $P < 0.01$). The first two *RL-D* leaves were significantly narrower and longer than those of the WT ($P < 0.01$): the first *RL-D* leaf was 41% narrower and 30% longer than the first WT leaf, while the second *RL-D* leaf was 41% narrower and 62% longer than the second WT leaf (Fig. 2b–c). The *RL-D* mutant was also significantly (19%) taller than the WT ($P < 0.01$; Fig. 2d). In addition, 100-grain weight and chlorophyll content were significantly greater than those of the WT: 100-grain weight increased by 8% ($P < 0.01$), and the chlorophyll content of the flag leaves increased by 9% ($P < 0.05$) (Fig. 2e–f). However, the seed setting rate and productive tiller number of the *RL-D* mutant were significantly lower than those of the WT ($P < 0.01$): seed setting rate decreased by 29% in the mutant, while productive tiller number decreased by 13% (Fig. 2g–h).

Consistent with the observed changes in gross leaf morphology in the *RL-D* mutant, internal leaf anatomy, as revealed by the free-hand sections, did not differ between the mutant and the WT until the end of the five-leaf stage. However, from the beginning of the six-leaf stage, the sclerenchyma tissues appeared to develop abnormally on the abaxial side of the leaf midribs in the *RL-D* mutant, although no abnormalities were observed around the leaf lateral veins. No other obvious differences in leaf anatomy were observed between the *RL-D* and the WT (Fig. 3a–e). Semi-thin and ultra-thin sections confirmed that only the development of sclerenchyma tissues on the abaxial side of the midribs was defective in the *RL-D* mutant (Fig. 3f–m).

Genetic Analysis and Molecular Mapping of the *RL-D* Locus

All plants in the F_1 generation produced by crossing the mutant *RL-D* (male parent) with the WT Nipponbare variety

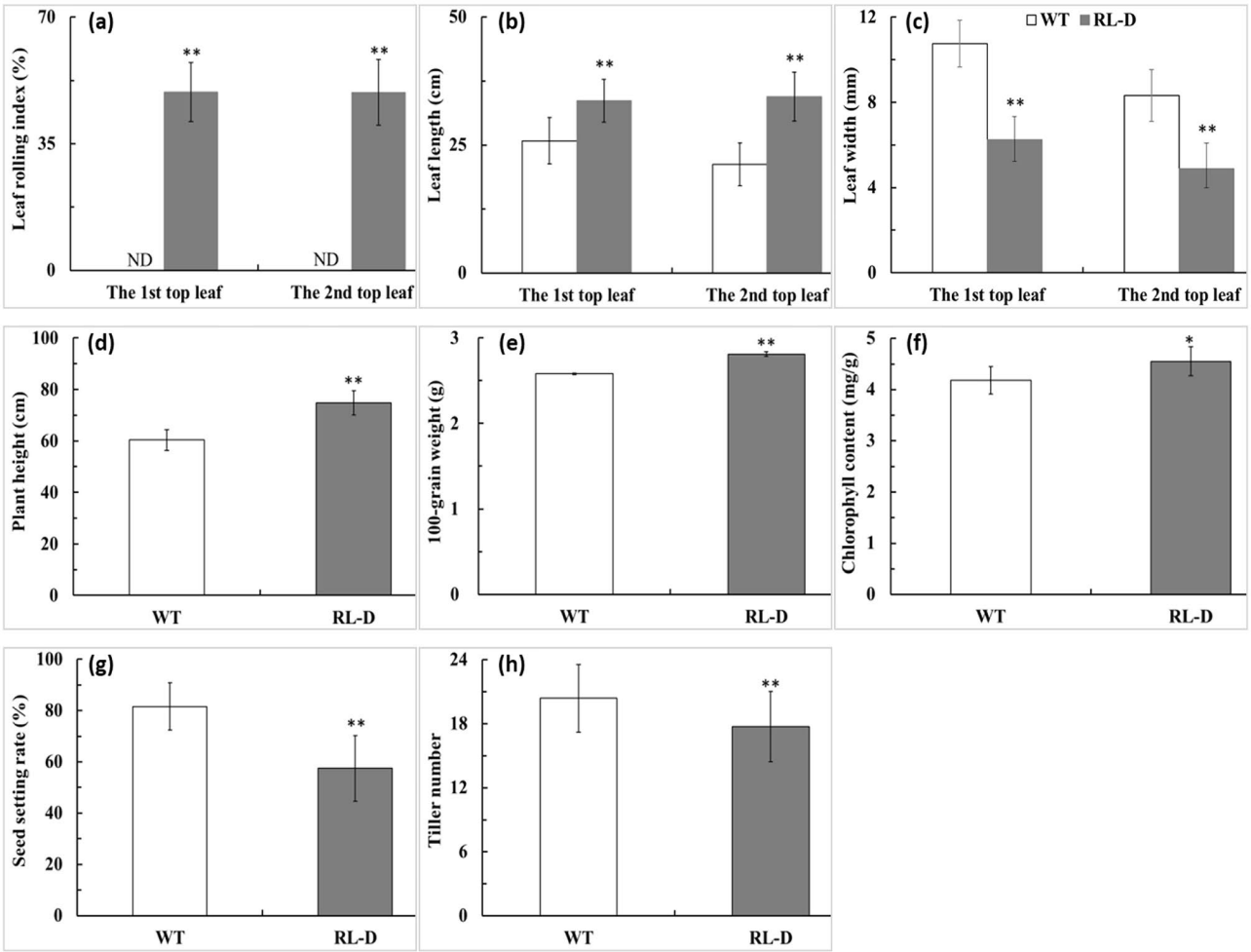
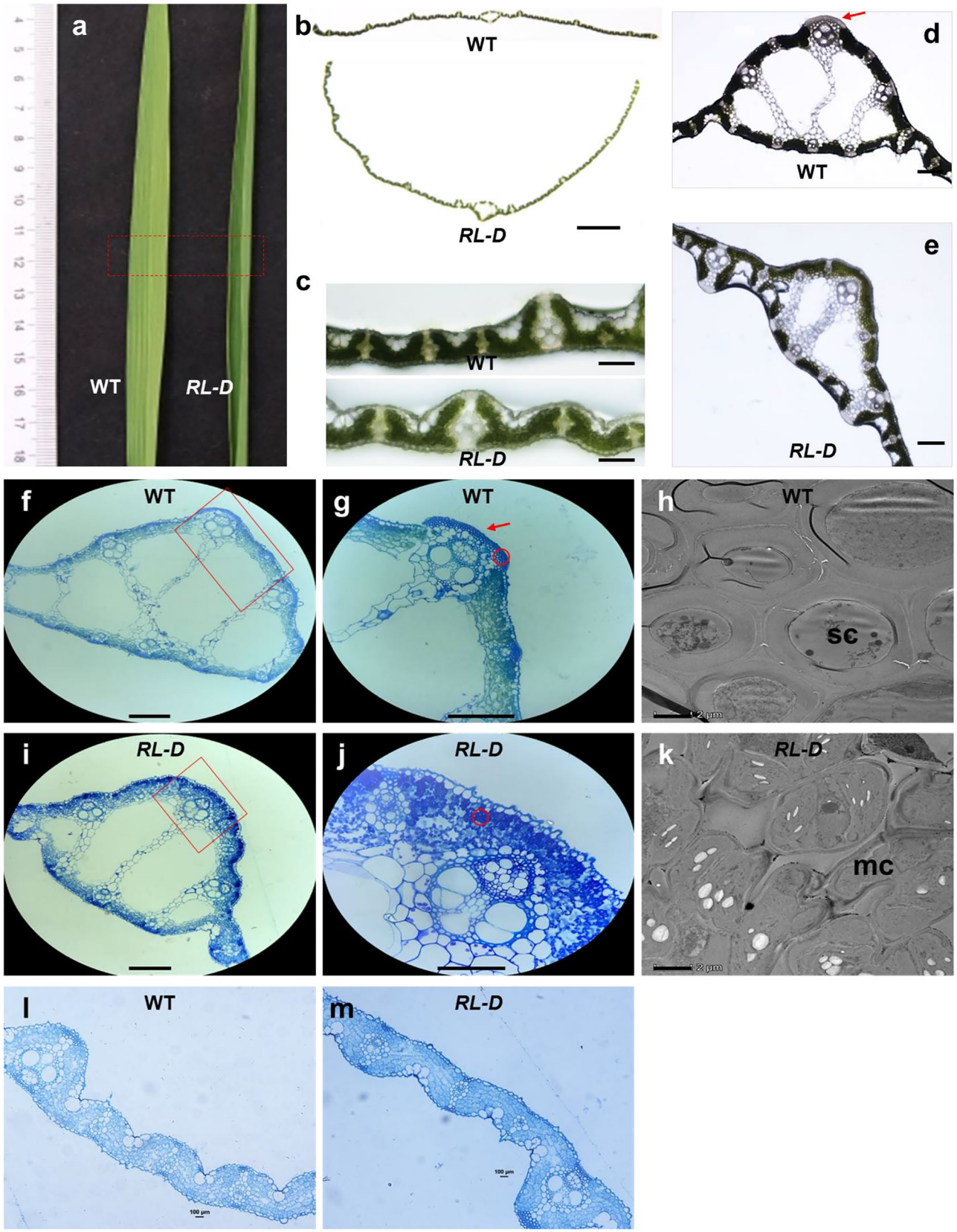


