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ARTICLE

Identification of a Novel *OsCYP2* Allele that Was Involved in Rice Response to Low Temperature Stress

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ABSTRACT

Cyclophilin (CYP) plays an important role in plant response to stress, and *OsCYP2*, one gene of cyclophlilin family, is involved in auxin signal transduction and stress signaling in rice. However, the mechanism that *OsCYP2* is involved in rice response to low temperature is still unclear. We identified a new *OsCYP2* allelic mutant, *lrl3*, with fewer lateral roots, and the differences in shoot height, primary root length and adventitious root length increased with the growth process compared to the wild-type plant. Auxin signaling pathway was also affected and became insensitive to gravity. The transgenic rice plants with over-expression of *OsCYP2* were more tolerant to low temperature than the wild-type plants, suggesting that *OsCYP2* was involved in the low temperature response in rice. In addition, *OsCYP2* negatively regulated the expression of *OsTPS38*, a terpene synthase gene, and was dependent on the *OsCDPK7*-mediated pathway in response to low temperature stress. *OsTPS38*-overexpressed transgenic line ox-2 was more sensitive to low temperature. Therefore, *OsCYP2* may negatively regulate *OsTPS38* through an *OsCDPK7*-dependent pathway to mediate the response to low temperature in rice. These results provide a new basis for auxin signaling genes to regulate rice response to low temperature stress.

KEYWORDS

Rice; low temperature; *OsCYP2*; *OsTPS38*; *OsCDPK7*

1 Introduction

Plants resist low temperature through a series of complex mechanisms [1]. Ca^{2+} is an important signaling molecule that can participate in the sensing mechanism of low temperature in plants [2–4]. The calcium dependent protein kinase (CDPK) system is an important Ca^{2+} transduction pathway in cells and can sense changes in external signals [5–12]. Previous studies have confirmed that CDPK is widely distributed. When plants are under low temperature stress, Ca^{2+} binds to the regulatory region of CDPK, activates the activity of CDPK and transmits signals to the downstream [13–18]. CDPK can positively regulate the expression of stress-related genes in the signal transduction pathway under low temperature

stress in rice [19]. Other studies have shown that the expression of *OsCDPK* genes has a circadian rhythm, which is not obviously induced in the early stage under low temperature treatment, but is activated after 18–24 h under low temperature treatment, and gradually plays its function with the increase of stress time [18]. *CDPK7* is a relatively conserved gene in the CDPK family and can be induced to express under low temperature stress [20]. Moreover, the sense *OsCDPK13*/*OsCDPK7* transgenic lines in rice had higher recovery rates after cold treatment than the control [21]. According to the previous studies, CDPK mediated the mechanism of plant response to low temperature by sensing Ca^{2+} signal.

Cyclophilin (CYP), a member of the immunophilic family, has the PPIase activity and can limit the folding and processing of rate-limiting proteins [22]. Cyclophilin is the target protein of cyclosporin A, which can bind to Ca^{2+} in the cytoplasm and plays an important role in Ca^{2+} signal transduction pathway [23]. Clones of cyclinoid fragments from beans, maize, *Arabidopsis thaliana* and rice indicate that cyclinoids are involved in the regulation of cell division and transcriptional regulation, and mediate signal transduction pathways, as well as play a key role in plant response to stresses [24–28]. Functional loss of OsCYP20-2 protein in rice can make the mutant sensitive to low temperature stress, and OsCYP20-2 protein in rice chloroplasts can promote the formation of homo-dimer of OsFSD2 to eliminate the effect on ROS under low temperature stress [29]. *CyPs1* plays a regulatory role in the infection and development of the pathogen, and 29 Cyclophilin genes found in *A. thaliana* are involved in the processes of photosynthesis, plant stress resistance and mRNA splicing [30]. Moreover, *CYP19-4s* (*AtCYP19-4* and *OsCYP19-4*) may affect the polarity of auxin transport and PIN localization [31]. *AtCYP18-3* is involved in the process of seedling decolorization [32]. The *dgt* (*LeCyp1* gene mutant) mutant in tomato showed few lateral roots [33,34]. The *lrt2* (lateral rootless 2, namely *OsCYP2* allele) mutant in rice showed similar phenotypes of auxin signal mutants, and *OsCYP2* interacts with OsSGT1 to participate in auxin signal transduction [35–37]. *OsCYP2* is believed to be a key regulator of ROS levels by regulating the activity of antioxidant enzymes at translational level, and may be involved in circadian rhythm regulation and signaling pathways of stress such as salt, heat, cold or ABA [38]. However, the mechanism of *OsCYP2* in response to low temperature in rice is still unclear.

Terpene synthase (TPS) is an important enzyme in terpene biosynthesis and has circadian rhythm [39–41], which is derived from mevalonic acid and can participate in plant defense response to stress [41,42]. 16 TPS genes have been identified in rice, of which *OsTPS37* (*Os08gLOC_04500*), *OsTPS38* (*Os07gLOC_11790*) and *OsTPS40* (*Os08gLOC_07100*) are mainly responsible for the synthesis of rice sesquiterpenes and participate in the defense system of rice [43]. At present, the molecular mechanism of *OsTPS38* involved in low temperature response in rice is not clear.

In this study, we identified a novel *OsCYP2* allelic mutant, *lrl3*, with few lateral roots. The auxin signaling pathway in *lrl3* was affected and *OsCYP2* regulated the low temperature response of *OsTPS38*. Moreover, *OsCYP2* negatively regulated *OsTPS38* expression and mediated the low temperature response mechanism in rice through the *OsCDPK7*-dependent pathway. These results will provide a new basis for auxin signaling genes to regulate rice response to low temperature stress.

2 Results

2.1 lrl3 Mutant Showed Few Lateral Roots and Insensitive Gravitropism

We screened the T-DNA insertion mutant pools of zh11 in *japonica* rice, and identified a lateral rootless mutant *lrl3* (lateral rootless 3) compared with the wild-type plant zh11, and further functional studies were conducted using homozygous mutants and BC_1F_2 generations.

In order to study the function of *lrl3* mutant in details, we performed phenotypic analysis on the wild-type plant zh11 and the mutant *lrl3* cultured in normal rice (*Oryza sativa*) medium for 7 days, and found that compared with the wild-type plant zh11, the mutant *lrl3* had short shoot and long primary roots, but a few adventitious roots and few lateral roots (Fig. 1A). Further stereoscopic observation revealed that no significant differences were showed between *lrl3* and zh11 in root hairs, root tips and root caps, but

lateral roots were significantly absent in *lrl3* (Fig. 1B). After statistical analysis, it was found that significant or extremely significant differences between *lrl3* and zh11 were detected in shoot height, primary root length, number of lateral roots and number of adventitious roots ($p < 0.05$ or $p < 0.01$) (Fig. 1D). These differences were further increased when *lrl3* and zh11 were cultured for 14 days (Fig. 1C).

