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Genome-wide transcriptomic analysis reveals the gene regulatory network that controlled by *SRL1* in regulating rice leaf rolling

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Abstract

Background

SRL1 (*SEMI-ROLLED LEAF 1*) also named as *CLD1* (*CURLED LEAF AND DWARF 1*), encoding a putative glyphopholipidininol-anchored membrane protein, has been characterized as a gene involved in the regulation of leaf morphology in rice. Mutants of *srl1-1* (point mutation) and *srl1-2* (transferred DNA insertion) exhibit defects in leaf development resulting in a phenotype with adaxially rolled leaves.

Results

To explore the gene regulatory network of leaf development that controlled by *SRL1* in rice, we created a homozygous *SRL1* knock out (KO) line by CRISPR/Cas9, which showed defects in leaf development with adaxially rolling. By comparing the leaf transcriptome of a homozygous *SRL1* KO line (*srl1*-KO) with the control, a total number of 3,178 genes were identified as differentially expressed genes, of which 1,216 genes were significantly up regulated, while 1,962 genes were down regulated. Further analyses indicated that, a group of known leaf rolling related genes, which involved in bulliform cells and cuticle development such as *OsZHD1*, *OsLBD3-7*, *RFS*, *ACL1*, *CFL1*, *SND1*, *OsCESA5* and *OsCESA6* were up or down regulated in the *srl1*-KO.

Conclusions

SRL1 might control leaf rolling by regulating a couple of genes that affecting cytological architecture of leaf cells such as bulliforms and cuticle of leaves.

Keywords: *SRL1/CLD1*, rolling leaf, RNA-seq, bulliform cells, cuticle

Background

Rice is one of the most widely cultivated crops feeding more than half of the population around the globe. As the major photosynthetic organ of plants, the photosynthetic efficiency of leaves has a great influence on rice yield (Walter et al 2009). Therefore, it is of great significance to study the mechanism of leaf development to improve rice yield. In recent years, many genes involved in leaf development have been identified (Xu et al 2018).

SRL1 (*SEMI-ROLLED LEAF 1*), which encodes a putative glycosylphosphatidylinositol (GPI)-anchored protein (GAP) has been cloned and characterized as a gene that involves in modulating leaf rolling. *SRL1* is expressed in various tissues and SRL1 protein is located predominantly at the plasma membrane. Both of *srl1-1* (point mutant) and *srl1-2* (transferred DNA insertion, knockdown mutant) displayed adaxially rolled leaves due to the increase of bulliform cells at the adaxial cell layer. Laser microdissection and microarray analysis with the cells that will become epidermal cells in wild type but bulliform cells in *srl1-1* indicated that the genes encoding vacuolar H^+-pyrophosphatase and H^+-ATPase were up regulated in *srl1-1*, suggesting that *SRL1* regulates leaf rolling through inhibiting the formation of bulliform cells by repressing the expression of the genes encoding vacuolar H^+-pyrophosphatase and H^+-ATPase (Xiang et al 2012). *CLD1* (*CURLED LEAF AND DWARF 1*) is allelic with *SRL1*. In addition to adaxially rolled leaves, the *cld1* mutant, which was controlled epigenetically through DNA methylation at the *SRL1* locus showed multiple morphological abnormalities, including the twisted rolling leaves, dark-green leaves, dwarf of plant architecture, and faster water loss. The

quantitative proteomic analysis showed that a group of proteins, which associated with cell wall formation, epidermis development and water stress, were differentially expressed between the *cld1* mutant and the wild type (Li et al 2017).

In addition to *SRL1*/*CLD1,* a group of genes that regulate leaf rolling by affecting number and/or area of bulliform cells have been cloned and functional characterized, including *YAB1* (*YABBY1*), *OsZHD1* (*ZINC FINGER HOMEODOMAIN 1*), *OsZHD2*, *LC2* (*LEAF INCLINATION 2*), *RFS* (*ROLLED FINE STRIPED*), *RL14* (*ROLLING LEAF 14*), *NRL1* (*NARROW AND ROLLED LEAF 1*), *OsMYB103L*, *ROC5* (*RICE OUTER CELL SPECIFIC 5*), *PFL* (*PROTODERMAL FACTOR LIKE 1*), *ACL1* (*ABAXIALLY CURLED LEAF 1*), *ACL2*, *REL1* (*ROLLED AND ERECT LEAF 1*), *REL2*, *OsLBD3-7* (*LATERAL ORGAN BOUNDARIES DOMAIN 3-7*), *NAL7* (*NARROW LEAF 7*), *NAL2/3*, *OsI-BAK1* (*BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1*), *OsARF18* (*AUXIN RESPONSE FACTOR 18*), *SLL2* (*SHALLOT-LIKE 2*) and *OsRRK1* (*RECEPTOR-LIKE CYTOPLASMIC KINASE 1*) (Chen et al 2015, Cho et al 2018, Cho & Paek 2016, Dai et al 2007, Fang et al 2012, Fujino et al 2008, Hu et al 2010, Huang et al 2016, Khew et al 2015, Li et al 2016, Li et al 2010, Ma et al 2017, Xu et al 2014, Yang et al 2014, Yang et al 2016, Zhang et al 2015, Zhao et al 2010, Zou et al 2011). Moreover, genes involved in regulation of leaf polarity such as *ADL1* (*ADAXIALIZED LEAF 1*), *SLL1* (*SHALLOT-LIKE 1*) and *OsAGO7* (*ARGONATUE 7*) (Hibara et al 2009, Shi et al 2007, Zhang et al 2009); genes related to development of sclerenchymatous cells such as *NRL2*/*SRL2*, *SLL1* and *RL14* (Liu et al 2016, Zhang et al 2009, Zhao et al 2016); genes related to cuticle development such as *CFL1* (*CURLY FLAG LEAF 1*) and *OsMYB103L* (Wu et al 2011, Yang et al 2014); miRNs (miR160 and miR166) with their targets (*OsARF18* and *OsHB4* (*HOMEODOMAIN CONTAINING PROTEIN 4*)) and the genes involved

in processing miRNA (*DCL1* (*DICER-LIKE 1*), *OsAGO1*, *OsAGO7*) also have been proved to regulate leaf rolling (Huang et al 2016, Liu et al 2005, Shi et al 2007, Wu et al 2009).