Fig. 2 Quantitative agronomic traits of the rolled-leaf mutant rice (*RL-D*) as compared to the wild-type Nipponbare rice (WT). Data shown represent means ± SD ($n = 30$). **a** Leaf rolling index (LRI). **b** Leaf length. **c** Leaf width. **d** Plant height. **e** 100-grain weight. **f** Chlorophyll content. **g** Seed setting rate. **h** Productive tiller number. *$P < 0.05$, **$P < 0.01$; Student's *t* test


a
WT
RL-D
b
WT
RL-D
c
WT
RL-D
d
WT
e
RL-D
f
WT
g
WT
h
WT
sc
2 μm
i
RL-D
j
RL-D
k
RL-D
mc
2 μm
l
WT
100 μm
m
RL-D
100 μm

◂**Fig. 3** Histological observations of the leaves of wild-type Nipponbare rice (WT) and rolled-leaf mutant rice (*RL-D*) at the booting stage. **b**–**e** Free-hand cross-sections at the middle of the flag leaf as indicated by the red dashed-line box in **a**. Close-ups of free-hand cross sections of lateral veins (**c**) and midribs (**d**, **e**). Semi-thin cross sections of midribs (**f**, **i**) and lateral veins (**l**, **m**) of the flag leaf. **g**, **j** Close-ups of the solid-line boxed sections of **f** and **i**, respectively. **h**, **k** Ultra-thin cross sections of the midribs, corresponding to the circled areas in **g** and **j**, respectively. Red arrow indicates normal sclerenchyma tissues. sc, sclerenchyma cells; mc, mesophyll cells. Scale bar in **b**, 1 mm; scale bars **c**–**e**, **f**–**g**, **i**–**j**, and **l**–**m**, 100 μm; scale bars in **h** and **k**, 2 μm