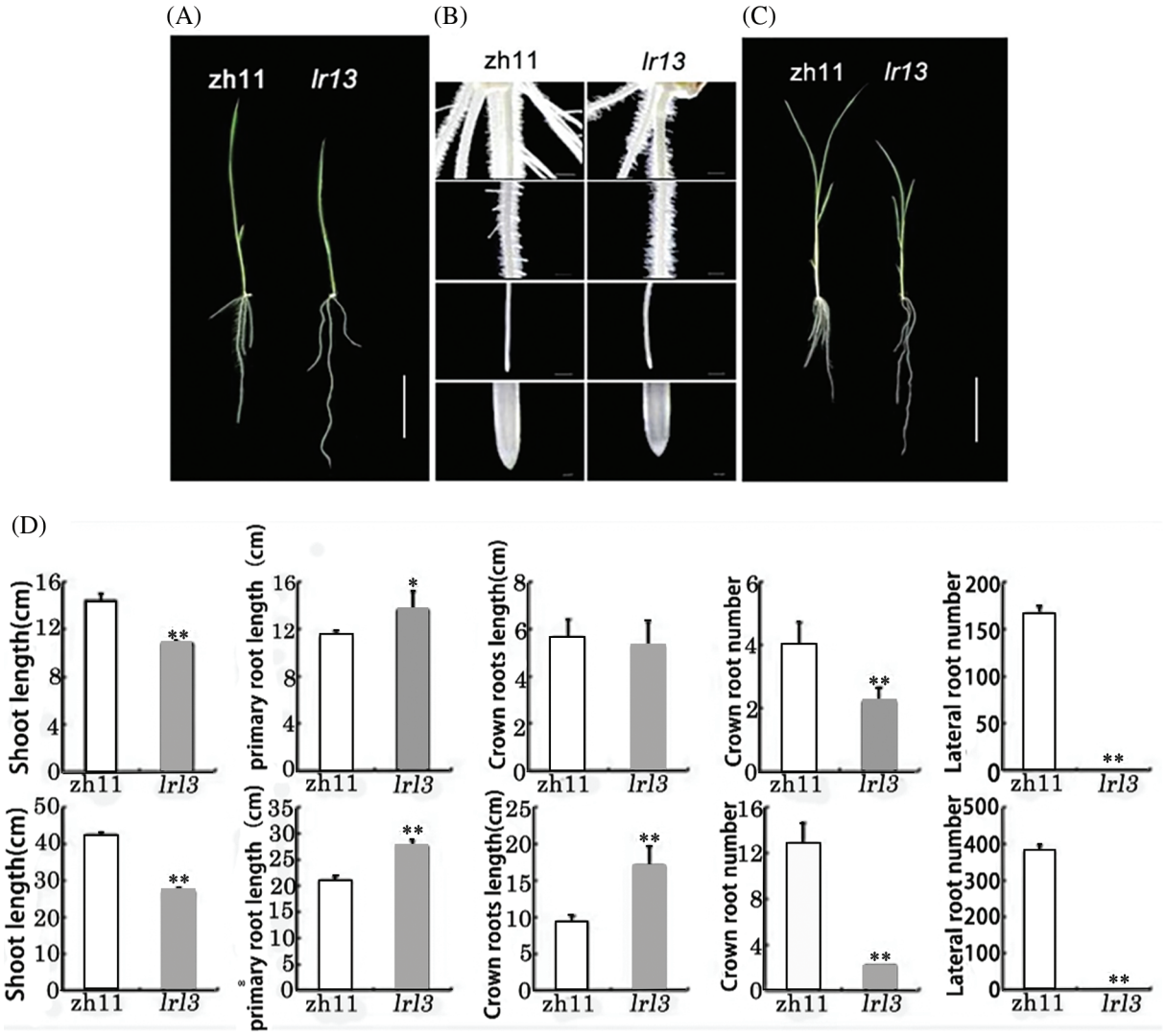

Figure 1: Identification and analysis of the phenotype of the wild-type plant and the mutant. (A) Phenotype of the wild-type zh11 and the mutant *lrl3* at 7-day-old seeding stage. Bar is 5 cm. (B) Root hair observation at 7-day-old seeding stage. Bar is 1 mm. The first line is the rhizome junction; the second lined type zh11 is the mature region of primary root; the third line is the primary root tip; the fourth line is primary root cap. (C) Phenotype of the wild-type zh11 and the mutant *lrl3* at 14-day-old seeding stage. Bar is 5 cm. (D) The phenotype parameters of the wild-type zh11 and the mutant *lrl3* cultured in normal nutrient solution for 7 (top line) and 14 (bottom line) days, * and ** indicate significant differences at 5% and 1% levels, respectively

These results suggest that the mutation of *lrl3* controls shoot elongation, primary shoot, and adventitious root elongation, as well as the number of adventitious roots and lateral roots, and that the differences are more significant along with the growth process.

To investigate the root gravitropism, we horizontally placed the primary root of *lrl3* T2 lines and zh11 two days after sowing, and found that *lrl3* was slow in response to gravity and barely bent compared with zh11 (see Supplementary Fig. 1). This suggests that the mutation site of *lrl3* regulates the response of rice root to gravity.

2.2 The Auxin Signaling Pathway of lrl3 Was Disrupted

To understand the physiological mechanism of root development, we treated *lrl3 and* zh11 with auxin polar transport inhibitor N-1-naphthylphthalamic (NPA) and Synthetic hormones 1-naphthlcetic acid (NAA). The results presented that *lrl3 and* zh11 showed similar responses to NPA, while *lrl3* showed significant resistance to NAA compared with zh11 under the treatment of 0.1 μM NAA (Fig. 2). These results suggest that the mutation locus of *lrl3* is involved in auxin signaling during lateral root formation in rice, rather than auxin polar transport.

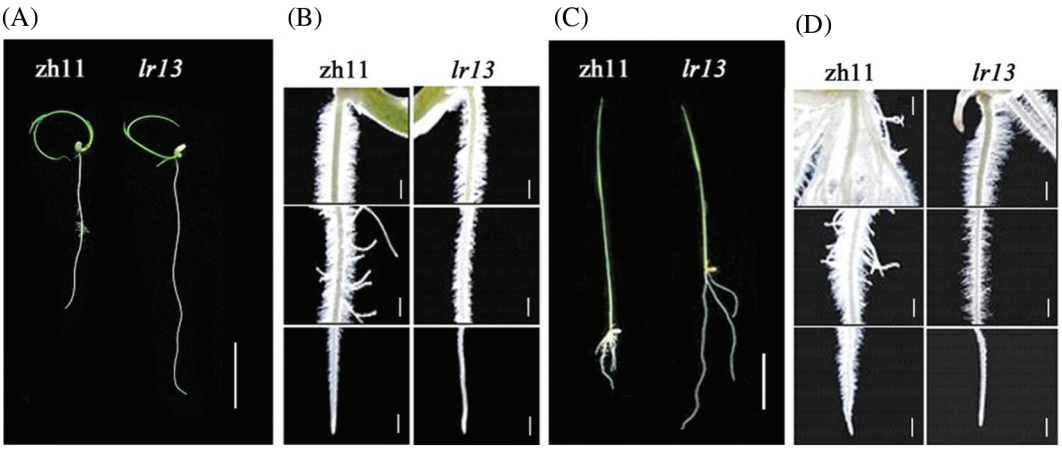

Figure 2: Phenotype of the wild-type plant and the mutant under NPA and NAA treatments. (A) and (C) Phenotype of the wild type zh11 and the mutant *lrl3* under 1 μM NPA and 0.1 μM NAA treatment at 7-day-old seeding stage. Bar is 2 cm. (B) and (D) Phenotype of the wild-type zh11 and the mutant *lrl3* under 1 μM NPA and 0.1 μM NAA treatment at 7-day-old seeding stage. The top, middle and bottom line are root hair phenotype of rhizome junction, mature zone and root tip of primary root, respectively. Bar is 1 mm

2.3 LRL3 Gene Mapping and Genetic Complementation Verification

Genetic analysis of BC_1F_2 populations of *lrl3 and* zh11 showed that the ratio of wild-type individuals to mutant individuals was in accordance with the expected value of 3:1, suggesting that *lrl3* lateral rootless mutation is controlled by a single recessive gene (Table 1).