The gene regulatory network controlling by *SRL1*/*CLD1* have been studied previously using transcriptomic analysis (microarray) with *srl1-1* and the iTRAQ (isobaric tags for the relative and absolute quantitation) base proteomic analysis with *cld1*. However, the previous transcripomic study focused on the genes expression difference in a group of cells that abutting the midrib at the adaxial surface of leaf blades which would become epidermal cells in wild type and probably bulliform cells in the *SRL1* point mutant *srl1-1*, this may lead to a incomplete understanding of the *SRL1* regulation mechanism in leaf development due to the limit of the sampling location and the limit of the technology for gene expression profiling (microarray). While the previous proteomic study was with *cld1*, which exhibited extra defects in addition to adaxially rolled leaves. Part of the differentially expressed proteins obtaining from this study may not relate to the leaf development but the other developmental processes due to the multiple defects in *cld1*. To obtain a more complete view of the *SRL1* regulation mechanism in leaf development, we created a *SRL1* knockout mutant by CRISPR/Cas9, and compared the gene expression profiles of the mature leaves between the knockout mutant and its control.

Results

Generation of *SRL1* KO lines by CRISPR/Cas9

In order to study the biological function of *SRL1*, we created *SRL1* knockout (KO) lines by CRISPR/Cas9, with the target site located in the first exon (Fig. 1a). We obtained 14 T_0 transgenic

100 plants. For each T_0 transgenic plants, the target sequence was identified by Sanger-sequencing of
101 the target-containing amplificons followed by decoding via DsDecodeM (Liu et al 2015). Half of
102 the T_0 transgenic plants (7 of 14) were identified as non-KO plants, in which contained the intact
103 *SRL1* and showed no defects in leaf development; the other 7 plants were identified as knock out
104 mutants with 1 bp insertion (T or C) in the first exon resulting in a frame shift with premature
105 transcription termination (Additional file 1: Table S1, Fig. 1b~c). All the knockout mutants
106 exhibited adaxially rolled leave which were similar to that of the *srl1-1* and *srl1-2* (Xiang et al
107 2012) indicating that we had successfully created the *SRL1* knockout line (*srl1*-KO) as well as the
108 control line (non-KO), which could be available for the further transcpritomic study (Fig. 1d~f).

109 **Transcriptomic analysis**

110 To understand the gene regulatory network that controlled by *SRL1*, we analyzed the
111 transcriptomic data that generated from the mature leaves (60 days after germination) of the *SRL1*
112 knockout mutants *srl1*-KO and the control non-KO. We carried out the RNA-seq with three
113 biological replicates of *srl1*-KO and non-KO respectively. After filtering out the unqualified reads
114 from the raw data, we obtained 62.85, 56.16 and 56.06 million clean reads for the three KO
115 libraries and 55.25, 58.70 and 58.91 million clean reads for the three non-KO libraries. For each of
116 the six libraries, at least 94.02% of the clean reads had a quality score of Q30. The GC content
117 was 54.45%, 54.63% and 54.54% for KO libraries and 55.04%, 54.55% and 54.44% for non-KO
118 libraries. Using HISAT2 software (Kim et al 2015), more than 89.71% of clean reads were
119 successfully and uniquely mapped to the rice genome (Table 1). Furthermore, we evaluated the
120 correlation between the samples by Pearson's correlation coefficient using deepTool2 (Ramirez et

al 2016). The correlation was higher for the samples within group than that from different groups. Taken together, all these results indicated the good quality of our sequencing data suggesting the high confidence for the subsequence transcriptomic analysis results (Fig. 2a).

We detected 32,668 genes expression by RNA-seq at least in one set of the samples (Additional file 2: Table S2), A total number of 3,178 genes were identified as differentially expressed genes (DEGs) with the cutoff for FDR (False Discovery Rate) < 0.01 and absolute $\log_2$FC (Fold Change) > 1. Of these DEGs, 1,216 genes were significantly up regulated in the *srl1*-KO line, while 1,962 genes were down regulated (Fig. 2b, Additional file 3: Table S3). Among these DEGs, 678 genes were annotated by KEGG (Kyoto Encyclopedia of Genes and Genomes) (Additional file 4: Table S4). Three hundred seventy eight of 678 DEGs were assigned to 101 KEGG pathways (Additional file 5: Table S5). Plant hormone signal transduction, plant-pathogen interaction and starch & sucrose metabolism were the top three largest groups of DEGs according to the KEGG pathways analysis (Fig. 2c). To further validate the RNA-seq data, the expression quantification of 12 known leaf rolling related genes were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) including *OsZHD1*, *LC2*, *RL14*, *ACL1*, *OsLBD3-7*, *YAB1*, *NAL7*, *CFL1*, *ADL1*, *OsAGO1c*, *OsAGO1d* and *NRL2*. The selected genes expression pattern determined by qRT-PCR were largely identical to those determined by RNA-seq, which further indicated the credibility of the transcriptomic data (Fig. 2d).

The expression profiles of leaf-rolling related genes

To better understand the molecular regulatory mechanism of *SRL1* on controlling the leaf rolling, we focused on the genes that previously known to relate to leaf rolling (Table 2). In our

transcriptomic study, many genes have been demonstrated to regulate leaf rolling by affecting cytological architecture of leaf cells such as bulliform cells or the cuticle of leaves. Four genes involved in the regulation of bulliform cells differentially expressed between the *srl1*-KO and its non-KO control. *OsZHD1* (FDR = 0.005, log_2FC = -1.402) and *OsLBD3-7* (FDR = 0.009, log_2FC = -1.158) were significantly down regulated, while *RFS* (FDR = 0.003, log_2FC = 1.516) was significantly up regulated. Moreover, *ACL1* (FDR = 0.011, log_2FC = -1.558) was up regulated. These results were consistent with the fact that *SRL1* regulates leaf rolling by affecting bulliform cells. In addition, *CFL1* (FDR = 0.011, log_2FC = -1.594), a gene related to leave cuticle development was down regulated, indicating that the defects in the cuticle development may take part responsibility for the leaf rolling in the *srl1*-KO mutant.