or Jingxian 89 (a flat-leaf variety) expressed *RL-D*-like leaf rolling phenotypes. In the two F_2 generations, the segregation ratios of rolled-leaf (mutant type) to flat-leaf (WT) plants were 563:181 = 3.11:1 ($\chi^2 = 0.15 < \chi^2_{0.05} = 3.84$), and 525:164 = 3.20:1 ($\chi^2 = 0.47 < \chi^2_{0.05} = 3.84$), respectively, consistent with the Mendelian ratio for one pair of alleles (3:1). This suggested that the *RL-D* locus was controlled by a single dominant gene. Using the F_2 population constructed by crossing *RL-D* (male parent) with Jingxian 89, we located the rolled-leaf locus *RL-D* in a region about 743 kb long, anchored by InDel15402194 and InDel16145546 on chromosome 3 (Fig. 4). Notably, in the F_1 and F_2 offspring obtained by crossing *RL-D* with the WT or Jingxian 89 varieties, no obvious defects were observed in the *RL-D* phenotypes (e.g., lower seed setting rate and/or tiller number), implying that the *RL-D* locus may not be responsible for these phenotypical defects.

Using the chromosome 3 sequence from the reference genome of the *japonica* Nipponbare variety (https://www.ncbi.nlm.nih.gov/nuccore/NC_029258.1), we identified 94 coding sequences (CDSs) in the mapped region. However, PCR amplification and re-sequencing of the *RL-D* and the WT identified only two genes in the mapped region with base mutations in the *RL-D* mutant: one substitution (G to A) was identified 1885 bp downstream of ATG in the sixth exon of *Os03g0389900*, corresponding to the conversion of serine to asparagine (Fig. 4 and Fig. S1), and two substitutions (G to T and G to A) were identified 13 bp and 63 bp, respectively, downstream of ATG in the first exon of *Os03g0395100* (Fig. 4 and Fig. S1). The G-to-T substitution in *Os03g0395100* introduced a termination codon (Fig. S1). Further analysis of the *Os03g0395100* coding sequence in the mutant showed that the mutated CDS may be translated from ATG, which was identified 94 bp downstream of the original start codon, producing a truncated form of this

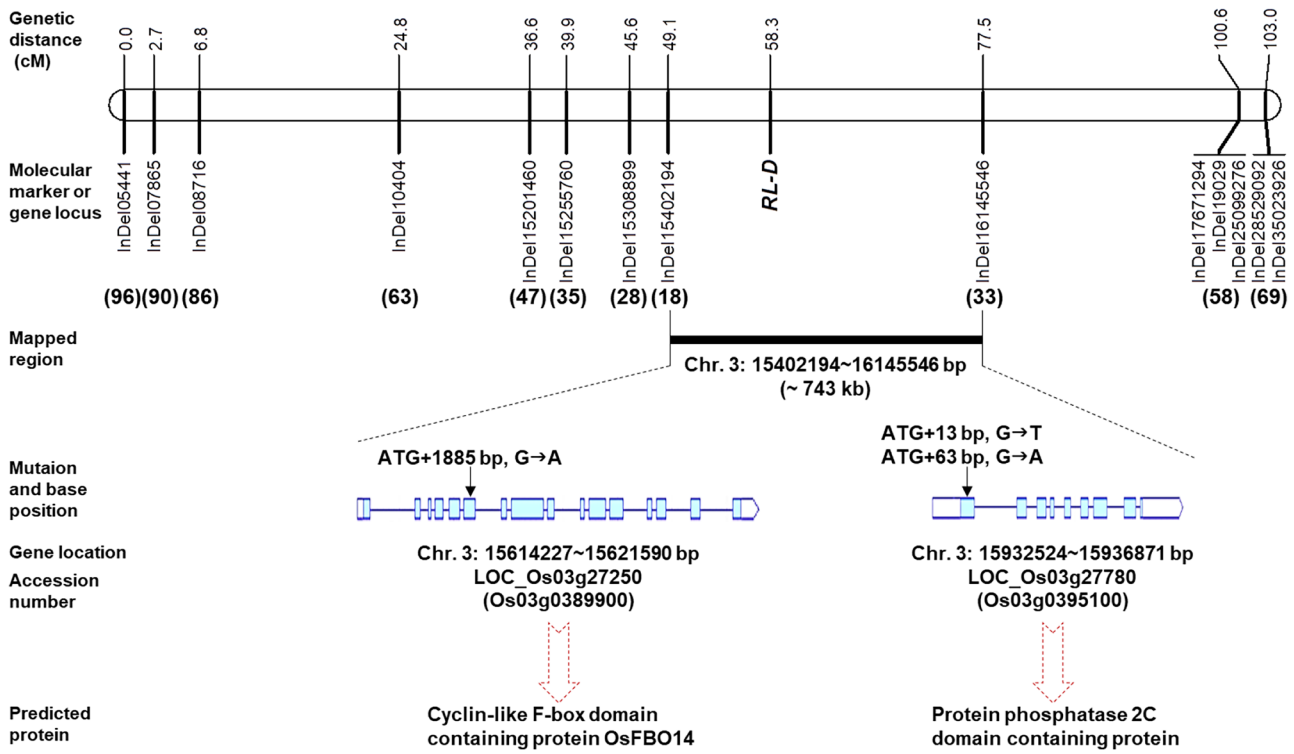


Fig. 4 Molecular map of the *RL-D* locus and the tentative candidate genes in the mapped region. The numbers in parentheses indicate the recombination events detected by each marker in the F_2 mapping population composed of 164 recessive (normal leaf) individuals (from Jingxian 89 × *RL-D*). Base positions in the mapped region and the locations of the base mutations in the tentative candidate genes are indicated based on chromosome 3 of the Nipponbare genome. Accession numbers in the format "LOC_Os×g××××××" pertain to the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu), while those in the format "Os×g××××××××" pertain to the International Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp)

protein. The re-sequencing results showed that the promoter regions of these two genes (i.e., the 2-kb region upstream of each start codon) were identical between *RL-D* and the WT. *Os03g0389900* encodes OsFBO14, a protein containing a cyclin-like F-box domain, while *Os03g0395100* encodes a protein herein named OsPP2C, containing a phosphatase 2C domain (Fig. 4).