Table 1: Genetic analysis

Population	Population size	Wild type individual	Mutant individual	Expected ratio	χ^2	$p_{(0.05,\ 0.01)}$
BC_1F_2	373	294	79	3:1	2.7	3.84, 6.63

We employed 30 mutant individuals in F_2 genetic population to detect polymorphisms by SIS2 (M2) and RM12368 markers in close linkage with *OsCYP2* gene regulating lateral root formation. The *lrl3* band was detected in all the 30 mutant individuals at SIS2 (M2) and in all the 29 mutant individuals at RM12368. The results indicated that the *LRL3* gene, the candidate gene *OsCYP2* for *lrl3* mutation, was preliminarily located

near the molecular markers SIS2 (M2) and RM12368 on the chromosome 2 (Fig. 3A). As reported, the gene *OsCYP2* encoding rice cyclophilin, participates in the protein folding, which is thought to be associated with auxin signaling [35], so the *OsCYP2* gene is regarded as the candidate gene for *lrl3* mutation. The coding region of the *LRL3/OsCYP2* gene is 519 bp between 1116066 bp and 1116971 bp on chromosome 2. Agarose gel electrophoresis and sequencing analysis on the mutant *lrl3* indicated that 59 basepairs of nucleotides were missing at *LRL3*/*OsCYP2* (Figs. 3B–3C). Further bioinformatics analysis showed that the 59-bp deletion at *LRL3*/*OsCYP2* led to the deletion of glutamate from 15th to 35th and frame-coding mutations, so that the *LRL3*/*OsCYP2* gene in *lrl3* encodes only 94 amino acids (Δ*OsCYP2* in Fig. 3C) because of frame-shift mutation. To confirm that *lrl3* is an *OsCYP2* allelic mutant, we crossed *lrl3* (deletion of 59 bp) with *lrt2* (deletion of 50 bp), and obtained positive double-mutant F_1 plants. Phenotypic analysis of the wild-type plant zh11, *lrl3*, double mutant F_1 between *lrl3* and *lrt2*, *lrt2* and the wild-type plant Nipponbare showed that the double mutant F_1, *lrl3* and *lrt2* all exhibited lateral rootless phenotype. This result of genetic complementarity suggests that *lrl3* lateral rootless phenotype is caused by *OsCYP2* deletion mutation (Figs. 3D–3E). In addition, the homology analysis showed that the four amino acids of *OsCYP2* were only present in the 12 plants analyzed, but not in human sapiens and yeast, suggesting that these four conserved amino acids are unique to plants during the evolution of the Cyclophilin family (see Supplementary Fig. 2).

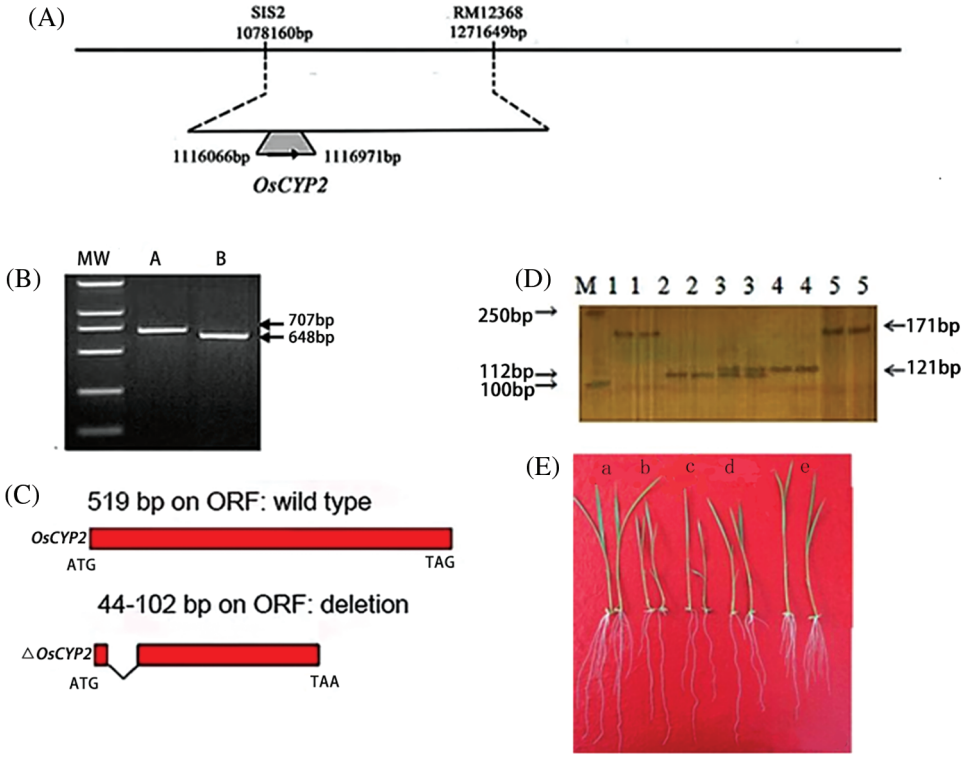

Figure 3: Gene mapping and genetic complementation analysis of *LRL3*/*OsCYP2*. (A) Map-based cloning of *LRL3* gene that is the candidate gene *OsCYP2* for *lrl3* mutation. (B) Agarose gel electrophoresis detection of *OsCYP2* allelic mutant. MW, DNA Marker DL2000; A the wild type plant zh11; B the mutant *lrl3*. (C) Schematic diagram of the *OsCYP2* allele structure. (D) The poly-acrylamide gel electrophoresis detection for genetic complementation analysis. M, DL2000 Marker; 1, zh11; 2, *lrl3*; 3, F_1; 4, *lrt2*; 5, Nipponbare. (E) Phenotype of 7-year-old seedlings of zh11, *lrl3*, F_1 of *lrl3* and *lrt2*, *lrt2*, and Nipponbare for genetic complementation analysis. a, zh11; b, *lrl3*; c, F1 of *lrl3* and *lrt2*; d, *lrt2*; e, Nipponbare. Bar is 2 cm

2.4 OsCYP2 is Involved in Low Temperature Response

There was no significant difference in leaf morphology and color between zh11 and *lrl3* before low temperature treatment. However, compared with the wild-type plant zh11, *lrl3* was more severe curled of leaf, yellowing of leaf tip and needle-like leaf shape after low temperature treatment (Figs. 4A–4B). Furthermore, because of *lrl3* low seed-setting, we investigated the response of the *OsCYP2* gene to low temperature using *OsCYP2*-overexpressed lines SSBM-OE and the wild-type plants SSBM. To clarify the different response of the *OsCYP2*-overexpressed lines SSBM-OE and the wild-type plants SSBM to low temperature stress, we carried out low temperature treatment of the *OsCYP2*-overexpressed lines SSBM-OE and the wild-type plants SSBM for 27 days, all the wild-type plants SSBM died while some of the *OsCYP2*-overexpressed lines SSBM-OE were still alive (Fig. 5). These results indicated that O*sCYP2* positively regulated resistance to low temperature.