In addition to the above genes, we checked the expression pattern of the leaf polarity related, sclerenchymatous cells related and miRNA related genes, which have also been demonstrated to play important roles in leaf rolling. However, we did not detect the significant expression change for these genes in our transcriptomic data, indicating that *SRL1* may not regulate these kinds of genes (Table 2).

In the previous *SRL1* study, it was suggested that genes encoding vacuolar H^+-ATPases and H^+-pyrophosphatases might play important roles in regulating the formation of bulliform cells. The microarray analysis revealed that ten vacuolar-related genes (*LOC_Os01g13130*, *LOC_Os01g42430*, *LOC_Os01g55260*, *LOC_Os01g59800*, *LOC_Os02g07870*, *LOC_Os02g24134*, *LOC_Os03g15650*, *LOC_Os03g58700*, *LOC_Os04g52190* and *LOC_Os04g55040*) were up-regulated in *srl1-1* mutant (Xiang et al 2012). In our study,

LOC_Os04g52190/Os04g0611400 (FDR = 2.55×10^{-4}, $\log_2$FC = 1.616) was significantly up regulated. Beside that, the other nine genes showed no significant change in our transcriptomic study (Table 3).

In the previous *CLD1* study (Li et al 2017), a couple of genes related to secondary cell wall cellulose were significantly down regulated (FC > 2) including a set of cellulose synthase gene (*OsCESA1*, *OsCESA2*, *OsCESA3, OsCSLD4/NRL1*) (Hu et al 2010, Li et al 2009, Luan et al 2011, Wang et al 2010) and a secondary cell wall-associate transcription factor (*SND1*) (Zhong et al 2006). In our study, *SND1* (FDR = 0.015, $\log_2$FC = -1.753) was down regulated. We did not detect obvious change for *OsCESA1*, *OsCESA2*, *OsCESA3* and *OsCSLD4/NRL1,* however, the other two cellulose synthase genes *OsCESA5* (FDR = 2.09×10^{-119}, $\log_2$FC = -4.168) and *OsCESA6* (FDR = 8.67×10^{19}, $\log_2$FC = -2.197), which did not change in the *cld1* mutant, showed remarkable changes in the *srl1*-KO mutant (Table 4). This may due to the different genetic background between the *srl1*-KO (Nipponbare, *sativa* L. ssp. *japonica*) mutant and the *cld1* mutant (93-11, *O. sativa* L. ssp. *indica*).

Discussion

It has been previously demonstrated that many genes regulated the leaf rolling by affecting bulliform cells, which located in the ridges of the vascular bundle near midrib on the adaxial epidermis of leaf with large, empty, colorless and bubble-shape. In our study, *OsZHD1*, *OsLBD3-7, RFS* and *ACL1* were differentially expressed between the *srl1*-KO and the non-KO. These genes are all related to the development of bulliform cells. Overexpression of *OsZHD1*, a zinc finger homodomain class transcription factor, leads to an increase of bulliform cells that

causes abaxial rolling of leaves (Xu et al 2014). *OsLBD3-7* and *ACL1* regulate leaf rolling by affecting both number and area of bulliform cells. Overexpressing *OsLBD3-7* induces adaxially rolled leaves (Li et al 2016), while overexpressing *ACL1* leads to abaxial rolling leaves (Li et al 2010). *RFS* has been characterized as a key gene affecting leaf rolling, chloroplast development and reactive oxygen species scavenging. The *rfs-1* mutants shows defects in vascular bundle and bulliform cells development (Cho et al 2018). In addition, *OsMYB103L*, a *R2R3MYB* transcription factor targeting *CESA* genes that are involved in the regulation of cellulose synthesis. Overexpression of *OsMYB103L* displays adaxially rolled leaves due to reduced size of bulliform cells (Yang et al 2014, Ye et al 2015). Although *OsMYB103L* did not show expression change in our study, *OsCESA5* and *OsCESA6*, the downstream targets of *OsMYB103L* showed significant change between the *srl1*-KO and the non-KO.

The plant cuticle, a chemically heterogeneous lipophilic layer, protects the plants from biotic and abiotic stress. It has been previously reported that defective cuticle development is responsible for the rolling of the leaves. *CFL1*, encoding a WW domain protein, regulates cuticle development by modulating the function of *HDG1,* which encodes a class IV homeodomain transcription factor. Overexpression of *CFL1* led to severely impaired cuticle development resulting in the phenotype of curly leaves (Wu et al 2011). In our study, *CFL1* was down regulated in the *srl1*-KO indicated that *SRL1* may also regulate the cuticle development.

In plants, the protons was pumped into vacuolar lumen by the electrochemical H^+ gradient which was created by vacuolar H^+-ATPases and H^+-pyrophosphatases to maintain the cellular ionic and metabolism homeostasis (Ratajczak 2000). *SRL1* may repress the expression of the genes

encoding vacuolar H^+-ATPases and H^+-pyrophosphatases to suppress the enlargement of vacuolar and formation of bulliform cells. Ten vacuolar-related genes were up regulated in the *srl1-1* mutant by microarray analysis (Xiang et al 2012), however, in our RNA-seq study, only one gene (*LOC_Os04g52190/Os04g0611400*) was significantly up regulated in the *srl1*-KO. The difference between the two transcriptomic analyses may due to the different regions of sampling. For the microarray analysis, cells abutting the midrib at the adaxial surface of leaf blades (5 days after germination) that will become epidermal cells in wild type and probably bulliform cells in *srl1-1* were isolated by laser microdissection and used for microarray analysis, respectively. In our RNA-seq, the mature leaves (60 days after germination) of the *srl1*-KO and the control non-KO were collected and subsequently used for the RNA-seq analysis. The precise sampling region for the microarray analysis may contribute to the more sensitive detection for the particular genes that involved in bulliform cells formation. As a coin has two sides, the restrictive sampling region may limit the detection of the leaf rolling-related genes that involved in the other developmental processes, which may be detected by our RNA-seq analysis.