Transcriptomic Analysis of the *RL-D* Mutant

To investigate the molecular mechanisms underlying the leaf-rolling phenotype of *RL-D*, we compared the whole genome transcriptomes of the *RL-D* mutant and the wild type (WT). In total, 551 differentially expressed genes (DEGs) (fold change ≥ 2.0, $P < 0.05$) were identified between the *RL-D* mutant and the WT at the early six-leaf stage, at which point the leaf-rolling phenotype is clearly visible in the mutant. Of these DEGs, 396 were upregulated in the RL-D mutant as compared to the WT, and 155 were downregulated (Table S3). Gene Ontology (GO) analysis showed that terms associated with programmed cell death (GO:0012501; GO:0008219), protein phosphorylation or modification (GO:0006468; GO:0016310; GO:0006796; GO:0043687), and the regulation of gene expression or various metabolic processes were overrepresented in the DEGs of *RL-D* (Table 1). KEGG pathway analysis indicated that pathways associated with the biosynthesis of secondary metabolites (ko01110), as well as various biomolecule biosynthesis and metabolic pathways, were most significantly enriched in the DEGs (Table 2).

Several previously described genes associated with leaf rolling were identified in the RNA-seq data (Table S4) and further verified using qRT-PCR (Fig. 5). The leaf polarity-related genes *OsAGO7* (Shi et al. 2007) and *OsYABBY1* (Toriba et al. 2007) were significantly upregulated in the *RL-D* leaves as compared with WT leaves, but *OsYABBY5* (Toriba et al. 2007) was significantly downregulated (Fig. 5). *SLL1*, a gene associated with the sclerenchyma tissue (Zhang et al. 2009), was also significantly upregulated in the *RL-D* leaves as compared to the WT leaves (Fig. 5). Finally, several other genes related to the secondary cell wall were significantly differently expressed in the *RL-D*

Table 1 Gene ontology (GO) terms significantly enriched in the genes differentially expressed in the leaves of the rolled-leaf mutant rice (*RL-D*) as compared to the leaves of the wild-type Nipponbare rice

GO	Description	Number of enriched DEGs	*P* value
GO:0010467	Gene expression	47	3.60E–10
GO:0006350	Transcription	42	3.60E–13
GO:0050789	Regulation of biological process	42	7.10E–10
GO:0045449	Regulation of transcription	41	2.00E–13
GO:0010468	Regulation of gene expression	41	6.40E–13
GO:0043687	Post-translational protein modification	34	9.10E–08
GO:0006796	Phosphate metabolic process	33	4.20E–07
GO:0006810	Transport	32	2.30E–07
GO:0051234	Establishment of localization	32	2.30E–07
GO:0051179	Localization	32	2.90E–07
GO:0016070	RNA metabolic process	31	5.80E–10
GO:0006468	Protein amino acid phosphorylation	31	1.40E–07
GO:0016310	Phosphorylation	31	7.40E–07
GO:0055114	Oxidation reduction	30	2.10E–47
GO:0006355	Regulation of transcription, DNA-dependent	28	8.30E–12
GO:0051252	Regulation of RNA metabolic process	28	9.60E–12
GO:0006351	Transcription, DNA-dependent	28	2.10E–11
GO:0006950	Response to stress	23	5.00E–09
GO:0055085	Transmembrane transport	17	4.00E–17
GO:0006952	Defense response	10	0.00036
GO:0012501	Programmed cell death	10	0.00062
GO:0006915	Apoptosis	10	0.00062
GO:0008219	Cell death	10	0.00075
GO:0006811	Ion transport	9	0.0028
GO:0006979	Response to oxidative stress	7	0.00023
GO:0006725	Cellular aromatic compound metabolic process	6	0.00064
GO:0000003	Reproduction	5	0.00056

Table 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly enriched in the genes differentially expressed between the leaves of the rolled-leaf mutant rice (*RL-D*) and the leaves of the wild-type Nipponbare rice

Pathway_ID	Description	Number of enriched DEGs	*P* value
ko01110	Biosynthesis of secondary metabolites	24	0.003022908
ko00940	Phenylpropanoid biosynthesis	6	0.008449121
ko00592	alpha-Linolenic acid metabolism	6	3.69E-05
ko00591	Linoleic acid metabolism	4	0.000130263
ko00904	Diterpenoid biosynthesis	3	0.002381846
ko00333	Prodigiosin biosynthesis	2	0.005200232
ko00780	Biotin metabolism	2	0.023908844
ko00402	Benzoxazinoid biosynthesis	1	0.048048117

leaves as compared to the WT leaves: *OsPAL7* (Tonnessen et al. 2015) and *OsCAD7* (Li et al. 2009) were upregulated, while *Os4CL3* (Gui et al. 2011), *Dirigent1* (Davin and Lewis 2005), *OsCesA4*, *OsCesA7*, and *OsCesA9* (Tanaka et al. 2003) were downregulated (Fig. 5).