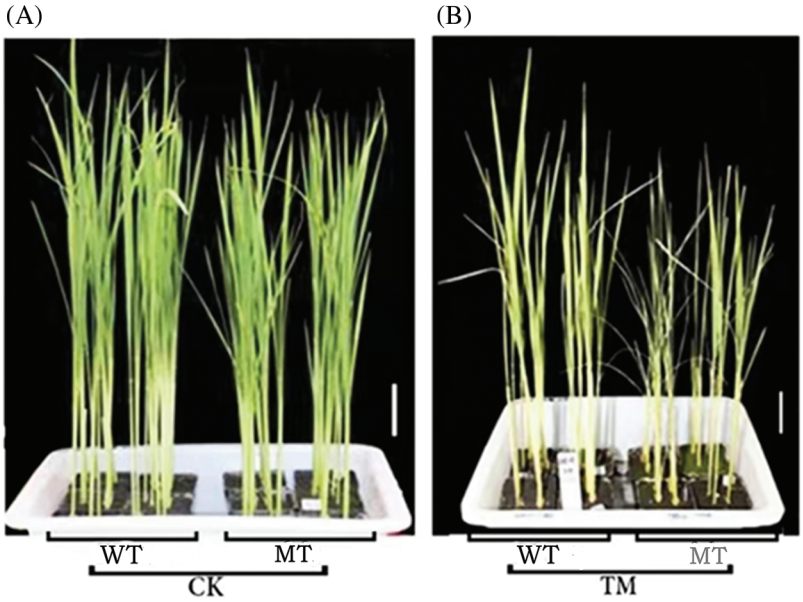

Figure 4: Phenotype of the wild-type plant and the mutant under low temperature treatment. (A) and (B) Phenotype of the wild-type plant zh11 and mutant *lrl3* before and after low temperature treatment, bar is 5 cm. WT, the wild-type plant zh11; MT, mutant *lrl3*; CK, before low temperature treatment; TM, after low temperature treatment

2.5 OsCYP2 Negatively Regulates OsTPS38 Response to Low Temperature through OsCDPK7-Dependent Pathway

In order to detect downstream genes regulated by *OsCYP2*, we performed transcriptome analysis on zh11 and *lrl3* before and after low temperature treatment, and found that a TPS gene *OsTPS38* (*Os08g0139700*) showed a significant difference in expression between zh11 and *lrl3* after low temperature treatment, which was contrary to the expression pattern of *OsCYP2* gene, confirmed by qRT-PCR (Figs. 6A–6B). This suggests that *OsTPS38* is negatively regulated by *OsCYP2* to mediate the mechanism of low temperature response in rice. Meanwhile, *OsCYP2* regulates the low temperature response mechanism in rice through *OsCDPK7*-dependent pathway (Fig. 6C).

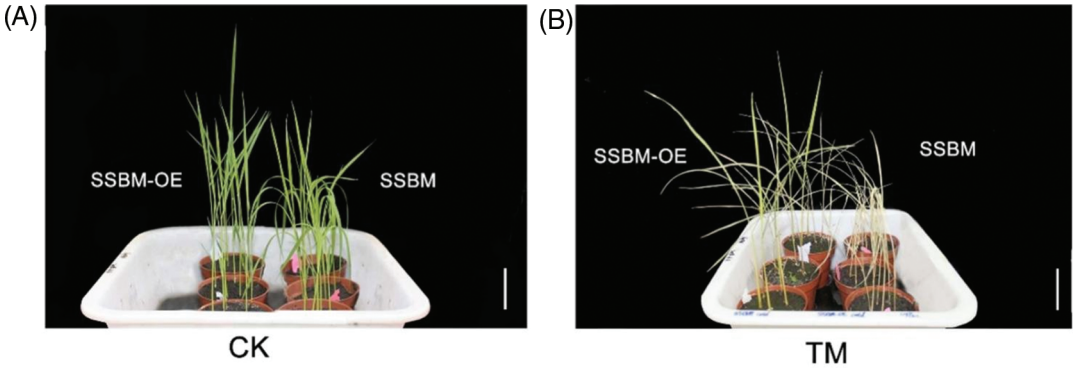

Figure 5: Phenotype of the *OsCYP2*-overexpressed lines SSBM-OE, the wild-type plants SSBM before and after under low temperature. The plants in the pot were the *OsCYP2*-overexpressed lines SSBM-OE (left), the wild-type plants SSBM (right). (A) Before low temperature; (B) After low temperature, bar is 2 cm. CK, before low temperature treatment; TM, after low temperature treatment

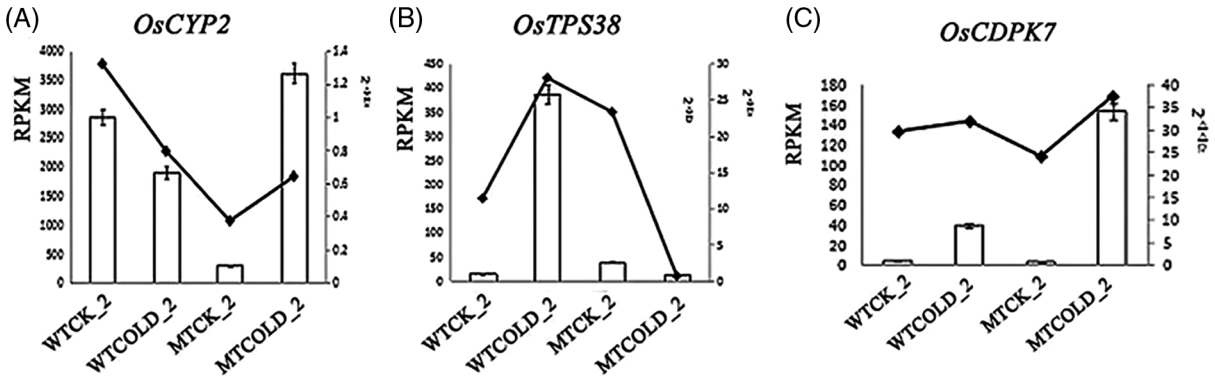

Figure 6: Expression of *OsCYP2, OsTPS38* and *OsCDPK7* in the wild-type and mutant under low temperature treatment by transcriptome analysis and qRT-PCR. WTCK_2, zh11 before low temperature; WTCOLD_2, zh11 after low temperature; MTCK_2, *lrl3* before low temperature; MTCOLD_2, *lrl3* after low temperature; RPKM, reads per kilobase per million mapped reads

2.6 OsTPS38 is Involved in Low Temperature Response

To further clarify the molecular mechanism of *OsTPS38* in response to low temperature, the overexpression lines ox-2 of *OsTPS38* and the wild-type plant zh11 were used to undergo low temperature treatment for 3 days at three-leaf stage, and it was found that the degree of leaf curling in ox-2 was higher than that in zh11 (Figs. 7A–7B). These results suggest that *OsTPS38* may negatively regulate the low temperature response in rice.

3 Discussion

3.1 The Mechanism of Auxin Pathway Regulating Low Temperature Response

Auxin is the first plant hormone that has been recognized and studied by humans. It almost participates in the whole process of plant growth and development, and plays an important role in the hormonal regulatory network at all stages of growth and development [44–46]. Interestingly, the auxin-signaling

mutants *axr1, tir1, gps2-1, gps1, gps2* and *gps3*, which show a decreased gravity response, responded to low temperature treatment [47–49]. In our study, *lrl3* mutant was also affected by gravity response and involved in response to low temperature, suggesting tha*t OsCYP2* may be involved in the low temperature response mediated by the auxin pathway. Low temperature stress may affect the function of the Pin-formed proteins (PINs) by inhibiting the transport or localization of intracellular proteins, and eventually cause slow root growth and gravitation anomaly [50–53]. However, we found significant differences in the expression of *OsPIN10a* in *OsCYP2* mutant compared with the wild type (see Supplementary Fig. 3). These results may suggest that *OsCYP2* also responds to low temperature through the auxin pathway. There are few studies on the mechanism of auxin pathway regulating low temperature response, but *WES1*, which encodes IAA amino acid synthase, is up-regulated in low temperature stress, and activates the expression of stress-related genes *Pathogenesis-related protein 1* (*PR-1*) and *C-repeat binding factors* (*CBFs*) by inactivating IAA, this indicates that the expression of the key genes *CBFs* in low temperature is directly regulated by auxin [54]. It has been reported that low temperature can affect the differential expression of some auxin response genes, such as *IAA20* [55,56], while we also found that the expression of *IAA20* gene was obviously affected by *OsCYP2* under exogenous IAA treatment (see Supplementary Fig. 4). Otherwise, another study has confirmed that OsCYP2 can interact with OsIAA11 [37]. In conclusion, we speculate that *OsCYP2* may be involved in the regulation of auxin response genes so as to mediate the cold response in rice (Fig. 8). However, the molecular mechanism of *OsCYP2* regulating rice response to low temperature through auxin pathway needs to be further improved.