Conclusions

In summarize, we generated the *SRL1* knock out line by CRISPR/Cas9, then carried out the RNA-seq for studying the regulatory network that controlled by *SRL1* in regulating the rice leaf rolling. A total number of 3,178 genes were identified as DEGs with 1,216 up-regulated genes and 1,962 down-regulated genes. Further more, a group of known leaf rolling related genes which involves in bulliform cell and cuticle development including *OsZHD1*, *OsLBD3-7*, *RFS*, *ACL1*, *CFL1*, *SND1*, *OsCESA5* and *OsCESA6* were up or down regulated in the *srl1*-KO, indicating that

SRL1 may control leaf rolling by regulating a couple of genes that affecting cytological architecture of leaf cells such as bulliforms and cuticle of leaves.

Methods

Plant materials and growth conditions

Rice (*Oryza sativa* L.) plants were grown in the greenhouse at 30°C for days and 25°C for night. The genetic background of the the transgenic plants was Nipponbare.

Vector construction

To knock out *SRL1*: Target site was designed online (http://cbi.hzau.edu.cn/crispr/), the optimal target site with low off-target score and high sgRNA score was selected. We prepared the CRISPR/Cas9 binary constructs as described previously (Ma et al 2015).

Sample collection and RNA extraction

Mature leaves (60 days after germination) from a single plant were collected, total RNA was extracted using a Plant RNA Purification Kit: RNeasy Plant Mini Kit (74904, QIAGEN, Germany) following the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels.

Library construction and Illumina sequencing

Library construction and RNA-seq were performed at Beijing BioMarker Technologies (Beijing, China) in accordance with the institute's protocols and briefly described here. RNA concentration

was measured using NanoDrop 2000 (Thermo). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNaseH. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 240 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on Illumina Hiseq Xten PE150 platform (paired end 150 bp) and paired-end reads were generated. All

the raw data have been submitted to the NCBI GEO database under accession number of GSE144729.

RNA-seq data analysis

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. These clean reads were then mapped to the reference genome sequence (Os-Nipponbare-Reference-IRGSP-1.0: http://rapdb.dna.affrc.go.jp/download/irgsp1.html). Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome. HISAT2 tools soft were used to map with reference genome (Kim et al 2015). Differential expression analysis of two groups (KO samples and Non-KO samples) was performed using the DESeq2 (Love et al 2014). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with FDR (false discovery rate) < 0.01 found by DESeq2 & absolute $\log_2$FC (Fold Change) > 1 were assigned as differentially expressed.

KO (KEGG Ortholog) and KEGG pathway enrichment analysis

KO analysis: Gene function was annotated based on the KEGG Ortholog database; KEGG pathway enrichment analysis: KEGG pathway enrichment analysis (http://www.genome.jp/kegg/)

was performed to identify differentially expressed genes (DEGs). KOBAS (Mao et al 2005) software was used to test the statistical enrichment of differential expression genes in KEGG pathways (E ≤ 1e-5).

qRT-PCR

First-strand cDNA was synthesized from 1 μg total RNA using the PrimeScript™ RT reagent Kit (with gDNA Eraser) (RR047A, Takara, Japan) according to the manufacturer's instructions. qRT-PCR were carried out using the ChamQTM SYBR® qPCR Master Mix (Q311-01, Vazyme, China) on the LightCycler® 480II (Roche) according to the manufacturer's instructions. Rice Ubiquitin (*Os03g0234200*) was used as internal reference and gene expression level was normalized to the Ubiquitin expression level. The primers were showed in Additional file 6: Table S6.

Additional file 1: Table S1. The target site sequence of the 14 T_0 transgenic plants

Additional file 2: Table S2. All genes expression profile

Additional file 3: Table S3. Differentially expressed genes

Additional file 4: Table S4. Genes assigned to KEGG

Additional file 5: Table S5. Genes assigned to KEGG pathway

Additional file 6:Table S6. Primers used for qPCR for this study

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List of Abbreviation

DEGs: Differentially Expressed Genes

FC: Fold Change

FDR: False Discovery Rate

KO: Knock Out

qRT-PCR: quantitative Reverse Transcription Polymerase Chain Reaction

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors have declared that no competing financial interests.

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Authors' contributions

J.J. and J.W. planned and designed the research. X.L., M.L., B.Z., J.B., L.Z. and P.X. performed experiments. J.J. wrote the manuscript. All authors read and approved the final manuscript.