Screening of Candidate Genes for the *RL-D* Locus

In RNA-seq analysis, *Os03g0395100* in the mapped region was significantly upregulated in the *RL-D* leaves as compared to the WT leaves, corresponding to a 1.5-fold increase in relative expression level ($P < 0.05$) (Table S4). However, *Os03g0389900* was not significantly differentially expressed in the *RL-D* mutant as compared to the WT (Table S4). To further screen the candidate gene for the *RL-D* locus, we quantified the expression levels of these two tentative candidate genes during early growth (just before and during the six-leaf stage, as well as during the seven-leaf stage) and during late growth (the tillering stage and the booting stage) using qRT-PCR. The expression levels of *Os03g0389900* did not differ significantly between *RL-D* and the WT at any tested stages (Fig. 6a). However, *Os03g0395100* was significantly upregulated in the *RL-D* mutant as compared to the WT at the five-leaf stage, just before the appearance of leaf rolling (Fig. 6b). This gene was also significantly upregulated compared to the WT at the six- and seven-leaf stages (Fig. 6b).

To explain the possible genetic causes of the *RL-D* mutation, which may be associated with mutations in *Os03g0395100* (*OsPP2C*), we compared the subcellular locations of the WT and mutated proteins. The base mutation in the first exon of *OsPP2C* in the *RL-D* mutant may produce a truncated form of OsPP2C protein (as described above) and alter the N-terminal amino acid sequences of the OsPP2C protein in the *RL-D* mutant. This may shift the subcellular location of the protein. In the WT, the fluorescence signal of the p35S-OsPP2C-GFP fusion protein was observed only in the nucleus (Fig. 7b), in contrast to the

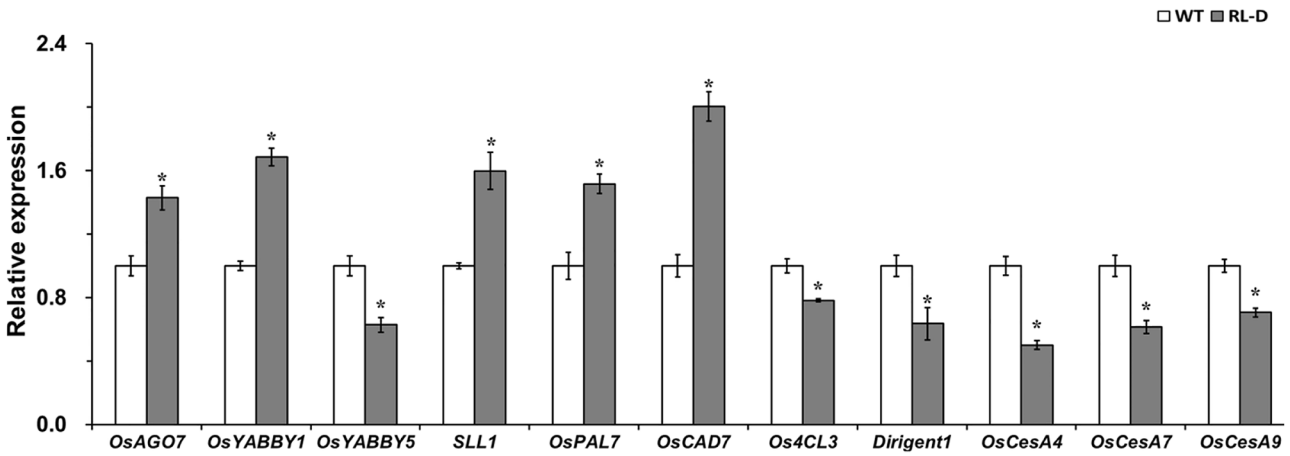


Fig. 5 qRT-PCR verification of the RNA-sequencing-detected genes associated with leaf development. Relative gene expression was measured in the top leaves. The 1st top (half-expanded) leaves from rice plants of the rolled-leaf mutant (*RL-D*) were compared to the wild-type Nipponbare (WT) at the early six-leaf stage. *OsActin2* (*Os10g0510000*) was used as the internal standard, against which the expression levels of the analyzed genes were normalized. Expression levels in the WT were set to 1. Data shown represent means ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$; Student's *t* test