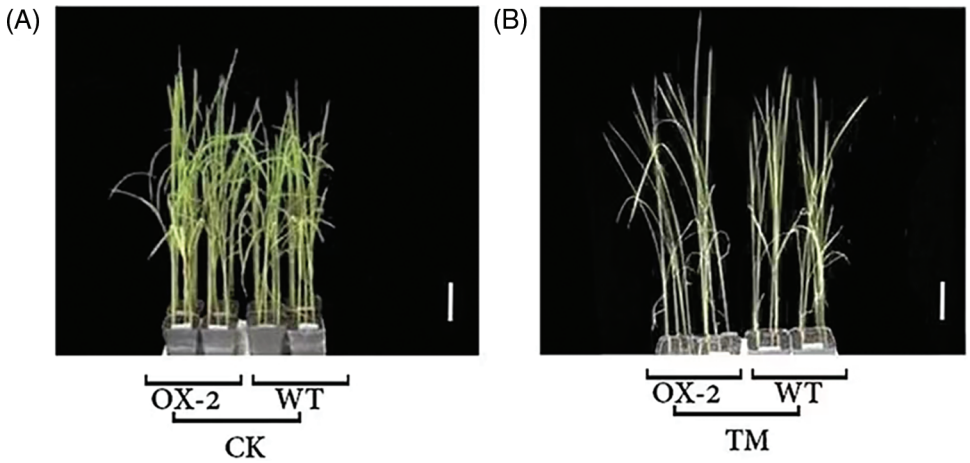

Figure 7: Response of *OsTPS38* to low temperature. (A) and (B) Phenotypes of transgenic lines and zh11 before and after low temperature treatment. WT, the wild-type plant zh11; OX-2, overexpression lines ox-2 of *OsTPS38*; CK, before low temperature treatment; TM, after low temperature treatment. Bar is 2 cm

3.2 The Low Temperature Response Pathway Depending on the ICE-CBF-COR Transcriptional Cascade to Sense Ca^{2+} Signals

Plants can regulate the corresponding physiological and biochemical responses to adapt to low temperature by activating signal transduction pathways, and the response to low temperature can be divided into ABA-dependent pathway, Ca^{2+} sensing pathway and ROS sensing pathway [57]. The low temperature environment causes the hardening of the plasma membrane in rice, which leads to the Ca^{2+} channel opening and the extracellular Ca^{2+} entering the cytoplasm, resulting in the difference of

intracellular and extracellular Ca^{2+} concentration. However, protein kinases such as CDPKs can sense the change of endogenous Ca^{2+} level and carry out phosphorylation reaction and activate the transcription factors of each downstream family, ultimately affecting the expression of COR genes. In this experiment, *OsCYP2* is involved in the pathway in response to low temperature by CDPK7-mediated Ca^{2+} signaling, which is also known as the ABA-independent ICE-CBF-COR transcriptional cascade pathway [58,59]. Under low temperature stress, the AP2 conserved domain of *CBFs* can bind CCGAC (CRT, C-repeat), the core elements of CORs initiation region, to activate CORs at the transcriptional level, while CBF genes are usually regulated by *ICE1*. The transcription factor OsDREB1F may be activated by *OsICE1*, which then activates the expression of downstream low-temperature responsive COR genes harboring DRE/CRT domain [60]. In addition, *OsICE1* is regulated by phosphorylation, sumoylation, and ubiquitination mediated by E3 ubiquitin ligase, and simultaneously binds to cis-elements on the promoter of *CBF3*/*DREB1* to participate in the low temperature response [61]. However, some studies have suggested that *CBF3* inhibition in *ice1-1* is gene silencing caused by T-DNA-triggered methylation [62]. *OsICE2* over-expression is involved in low temperature response of *CBF1/DREB1* [63]. *CBF2* is a negative regulator of *CBF1* and *CBF3*, but not depending on the expression of *CBF1* and *CBF3* [64–66]. *OsMYB3R-2* and *OsMYB2* involved in cold resistance up-regulates the expression of *OsDREB2A*, while MYBS3 negatively down-regulates the expression of *OsDREB1* and *OsDREB2A* [67–69]. Taken together, we propose that rice response to low temperature may occur through the following pathways (Fig. 8).

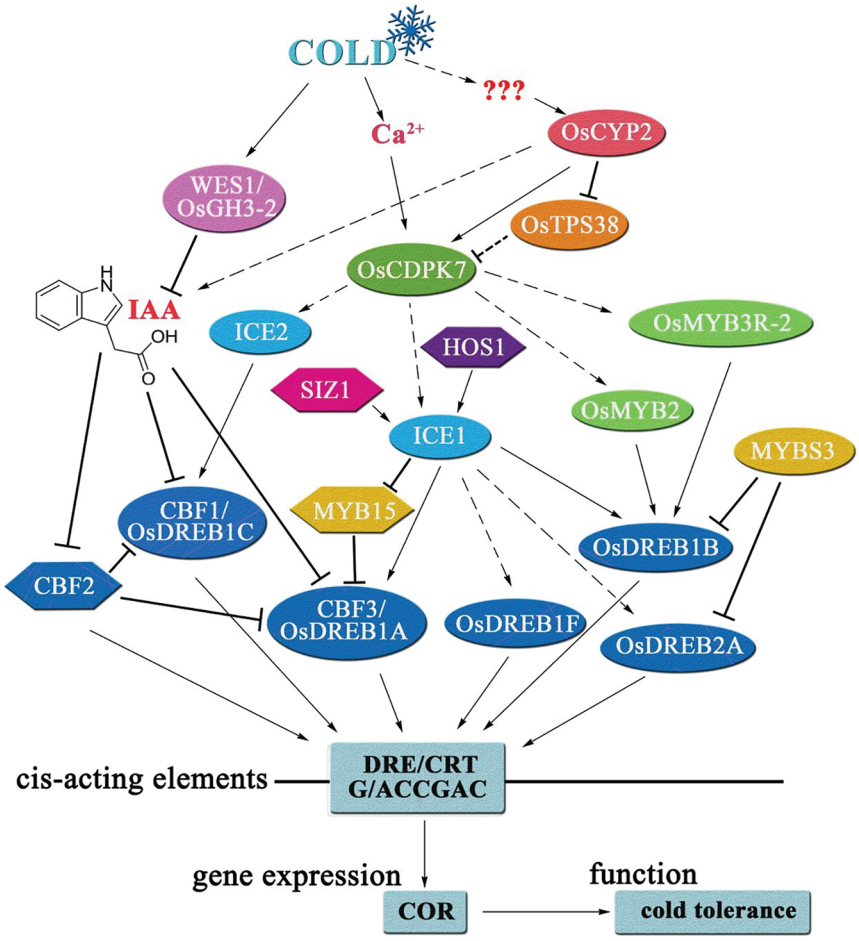

Figure 8: Cold sensing and responsive pathway. Solid arrows indicate positive regulation; T-shaped lines indicate negative regulation; broken arrows indicate predicted activation. The genes in the oval frame are from rice; the genes in the hexagonal frame are from *A. thaliana*

4 Methods

4.1 Plant Materials and Growth Conditions

The rice seeds of zh11 (the wild-type cultivar in *japonica* background), *lrl3, Oscyp2-1*, SSBM (the wild-type cultivar in *japonica* background), SSBM-OE, Kasalath (the wild-type cultivar in *indica* background) and *Oscyp2-2* in cold treatment were provided by Professor Xiaorong Mo of Zhejiang University, SSBM-OE transgenic lines were obtained by introducing the *OsCYP2* CDS sequence into the vector 35S-pCAMBIA1300 (see Supplementary Fig. 5) and then the transformation of the above construct into SSBM wild-type calli by EHA105, the seeds of *lrt2* and Nipponbare in allelic verification were provided by Professor Jianru Zuo of Chinese Academy of Sciences. The F_1 of *lrl3* crossed by *lrt2* was obtained in the Growth Chamber of Northeast Agricultural University.