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Not applicable

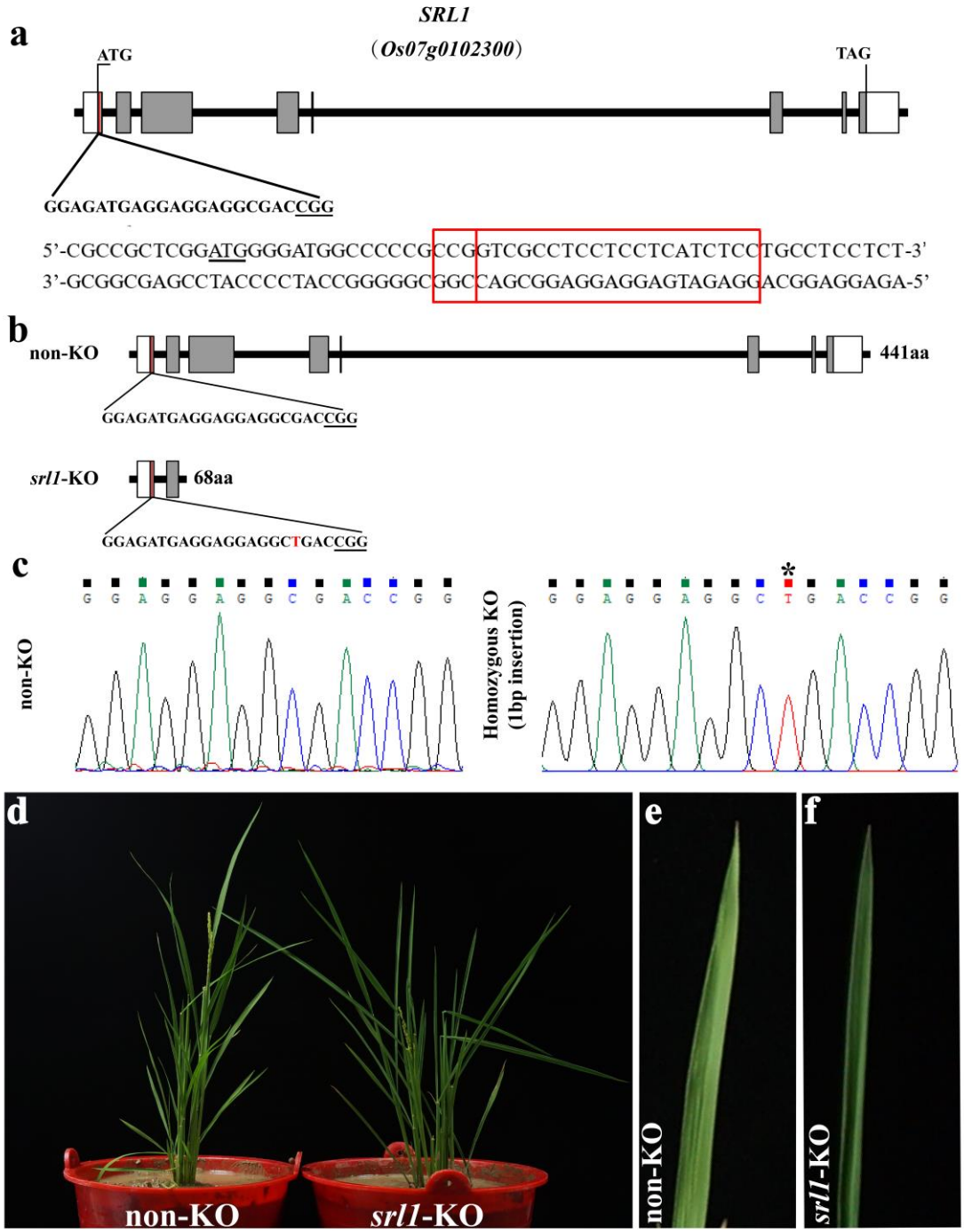


Figure 1. The CRISPR/Cas9 target site of *SRL1* and phenotype of the KO plants

(a) Schematic of *SRL1* gene structure and the CRISPR/Cas9 target site. The exon was indicated by gray rectangles, the untranslated region was indicated by white rectangles. The translation initiation codon (ATG) and termination codon (TGA) are shown. The target site nucleotides are shown in capital letters, and the protospacer adjacent motif (PAM) site is underlined. (b) Genomic structures at the *SRL1* locus in KO and non-KO line. (c) The original sequencing chromatograms of the target site for *SRL1* in non-KO and homozygous KO line. The protospacer adjacent motif (PAM) site is underlined, the "T" marked with an asterisk was the inserted in the *srl1*-KO. (d) Whole plant of non-KO and *srl1*-KO (e, f) Leaf blade phenotype of non-KO and *srl1*-KO.