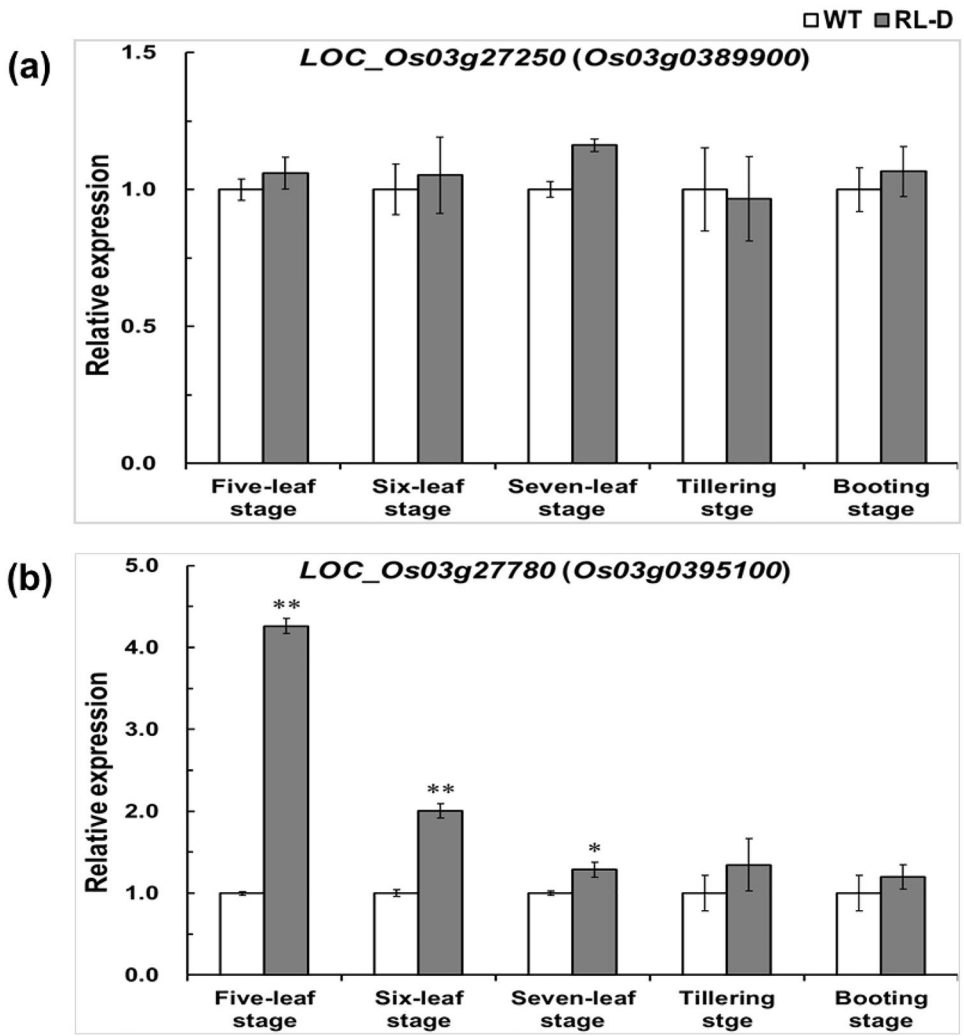


Fig. 6 Relative expression of candidate genes for the *RL-D* locus in the top leaves from rolled-leaf mutant rice (*RL-D*) and wild-type Nipponbare rice (WT), as determined using qRT-PCR. (a) *Os03g0389900*. (b) *Os03g0395100*. *OsActin2* (*Os10g0510000*) was used as the internal standard, against which the expression levels of the analyzed genes were normalized. Expression levels in the WT were set to 1. Data shown represent means ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$; Student's *t* test

GFP control (Fig. 7a). However, in the *RL-D* mutant, the fusion protein was expressed in both the nucleus and the cytoplasm (Fig. 7c).

Discussion

RL-D Represented a Novel Dominant Rolled-Leaf Germplasm Useful for Rice Breeding

In rice, moderate leaf rolling increases yield by improving photosynthetic efficiency, ventilation, and light transmission (Xu et al. 2018). Therefore, rolled-leaf germplasms are of great importance for the breeding of high-yield rice. At present, almost 50 leaf-rolling rice germplasms are available, but most of the reported alleles are recessive, which inhibits the effective large-scale application of the leaf-rolling trait in hybrid rice breeding. The few dominant rolled-leaf rice mutants available to date include *REL1*, in which the leaves roll from the seedling stage to maturity, but grain yield is reduced (Chen et al. 2015); *CFL1*, in which the leaves roll only from the late tillering stage (Wu et al. 2011); *R05*, in which leaf rolling increases only in the later vegetative stages, and chlorophyll content in the leaves is unchanged as compared to wild type (Shi et al. 2007); and *ACL-D*, which is clearly distinguishable at the three-leaf stage, but exhibits an abaxially rolled phenotype (Xu et al. 2014). Unlike these mutants, the leaves of *RL-D* rolled adaxially (inward) and were clearly identifiable beginning at the six-leaf stage. In the *RL-D* mutant, most of the cells in leaf cross sections were normal, except for the sclerenchymatous cells. Moreover, chlorophyll content, grain weight, leaf length, and plant height were all greater in the *RL-D* mutant as compared to the WT. The mutant also exhibited several negative characteristics, including a lower seed setting rate and a lower tiller number. However, hybrid breeding is usually a process of pyramiding favorable agronomic traits into one target cultivar (Wang et al. 2005). Therefore, *RL-D* not only represents a novel dominant rolled-leaf rice germplasm, but it also confers several beneficial agronomic traits that may