In the hydroponic experiment, the pH was adjusted to 5.5 with 1 N NaOH in normal rice (*O. sativa*) medium [70]. Rice seeds were washed with distilled water, treated to break dormancy by 0.6% HNO_3 for 16 h at room temperature, and transferred to germinate in the incubator at 37°C. The germinated seeds were planted in normal rice (*O. sativa*) medium (3 L) on a nylon mesh floating, in rice culture chamber (day/night: 30°C/22°C, 12/12 h; Rh80%, 450 μmol photons m^{-2} s^{-1}).

In order to study the effect of auxin on root growth and development, the seeds of zh11 and *lrl3* were cultured under 0.1μM NAA (Sigma Aldrich, http://www.sigmaaldrich.com/) liquid nutritious medium. IAA and NPA were used in short-term auxin treatment.

4.2 Low Temperature Treatment

The seeds of the wild-type zh11, *lrl3* mutant, *OsCYP2*-overexpressed lines SSBM-OE and the wild-type plants SSBM were germinated and cultured in hydroponic solution. Then the one-leaf seedlings were transplanted into 6 cm × 6 cm × 6 cm culture pots in the incubator (HPG-280, Harbin Donglian Electronic Technology Development Co., Ltd., Harbin, China). The culture conditions were as follows: the diurnal temperature was 25°C/22°C, 12/12 h, and the relative humidity was 80%. Sampling for 0 h was conducted before chilling treatment, and zh11 and *lrl3* were subsequently treated at 17°C at three-leaf stage. The culture conditions were as follows: the day and night temperature was maintained at 17°C/17°C, 12/12 h, and the relative humidity was 80%.

4.3 Root Parameter Analysis

Seedling roots of zh11 and *lrl3* were sampled on 7 and 14 days after sowing, and 5 samples were taken from each treatment for statistical analysis. Shoot height, primary root length and adventitious root length of the plants were measured with a scale. The number of adventitious roots was counted visually. Transmission Scanner STD1600 Scanner (Epson, Nagano Prefecture, Japan) was used to scan the primary roots of rice. WinRhizo (Regent Instruments Inc., Quebec, Canada) image analysis system was employed to calculate the number and length of lateral roots of the root system.

4.4 Analysis of Root Gravitation Response

The seeds of zh11 and *lrl3* were germinated and cultured on a nylon mesh in 5 L rice culture solution for two days, then the seeds were transferred to a plastic plate (11.7 cm × 11.7 cm) with the nutritious solution soaked in blue phosphorus-free paper, and the main roots were placed in parallel horizontally.

4.5 LRL3 Gene Mapping and Genetic Complementation Verification

F_1 individuals were obtained by crossing *lrl3* mutant (*japonica* rice zh11) as female parent and *indica* rice Kasalath as male parent. F_1 individuals self-cross to obtain F_2 genetic population. In 30 F_2 mutant plants, *LRL3* gene responsible for *lrl3* mutation was mapped to molecular markers SIS2 and RM12368. *OsCYP2*

controlling lateral root formation was detected in this region and named the candidate gene *LRL3*/*OsCYP2*. Supplementary Tables 1–2 provide details of all the mapping markers.

To test the function of *LRL3*/*OsCYP2* for lateral root formation of *lrl3* mutant, we crossed *lrl3* by *lrt2* mutant with 50 bp deletion in *OsCYP2* to obtain double mutant F_1, and to verify the allelism of *lrl3* and *lrt2* genetically.

4.6 OsCYP2 Homology Analysis

ClustalX 1.81 and Genedoc3.2 software were used to analyze the protein sequences of *OsCYP2* with the homologous proteins of the biological cyclophilin-ABH-like domain, and the homology of their conserved domains was compared. Then the phylogenetic tree was constructed by Neighbor-joining method with MEGA3.1 software. The evolutionary standard bootstrap was 1000, and the results were output with MEGA3.1 software.

4.7 Semi-Quantitative and Quantitative RT-PCR

Total RNA was extracted using Trizol D0410 reagent according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com/). The first cDNA strand was synthesized after 5 μg of total RNA was treated with Invitrogen II reverse transcriptase. Semi-quantitative RT-PCR and quantitative RT-PCR are described above [70]. Details of primers are shown in Supplementary Table 3. RT-PCR was performed at least three times with independent biological replicates, qRT-PCR was performed at least three times with independent technical replicates.

4.8 Transcriptome Analysis

Rice seedlings were treated at low temperature and the leaves of zh11 and *lrl3* seedlings under the normal and chilling treatment were sampled and quickly frozen in liquid nitrogen and stored at −80°C for later analysis. Novogene Bioinformatics Technology Co., Ltd., China was entrusted to complete RNA extraction, quality control, database construction and Illumina HiSeQTM sequencing.

4.9 Construction of OsTPS38-Overexpressed Vector

According to the known sequence of *OsTPS38* gene (http://rice.uga.edu/cgi-bin/ORF_infopage.cgi?orf=LOC_Os08g04500.1) and the cloning site of plant expression vector pBWA(V)HU-45001OE-Gus, specific primers (45001OE-F: cagtCACCTGCaaaacaacatggcaacctctgttccgagtgtacta; 45001OE-R: cagtCACCTGCaaaatacattaaacagagaggatgtagatggagtg) were designed. Then the *OsTPS38* gene was amplified and introduced into the vector pBWA(V)HU-45001OE-Gus (see Supplementary Fig. 6). The construction and genetic transformation of *OsTPS38*-overexpressed vector were entrusted to Wuhan Biorun Biological Technology Co., Ltd. (China) to obtain *OsTPS38*-overexpressed positive lines in zh11 background.

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Author Contributions: Hongxiu Gao, Tianqi Liu, Lin Zhu and Zhenxing Zhu performed phenotypic observation and measurement. Hongxiu Gao, Tianqi Liu, Lin Zhu, Haitao Lv and Zhongchen Zhang performed gene mapping and data analysis. Lin Zhu and Zhongchen Zhang performed genetic complementation verification. Tianqi Liu and Lin Zhu performed RNA data analysis. Tianqi Liu, Xueyu