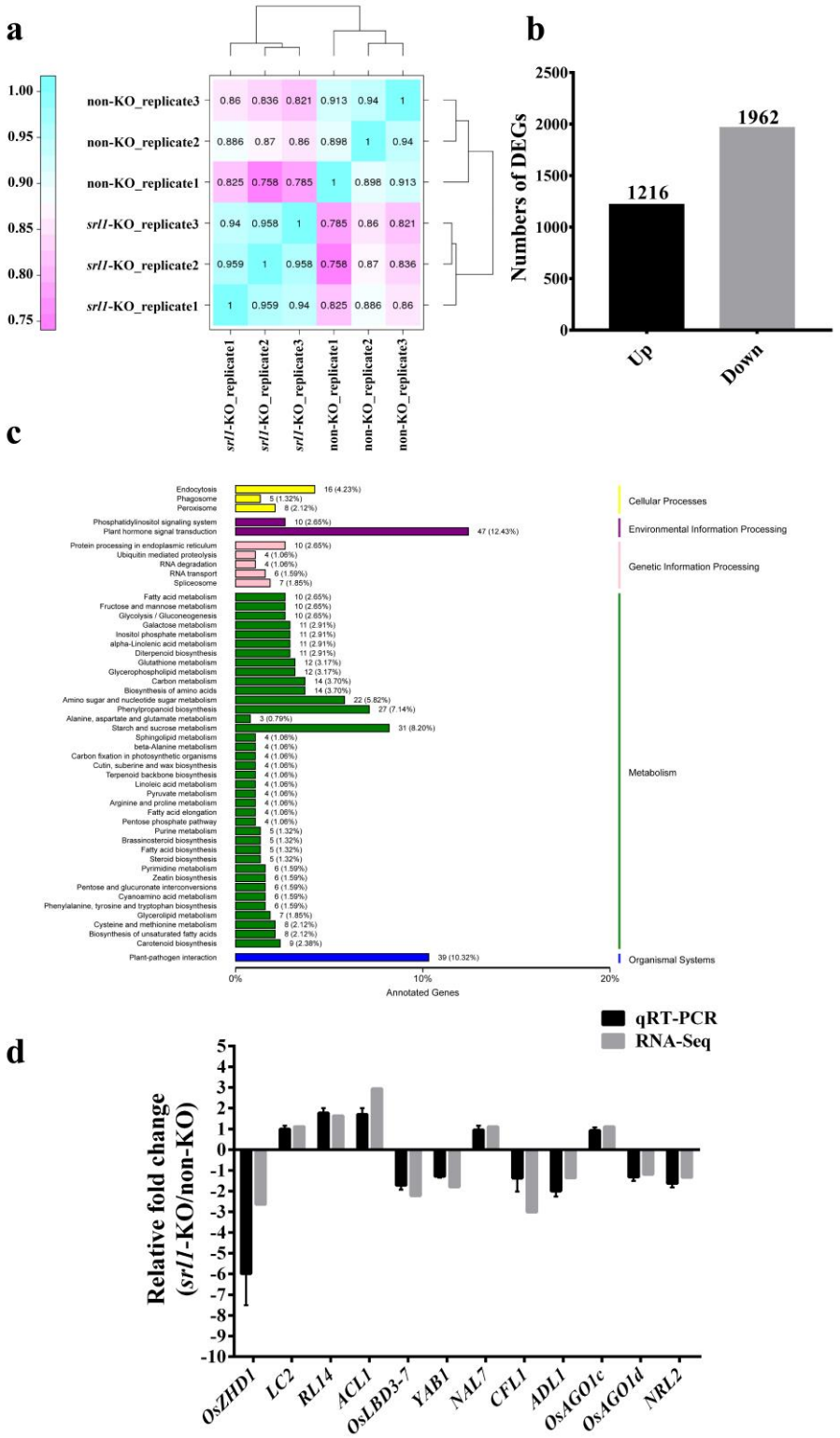


Figure 2. Overview of the DEGs

(a) Correlation between any two replicates was calculated by Pearson's Correlation Coefficient value. (b) Numbers of up-regulated and down-regulated DEGs. (c) KEGG pathway assignment of the assembled unigenes (top 50 pathways according to enrichment factor). The vertical axis represents the enriched KEGG pathways and the horizontal axis represents the number of unigenes in each pathway. (d) The expression profiles of twelve leaf rolling related genes were validated by qPCR. Values are shown as means ± s.d. (n = 3)

Table1. Statistical analysis of transcriptomic sequencing data

Sample	Total reads	Mapped reads	Mapped ratio	Uniq mapped reads	Uniq mapped ratio	GC content	%≥Q30
***srlI*-KO_replicate1**	62,850,674	59,408,986	94.52%	56,381,278	89.71%;	54.45%	94.20%
***srlI*-KO_replicate2**	56,155,420	53,346,014	95.00%	51,863,636	92.36%	54.63%	94.02%
***srlI*-KO_replicate3**	56,057,300	53,255,734	95.00%	51,919,302	92.62%	54.54%	94.19%
non-KO_replicate1	55,252,706	52,949,414	95.83%	51,221,015	92.70%	55.04%	94.28%
non-KO_replicate2	58,704,322	56,170,576	95.68%	54,691,345	93.16%	54.55%	94.50%
non-KO_replicate3	58,905,796	56,352,819	95.67%	54,756,848	92.96%	54.44%	94.49%

Total reads: number of clean reads (single end); Mapped reads: number of clean reads which could be mapped to the reference genome; Mapped ratio: the percentage of the mapped reads; Uniq mapped reads: number of mapped reads which could uniquely be mapped to the reference genome; Uniq mapped ratio: the percentage of the uniq mapped reads; GC content: the content of G and C base in the clean reads. %≥Q30: the percentage of clean reads whose the quality score were not less than 99.9%.

Table 2. The expression change of leaf rolling related genes in *srl1*-KO

Gene function	Gene symbol	Gene ID (rap-db)	FDR	log_2FC
Leaf Polarity (Adaxial Abaxial)	*ADL1*	*Os02g0709400*	0.015505392	-0.44226
	SLL1	*Os04g0432600*	0.424450406	-0.2106
	OsAGO7	*Os03g0449200*	0.971027564	0.061401
Bulliform Cells (Number and Size)	*SRL1*	*Os07g0102300*	0.010497713	-0.74096
	RFS	*Os07g0497100*	0.002628479	1.515866
	OsHox32	*Os03g0640800*	0.542687657	-0.22059
	YAB1	*Os07g0160100*	0.284669838	-0.84637
	OsZHD1	*Os09g0466400*	0.00494603	-1.40154
	OsZHD2	*Os08g0479400*	0.568782633	-0.6904
	LC2	*Os02g0152500*	0.767437117	0.165023
	RL14	*Os10g0558900*	0.228677074	0.710937
	NRL1	*Os12g0555600*	0.92137239	-0.14938
	OsMYB103L	*Os08g0151300*	0.672191693	0.689681
	ROC5	*Os02g0674800*	0.000274182	-0.52318
	PFL	*Os06g0553200*	0.158981616	-1.56833
	ACL1	*Os04g0415000*	0.01109317	1.557548
	ACL2	*Os02g0536500*	0.610385337	0.705774
	REL1	*Os01g0863500*	ND	ND
	REL2	*Os10g0562700*	0.524590455	-0.52246
	OsLBD3-7	*Os03g0790600*	0.009481005	-1.15808
	NAL7	*Os03g0162000*	0.647658033	0.153462
	NAL2	*Os11g0102100*	ND	ND
	NAL3	*Os12g0101600*	ND	ND
	OsI-BAK1	*Os03g0440900*	0.470309835	0.237016
	OsARF18	*Os06g0685700*	0.460616962	0.201005
	SLL2	*Os07g0574400*	8.44236E-05	-1.02348
	OsRRK1	*Os06g0693200*	0.88057517	-0.19566
Sclerenchymatous Cells	*SLL1*	*Os09g0395300*	0.990395328	0.02204
	NRL2/SRL2	*Os03g0308200*	0.0352642	-0.42714
	RL14	*Os10g0558900*	0.228677074	0.710937
Cuticle Development	*CFL1*	*Os02g0516400*	0.010961277	-1.59358
	OsMYB103L	*Os08g0151300*	0.672191693	0.689681
miRNAs	*OsAGO7*	*Os03g0449200*	0.971027564	0.061401
	OsAGO1a	*Os02g0672200*	0.312503448	-0.30481
	OsAGO1b	*Os04g0566500*	0.825927752	-0.08416
	OsAGO1c	*Os02g0831600*	0.533440632	0.157662
	OsAGO1d	*Os06g0729300*	0.340339116	-0.26243
	OsARF18	*Os06g0685700*	0.460616962	0.201005
	OsHB4	*Os03g0640800*	0.542687657	-0.22059
	DCL1	*Os03g0121800*	0.669179001	-0.5769