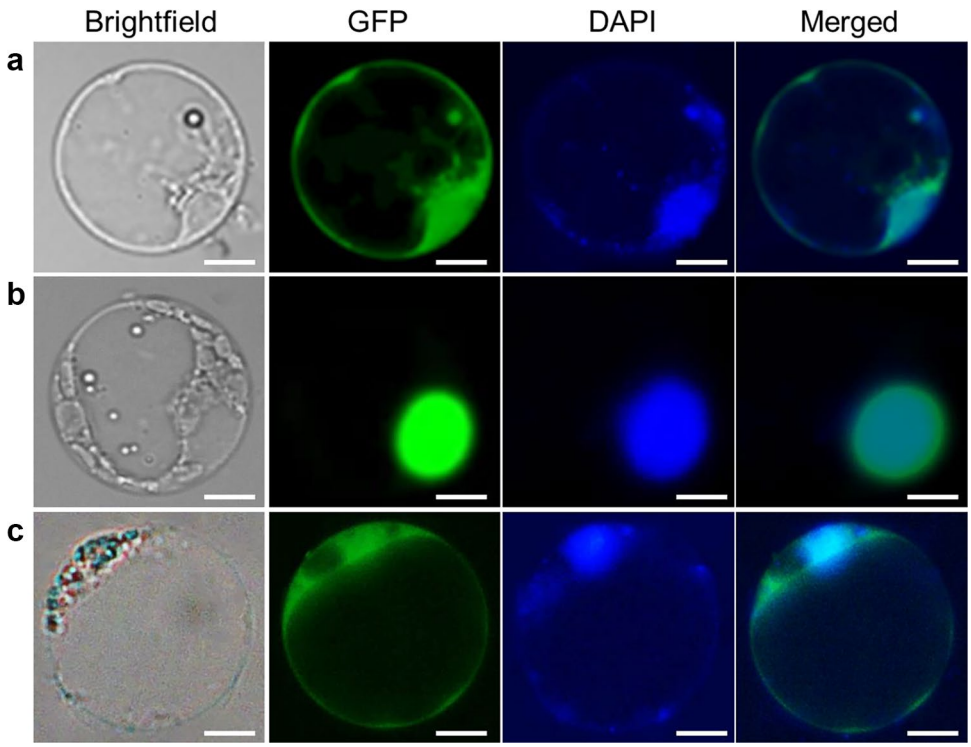


Fig. 7 Subcellular locations of the protein phosphatase-green florescence protein (OsPP2C-GFP) in leaf sheath protoplasts isolated from 2-week-old Zhonghua11 (ZH11, *Oryza sativa* L. ssp. *japonica*) rice seedlings. Brightfield, GFP, DAPI (4′,6-diamidino-2-phenylindole), and merged images of protoplasts are shown. **a** *GFP* control. **b** *OsPP2C* (WT)*::GFP*. **c** *OsPP2C* (*RL-D*)*::GFP*. Scale bars, 10 μm

support the future development of rice plants with improved phenotypes. In addition, because *RL-D* is a dominant moderate leaf-rolling mutation, it is easily identified in growing seedlings. Early identification is helpful when attempting to introduce favorable leaf traits into high-yield rice hybrids. Furthermore, moderate leaf-rolling helps to increase rice planting density and ventilation in the paddy field, which may increase rice yields in agricultural production.

Several Pathways, Including Leaf Polarity Regulation, Secondary Cell Wall Formation, and Protein Phosphorylation, May Participate in *RL-D* Formation

Previous studies have suggested that rolled-leaf formation in rice is influenced by several factors, including leaf polarity establishment (Xu et al. 2018), bulliform cell development (Xu et al. 2018), auxin (IAA) biosynthesis (Fujino et al. 2008), sclerenchyma tissue development (Zhang et al. 2009; Liu et al. 2016; Zhao et al. 2016), and leaf cuticle formation (Wu et al. 2011). In the *RL-D* mutant, we observed defective sclerenchyma tissues on the abaxial side of the leaf midribs (Fig. 3). Transcriptomic analysis identified many genes significantly differentially expressed between *RL-D* and WT. In particular, genes associated with transcription regulation were overrepresented in the DEGs (Table 1), suggesting that the regulation of gene expression is fundamental to the leaf-rolling phenotype in *RL-D*. Genes in the *YABBY* family mainly regulate the expression of genes that control the fate of leaf abaxial cells (Liu et al. 2007a; Toriba et al. 2007). Using qRT-PCR analysis, transcriptional changes in two *YABBY* genes were identified between the *RL-D* mutant and the WT: *OsYABBY5* was downregulated in *RL-D*, while *OsYABBY1* was upregulated (Fig. 5). This was in consistent with a previous study, in which the mRNA levels of the *YABBY* genes were differentially expressed in *OsHox32*-overexpressing rice plants with narrow- and rolled-leaf phenotypes (Li et al. 2016a, b). OsAGO7 belongs to the Argonaute (AGO) protein family; AGO proteins help to guide the cleavage of the mRNA of downstream genes, which are usually associated with leaf polarity development (Shi et al. 2007). Here, *OsAGO7* was also upregulated in *RL-D* (Fig. 5). Notably, genes related to various relevant pathways, including programmed cell death, secondary cell wall formation, and protein phosphorylation or modification, were also overrepresented in the *RL-D* DEGs (Tables 1 and 2).

Sclerenchymatous cells are lignified dead cells with thickened secondary cell walls, and programmed cell death is necessary for the differentiation of mesophyll cells into sclerenchymatous cells (Zhang et al. 2009). Deficiencies in *SLL1*, *SRL2*, and *NRL2*, which directly regulate the development of sclerenchyma tissues, lead to the abnormal programmed cell death of the abaxial mesophyll cells, resulting in leaf rolling (Zhang et al. 2009; Liu et al. 2016; Zhao et al. 2016). Our histological observations identified defective abaxial sclerenchyma tissues around the leaf midrib in the *RL-D* mutant (Fig. 3). However, of the genes reported to be associated with sclerenchyma tissue development, only *SLL1*

was significantly upregulated in *RL-D* as compared to the WT (Fig. 5); the expression levels of the other genes did not differ between these lines (data not shown). This suggested that the mechanism of rolled-leaf formation in *RL-D* might differ from that previously proposed.

Transcriptional changes in genes associated with secondary cell wall formation were also identified in *RL-D*. Two genes related to lignin monomer synthesis (*Os4CL3* and *Dirigent1*; Gui et al. 2011; Davin and Lewis 2005), and three genes encoding cellulose synthase catalytic subunits (*OsCesA4*, *OsCesA7*, and *OsCesA9*; Tanaka et al. 2003), which are necessary for the synthesis of secondary cell wall, were downregulated in *RL-D* as compared to the WT. This suggested that, in *RL-D*, the synthesis of lignin and cellulose might be reduced, inhibiting the formation of the secondary cell wall. These results were consistent with previous studies, which demonstrated that cell wall formation was repressed in rolled-leaf mutants (Fang et al. 2012). However, both *OsPAL7* and *OsCAD7* were upregulated in *RL-D* as compared to the WT. *OsPAL7* encodes phenylalanine ammonia-lyase, a key enzyme in the phenylpropanoid pathway; lignin is one of the metabolites of this pathway (Tonnessen et al. 2015). *OsCAD7*, which encodes cinnamyl alcohol dehydrogenase, also plays an important role in lignin biosynthesis (Li et al. 2009). The upregulation of these genes in *RL-D* as compared to the WT suggested that lignin biosynthesis in *RL-D* might be increased, reinforcing the plant cell wall despite the downregulation of *Os4CL3*, *Dirigent1*, *OsCesA4, OsCesA7*, and *OsCesA9*. Thus, various genes previously shown to be associated with leaf rolling exhibited different or even contradictory expression patterns in *RL-D*. These inconsistencies, in conjunction with the fact that *RL-D* is a dominant mutant, implied that a novel molecular mechanism underlies the leaf-rolling phenotype of *RL-D*.