Leng, Wei Xie and Zhongchen Zhang wrote the manuscript. Hongxiu Gao and Lin Zhu contributed to the article equally. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI-SRA database under the BioProject no. PRJNA732107 and accession nos. SRR14629497, SRR14629496, SRR14629495, and SRR14629494 for the RNA-seq data.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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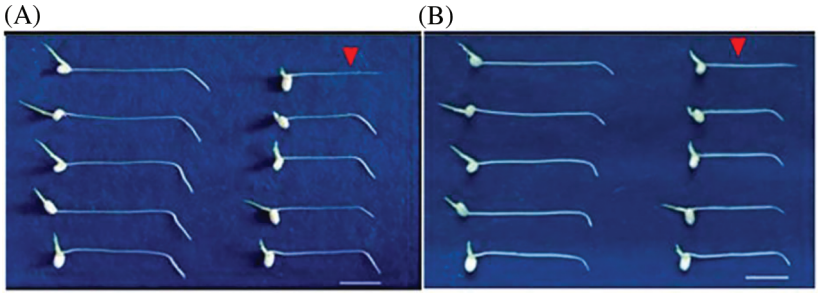
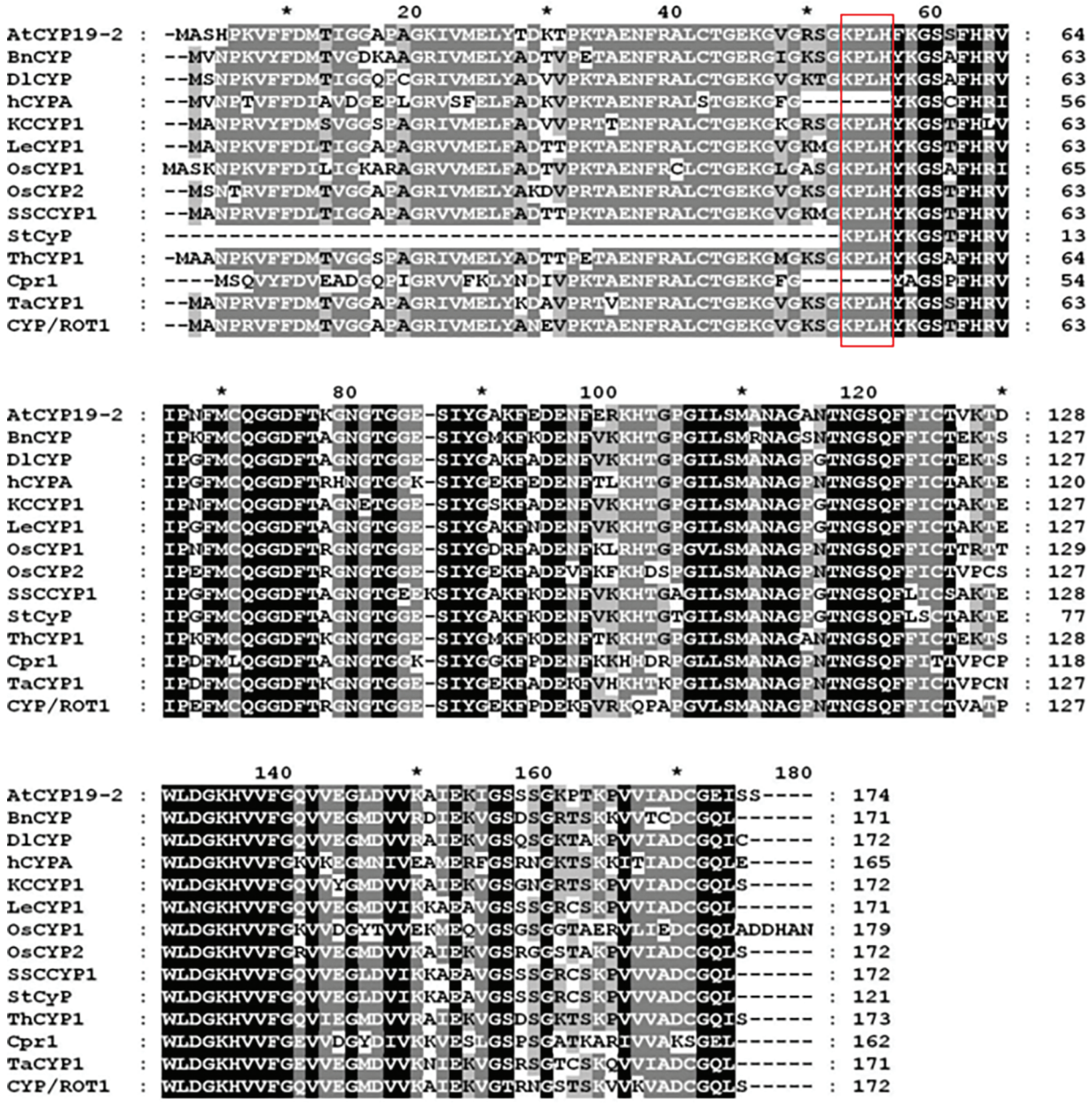
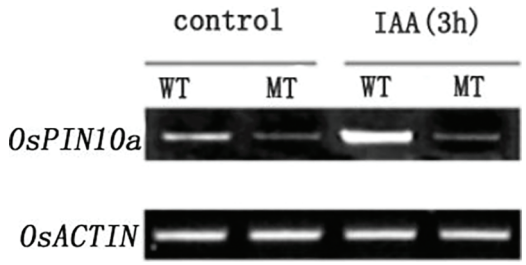
Supplementary Materials



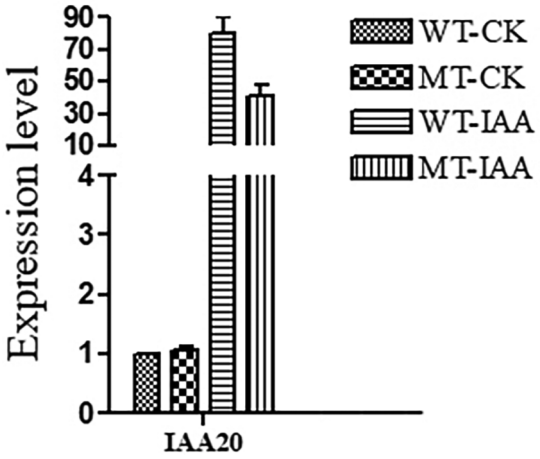
Supplementary Figure 1: Gravitropic response of the wild-type plant zh11 and the *lrl3* T2 generation. (A) and (B) are 12 h and 24 h primary root horizontal phenotypes of two-day-old seedlings, respectively. On the left are the wild-type seedlings zh11, and on the right are *lrl3* T2 generation lines (arrows indicate *lrl3* mutant, and others are wild-type individuals)