ND: not detected in the RNA-seq

Table 3. The expression change of some vacuolar related genes in *srl1*-KO

Gene ID	Annotation	FDR	log_2FC
LOC_Os01g13130/Os01g0232100	Tonoplast membrane integral protein ZmTIP4-3	0.149376	-0.87623
LOC_Os01g42430/Os01g0610100	Vacuolar ATP synthase 21-kD proteolipid subunit C	0.347985	0.20785
LOC_Os01g55260/Os01g0757400	SKD1 protein (Vacuolar sorting protein4b)	0.681726	-0.36565
LOC_Os01g59800/Os01g0813500	Vacuolar protein sorting36 family protein	0.95445	0.017811
LOC_Os02g07870/Os02g0175400	Vacuolar proton-ATPase subunit A	0.028012	0.643565
LOC_Os02g24134/Os02g0437800	Vacuolar protein-sorting protein45 homolog (AtVPS45)	0.158677	0.46179
LOC_Os03g15650/Os03g0262900	Vacuolar sorting protein9 domain-containing protein	1.02E-08	-0.98033
LOC_Os03g58700/Os03g0801600	Vacuolar protein sorting-associated protein35 family protein	0.018231	0.416066
LOC_Os04g52190/Os04g0611400	Vacuolar sorting receptor homolog	0.000255	1.615591
LOC_Os04g55040/Os04g0643100	Vacuolar ATP synthase subunit D (EC 3.6.3.14)	0.682713	0.116245

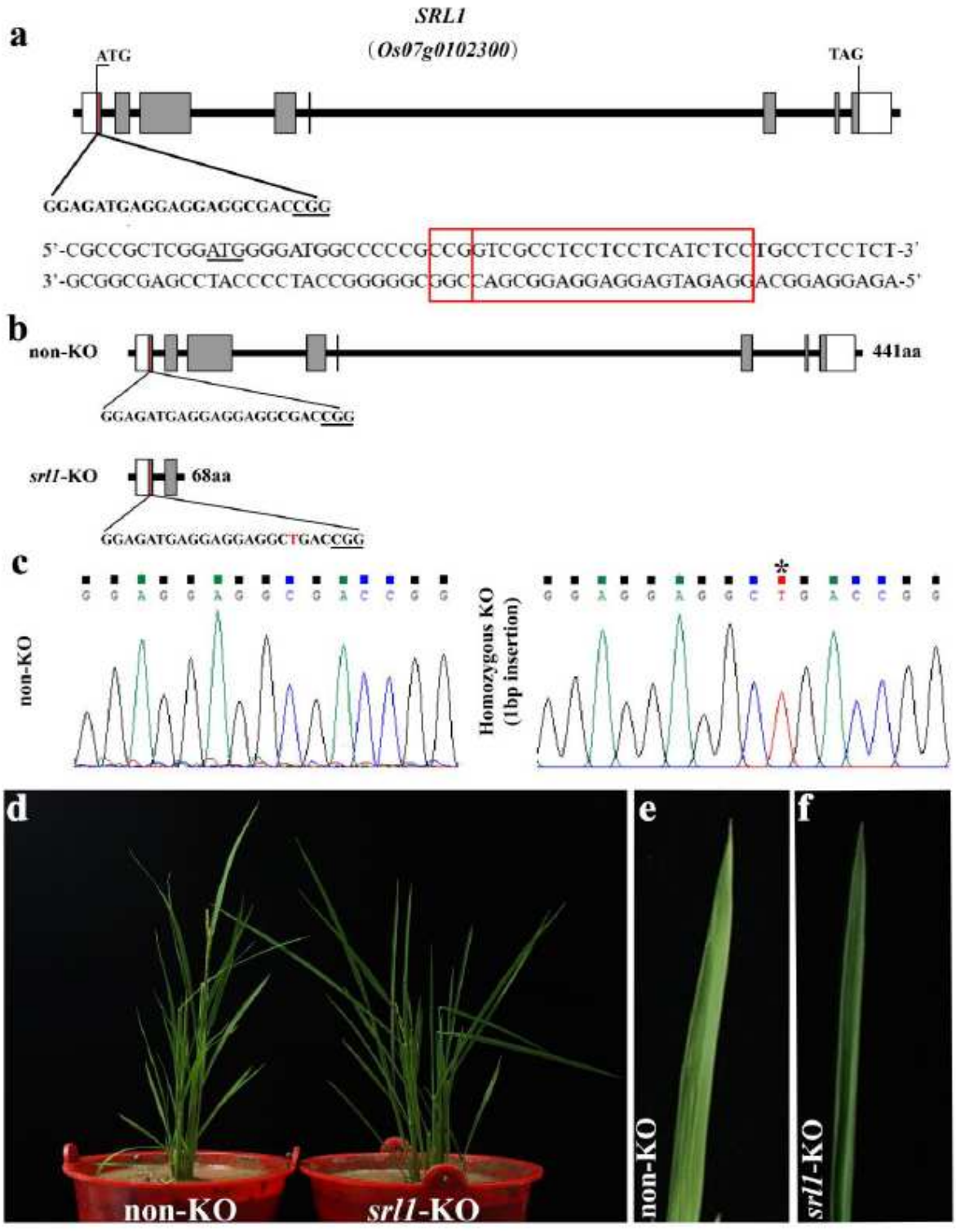
These genes were up regulated in the *srl1-1* mutant