The tentative candidate *PP2C* gene (herein named *OsPP2C*) was identified in the mapped region (Fig. 4). Re-sequencing identified base variations in the first exon of the *OsPP2C* (*Os03g0395100*) gene in the *RL-D* mutant that may lead to premature translation termination or the production of a truncated form of the OsPP2C protein (Fig. S1). This mutation may alter the subcellular location of the OsPP2C protein in the *RL-D* mutant (Fig. 7). Moreover, processes associated with protein phosphorylation or modification were significantly enriched in the genes differentially expressed in *RL-D* (Table 1). The *OsPP2C* gene encodes a protein phosphatase, which may participate in the regulation of multiple signaling transduction pathways via the dephosphorylation of cyclin-dependent kinase (CDK) (Tougane et al. 2010; Umbrasaite et al. 2010; Fan et al. 2013). In rice, PP2C phosphatases may participate in pollen germination, disease resistance, and responses to drought and oxidative stress (Fujii and Toriyama 2008; Hu et al. 2009; You et al. 2014). Although PP2C phosphatases have not previously been implicated in rolled-leaf formation, our results indicated that the leaf-rolling phenotype may be associated with the truncation of PP2C phosphatase or the alteration of its subcellular location in *RL-D*, although this possibility requires further study. Interestingly, qRT-PCR analysis showed *OsPP2C* was significantly upregulated just before the appearance of leaf rolling in the mutant plants (that is, at the five-leaf stage) (Fig. 6b). However, no base variations were observed in the promoter region of the *OsPP2C* gene in *RL-D*. We speculated that the alteration of subcellular location or the inactivation of the OsPP2C protein in *RL-D* may stimulate the production of additional *OsPP2C* mRNA due to a feedback loop. In addition, because *RL-D* is a "gain-of-function" dominant mutant, the genetic complementation of the *RL-D PP2C* gene into the WT and experiments knocking-out the WT *PP2C* gene are needed to confirm *OsPP2C* as the candidate gene for *RL-D*.

In the present study, the mapped interval (15,402,194–16,145,546 bp) of the *RL-D* locus is adjacent to the centromere region (17,882,716–20,185,397) of chromosome 3 (http://plants.ensembl.org/Oryza_sativa/Location/Overview?db=core;r=3:15402194-16145546), which may be one of coldspots on Chr. 3, thus leading to low recombination rate within the mapped interval. The previous study showed that the average physical distance per centimorgan is observed to be 244 kb for the rice genome, which varies with position along the chromosome, with centromere regions exhibiting > 1 Mb/cM (Chen et al. 2002). Generally, the genetic recombination in centromere regions and other heterochromatic regions is severely suppressed (Chen et al. 2002). The previous study also indicated that many low (coldspot)-recombinaniton regions outside centromeric regions were scattered along chromosomes, which exhibited low recombination rate (Wu et al. 2003). However, the repetitive content and gene density in these coldspots are similar to those of regions with normal recombination rates, and the factors that suppress genetic recombination in these regions remain unknown (Wu et al. 2003). Another study indicated that the locus of the rice *dwarf1* gene was adjacent to the centromere of chromosome 5, and the mapped region of the locus exhibited low recombination frequency. Finally, the authors used an F_2 mapping population with a size as large as 13,000 individuals, including 3,185 recessive (dwarf) plants, to fine-map the *dwarf1* locus in rice (Ashikari et al. 1999). In fact, none of the markers we developed in the current mapped interval was recombined with the *RL-D* locus. As a result, the *RL-D* locus was only roughly mapped in a 743-kb region (Fig. 4).

We supposed that the size of mapping population used in the present study was not large enough for fine mapping.

Conclusions

In summary, our results indicated that the dominant rolled-leaf phenotype of the *RL-D* mutant represents a novel rolled-leaf germplasm with beneficial agronomic traits, which can be used to develop desired phenotypes. Genetic analyses and re-sequencing assays suggested that *OsPP2C* (*Os03g0395100*) may be the candidate gene for the *RL-D* locus. We propose that the "gain of function" caused by the mutation of the *OsPP2C* gene in *RL-D* may regulate the rolled-leaf phenotype by affecting the development of the secondary cell wall, including sclerenchyma tissues and leaf polarity via dephosphorylation in a complex manner; this process may present a novel mechanism underlying leaf rolling in rice. However, the details of *OsPP2C* function, as well as the molecular mechanisms underlying the association of this gene with the development of sclerenchymatous cells, remain to be investigated. Our results help to clarify the leaf-rolling mechanism in rice, and also lay a foundation for further studies of *RL-D* and its application in rice breeding.

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Author Contribution XMG and FHW carried out the experiment and prepared the figures and tables. JCY designed the research, analyzed the data, and wrote the manuscript. HMC, XLL, and SCZ contributed to plant materials management and data evaluation. JLZ contributed for critically analyzing the data and reading this manuscript. All authors approved the final version of manuscript.

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Availability of Data and Materials All data supporting the conclusions of this article are provided with the article and its supplementary information files.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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