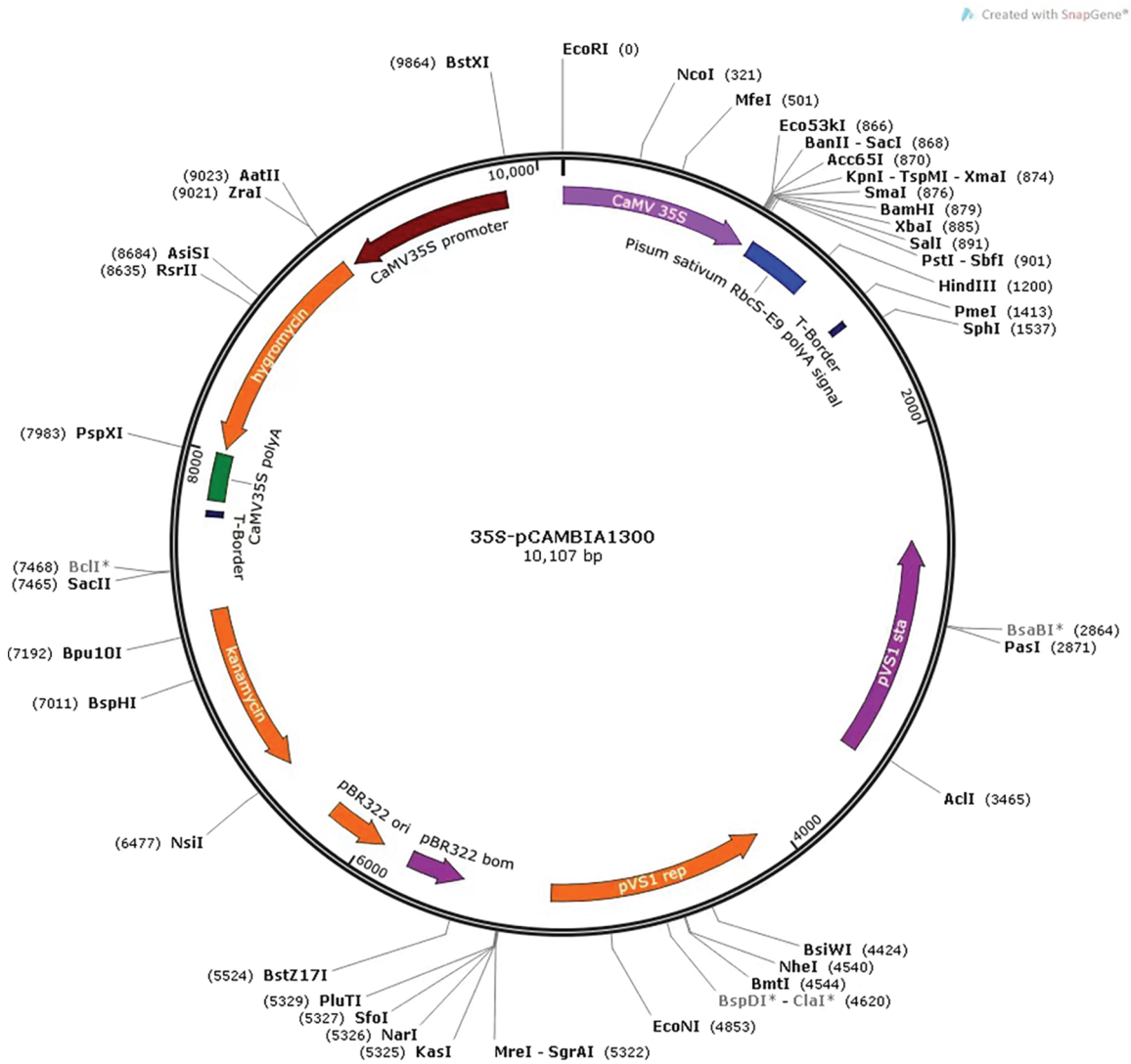
Supplementary Figure 2: Protein alignment between rice and other organisms with cyclophilin_ABH_like domain. AtCYP19-2, *A. thaliana* (gi: 98960923); BnCYP, *Brassica napus* (gi: 1345921); DlCYP, *Digitalis lanata* (gi: 1563719); hCYPA, *Human sapiens* (gi: 13543666); KCCYP1, *Kandelia candel* (gi: 37722431); LeCYP1, *Lycopersicon esculentum* (gi: 170439); OsCYP1, *Oryza sativa* (gi: 600764); OsCYP2, *Oryza sativa* (gi: 600768); Cpr1, *S. cerevisiae* (gi: 6320359); SSCCYP1, *Solanum commersonii* (gi: 1928938); StCyP, *Solanum tuberosum* (gi: 62529356); ThCYP1, *Thellungiella halophila* (gi: 38708271); TaCYP1, *Triticum aestivum* (gi: 13925734); CYP/ROT1, *Zea mays* (gi: 118104)



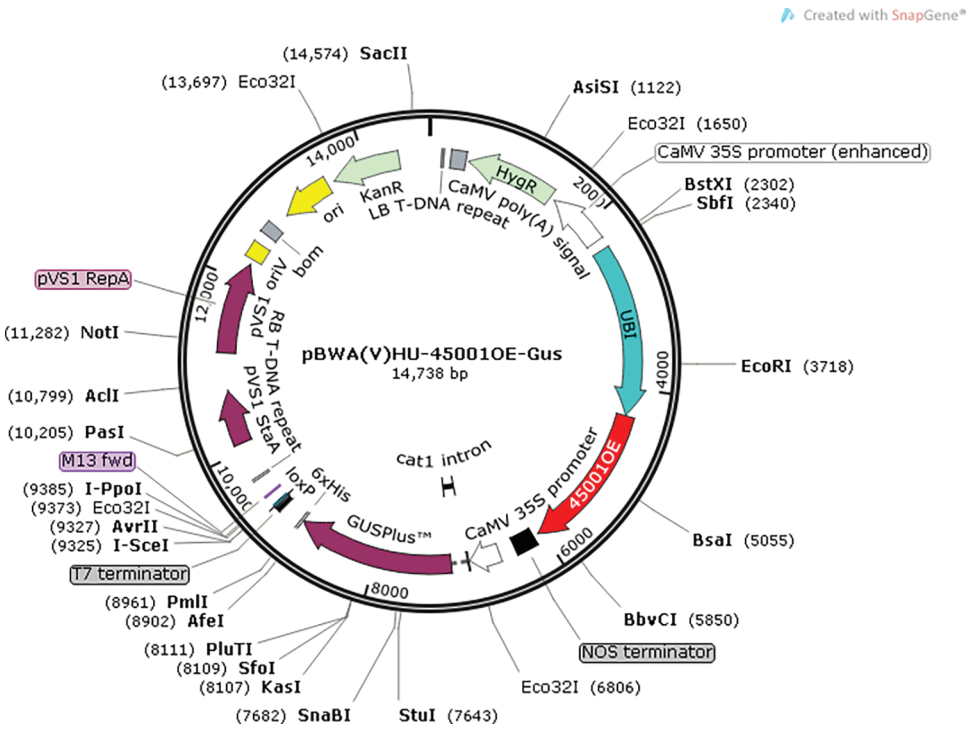
Supplementary Figure 3: Semi-quantitative RT-PCR of *OsPIN10a* in Kasalath and *Oscyp2-2*. WT and MT are seedling roots of Kasalath and *Oscyp2-2* under normal condition (control) and 10 μM IAA (IAA (3h)) for 3 h



Supplementary Figure 4: Quantitative RT-PCR expression in roots of Kasalath and *Oscyp2-2*. WT-CK and MT-CK are seedling roots of Kasalath and *Oscyp2-2* under normal condition, WT-IAA and MT-IAA are seedling roots of Kasalath and *Oscyp2-2* under 10 μM IAA for 3 h



Supplementary Figure 5: The vector of 35S-pCAMBIA1300



Supplementary Figure 6: The vector of pBWA(V)HU-45001OE-Gus

Supplementary Table 1: Primers used in gene mapping

Marker name	Marker type	Primer sequence(5'-3')	Restriction enzymes	Product size(bp) in *lrl3*	Product size(bp) in Kasalath
SIS2 (M2)	STS	F:CTCTTGGGAGTCCTAACT	no	269	281
		R: GCATGGTCCAAATGGTAT			
RM12368	SSR	F : GAGATAAGTGCCAC GATTGATTGC	no	152	162
		R: GGAGCCGTAC GAGTAATC TC TGC			

Note: The information of RM12368 used in this table is derived from the reported research [35].

Supplementary Table 2: Primers used in genetic complementary verification

Marker name	Primer sequence(5'-3')	Product size(bp) in zh11 and Nipponbare	Product size(bp) in *lrt2*	Product size(bp) in *lrl3*	Product size(bp) in F_1
cyp2-lrt2	F:ACGAGGGTGTTCTTCGACAT	171	121	112	112 and 121
	R:GAAGGTGCTCCCCTTGTAGT				

Supplementary Table 3: Primers used in gene expression

Marker name	Primer sequence(5'-3')	Product size (bp)	Function description
OsACTIN	F : TCCATCTTGGCATCTCTCAGC R:AGCCTTGGCAATCCACATCT	60	RT-qPCR
OsCDPK7	F :ACACCGAGATTCGTGATCTTATG R:GTTCCTCTCGCTCCAGTTTATT	114	RT-qPCR
OsCYP2	F : GTGGTGGTGGTGTTAGTCTTT R:GATCCAAGAACTCCGCCTAATC	93	RT-qPCR
OsTPS38	F : CTATGCCTCTCCAGATGTGTTC R: CTGAGATGGGCAGCATTGTA	117	RT-qPCR
OsPIN10a	F:CGGCTCTACCACAAGGGATTG R: TCATAGTCCAAGAAGGATGTAGTACA	143	RT-PCR
OSIAA20	F:CATCCTCGGCTCATACGC R: ATCGTGCCCATCCTCTTG	79	RT-qPCR

Note: The information of *OsIAA20* used in this table is derived from the reported research [35].