488 **Table 4. The expression change of the Secondary cell wall cellulose related genes in *srl1*-KO**

Gene function	Gene symbol	Gene ID (rap-db)	FDR	log_2FC
Secondary cell wall cellulose related	*OsCSLD1*	*Os10g0578200*	0.6927607	0.599779
	OsCSLD2	*Os06g0111800*	0.0005026	-0.88051
	OsCSLD3	*Os08g0345500*	ND	ND
	OsCSLD4/NRL1	*Os12g0555600*	0.9213724	-0.14938
	OsCSLD5	*Os06g0336500*	ND	ND
	OsCESA1	*Os05g0176100*	9.30E-05	-0.89603
	OsCESA2	*Os03g0808100*	3.54E-28	-1.9736
	OsCESA3	*Os07g0424400*	0.2319069	0.35963
	OsCESA4	*Os01g0750300*	0.2985569	-0.78159
	OsCESA5	*Os03g0837100*	2.09E-119	-4.16828
	OsCESA6	*Os07g0252400*	8.67E-19	-2.19716
	OsCESA7	*Os10g0467800*	0.6748408	-0.65668
	OsCESA8	*Os07g0208500*	0.7860391	-0.15179
	OsLAC17	*Os10g0346300*	0.3431087	1.111629
	SND1	*Os06g0131700*	0.0014968	-1.75336
	VND4	*Os06g0104200*	0.8699304	-0.29791
	OsDRP2B	*Os02g0738900*	0.3850803	0.282578

489 ND: not detected in the RNA-seq

Figures



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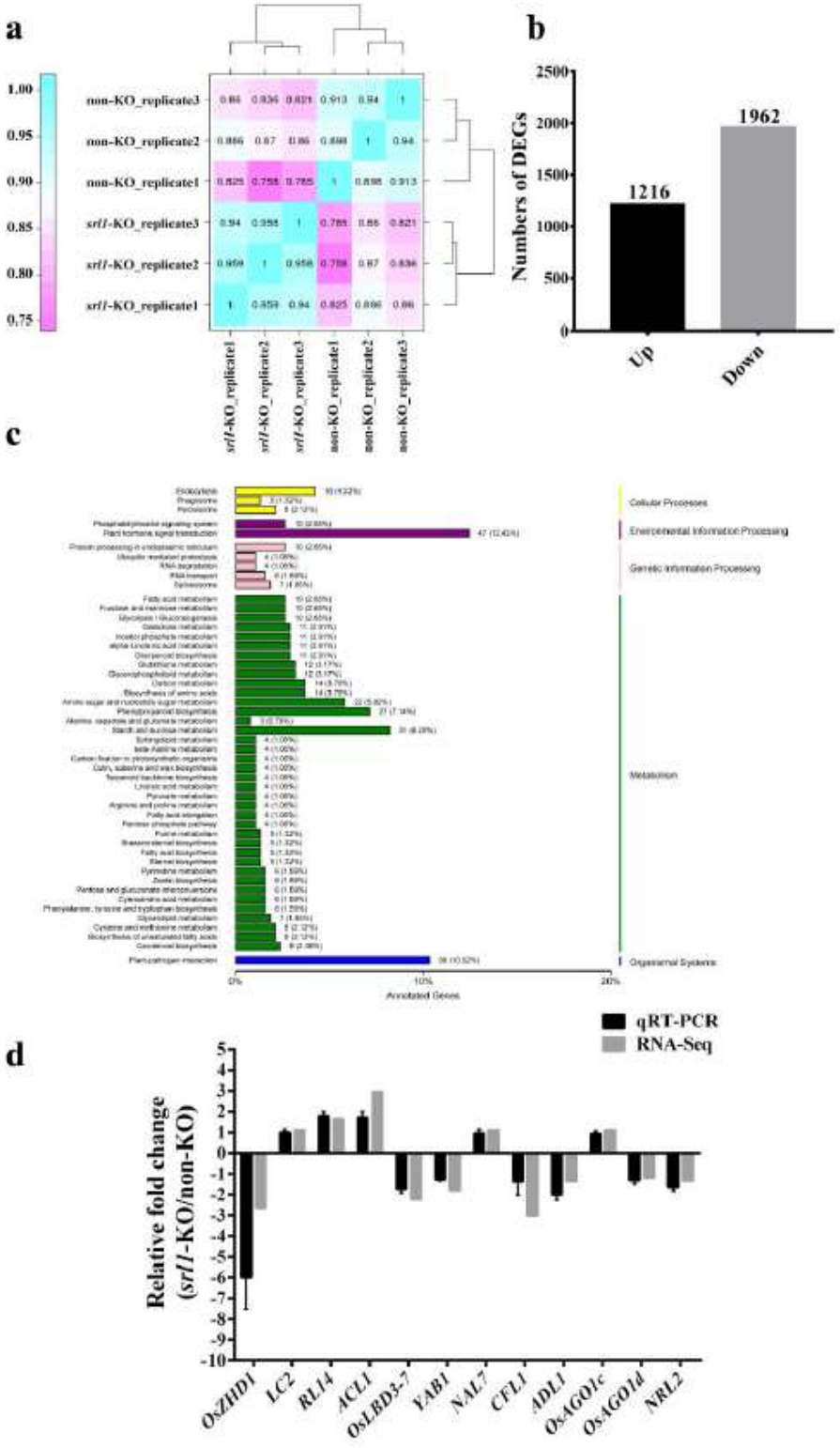

Figure 2

Overview of the DEGs. (a) Correlation between any two replicates was calculated by Pearson's Correlation Coefficient value. (b) Numbers of up-regulated and down-regulated DEGs. (c) KEGG pathway assignment of the assembled unigenes (top 50 pathways according to enrichment factor). The vertical axis represents the enriched KEGG pathways and the horizontal axis represents the number of unigenes in each pathway. (d) The expression profiles of twelve leaf rolling related genes were validated by qPCR. Values are shown as means ± s.d. (n = 3)

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