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ORIGINAL ARTICLE

Genome-wide identification and analysis of *Catharanthus roseus* RLK1-like kinases in rice

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Abstract

Main conclusion **A genome-wide survey of *Catharanthus roseus* receptor-like kinase1-like kinases (CrRLK1Ls) in rice revealed that the pattern of expression by some CrRLK1Ls is controlled by drought or circadian rhythms. This is probably accomplished through the functioning of *Gigantea* (*OsGI*). Such findings provide a novel angle for using CrRLK1Ls to study the drought-stress response and circadian regulation.**

Abstract The 17 CrRLK1L members of a novel RLK family have been identified in *Arabidopsis*. Each carries a putative extracellular carbohydrate-binding malectin-like domain. However, their roles in rice, a widely consumed staple food, are not well understood. To investigate the functions of CrRLK1Ls in rice, we utilized phylogenomics data obtained through anatomical and diurnal meta-expression analyses. This information was integrated with a large set of public microarray data within the context of the rice CrRLK1L family phylogenic tree. Chromosomal locations indicated that 3 of 16 genes were tandem-duplicated, suggesting possible functional redundancy within this family. However, integrated diurnal expression showed functional divergence between two of three genes, i.e., peak expression was detected during the day for *OsCrRLK1L2,* but during the night for *OsCrRLK1L3*. We found it interesting that *OsCrRLK1L2* expression was repressed in *osgigantea* (*osgi*) mutants, which suggests that it could function downstream of *OsGI*. Network analysis associated with *OsCrRLK1L2* and *OsGI* suggested a novel circadian regulation mechanism mediated by *OsGI.* In addition, two of five *OsCrRLK1L*s preferentially expressed in the roots were stimulated by drought, suggesting a potential role for this family in water-use efficiency. This preliminary identification of CrRLK1Ls and study of their expression in rice will facilitate further functional classifications and applications in plant production.

Keywords Circadian regulation · CrRLK1L family · GIGANTEA · Meta-profiling analysis · Rice

Abbreviations

DAT	Days after treatment
GO	Gene ontology
RGAP	Rice Genome Annotation Project
RLK	Receptor-like kinase
TAIR	The Arabidopsis Information Resource

Introduction

Protein kinases are essential for regulating growth and development in prokaryotes and eukaryotes. They can be grouped into three distinct classes: serine/threonine, tyrosine, and histidine kinases. In plants, receptor-like kinases (RLKs) comprise a large protein family with potential

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capacity for cell surface signaling (Cheung and Wu 2011). These transmembrane proteins have putative amino-terminal extracellular and carboxyl-terminal intracellular kinase domains, and their domain organization is strikingly similar to that of animal RLKs, such as the presence of an epidermal growth factor receptor (Shiu and Bleecker 2001).

CrRLK1L, is a novel RLK-type from *Catharanthus roseus* that has a unique extracellular domain (Schulze-Muth et al. 1996). In contrast to other plant RLKs, this one utilizes an intra- rather than an inter-molecular phosphorylation mechanism for regulation of kinase activity (Schulze-Muth et al. 1996). Studies with *Arabidopsis* have identified 17 CrRLK1L members that contain a signal peptide, a putative extracellular carbohydrate-binding malectin-like domain, a transmembrane domain, and a kinase domain (Boisson-Dernier et al. 2011; Cheung and Wu 2011; Lindner et al. 2012). In particular, the existence of a malectin-like domain supports the roles this family has in mechanisms associated with cell wall surveillance (Lindner et al. 2012). Functions have been reported for some *Arabidopsis CrRLK1L*s in cell elongation, coordination of cell wall integrity, cell wall sensing, and cell-to-cell communication between male and female gametophytes (Lindner et al. 2012). Among these, expression of *FERONIA* (*FER*), *THESEUS1* (*THE1*), *HERCULES1* (*HERK1*), and *HERCULES2* (*HERK2*) is stronger in regions within vegetative tissues where cells are elongating, and are also up-regulated by brassinosteroids to modulate growth and development (Guo et al. 2009b). Loss-of-function studies have presented elongation defects in the leaves and stems of *fer* single mutants, *the1/herk1* double mutants, and a *the1/herk1/herk2* triple mutant, all indicating that *CrRLK1L* members have somewhat redundant roles in that process during the vegetative growth phase. FER is also involved in polar growth of root hairs and pollen tube reception through cell-to-cell communications between gametophytes (Escobar-Restrepo et al. 2007; Duan et al. 2010).

Within the *CrRLK1L* family, *ANXUR1* (*ANX1*) and *ANXUR2* (*ANX2*) are the closest *FER* homologs in *Arabidopsis*, and are preferentially expressed in pollen, playing redundant roles in controlling pollen tube integrity (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). In addition, the pollen-expressed NADPH oxidases function downstream of *ANX1* and *ANX2* to coordinate cell wall integrity in tip-growing cells via ROS production, Ca^{2+} homeostasis, and exocytosis (Boisson-Dernier et al. 2013). Pollen tube rupture and sperm release appear to be mediated by high levels of ROS at the entrance to the female gametophyte (Duan et al. 2014). The FER signaling pathway is required in calcium responses and for coupling the programmed cell death of pollen tubes and the receptive synergid to control plant sperm delivery (Ngo et al. 2014). In addition to ROS and Ca^{2+}, a secreted peptide—rapid alkalinization factor (RALF)—suppresses cell elongation in primary roots. This is mediated by activating FER through a ligand and receptor pair (Haruta et al. 2014).

The molecular and mechanical properties of cell walls enable them to adapt to extracellular abiotic and biotic stresses through ROS regulation. This possibly occurs when the expression of the CrRLK1L family genes is controlled (Lindner et al. 2012). Interestingly, the *CrRLK1Ls, At5g38990* and *At5g39020* are up-regulated by elicitor treatments and biotic stresses but down-regulated by drought (Lindner et al. 2012). Unlike in *Arabidopsis*, none of the *CrRLK1L*s has been characterized in *Oryza sativa* (rice), a model crop plant. We speculate that this manipulation of cell wall elongation and integrity through ROS regulation might be very critical to the maintenance of crop productivity.

Oscillations within the 24-h circadian clock drive diurnal rhythms. Those circadian rhythms have been studied in various organisms including higher plants such as *Arabidopsis* and rice (McClung 2006). Chen (2013) has reported that the plant circadian clock has three interlocked transcriptional feedback loops. The central loop includes Circadian Clock-Associated 1 (CCA1)/Late Elongated Hypocotyl (LHY) and Timing Of Cab Expression 1 (TOC1)/Pseudo-Response Regulator 1 (PRR1), which reciprocally regulate CCA1/LHY, CCA1 Hiking Expedition (CHE), Early Flowering 3 (ELF3), ELF4, and Phytoclock 1 (PCL1). The morning loop comprises PRR5, PRR7, PRR9, and casein kinase II (CK2), while the evening loop includes Gigantea (GI), Zeitlupe (ZTL), and PRR3. Rice plants are day length-sensitive, and their circadian clock controls key developmental processes such as flowering time (Yano et al. 2000; Izawa et al. 2011; Izawa 2012). A mutation of *OsGI*, an *Arabidopsis GI* ortholog, causes late flowering (Izawa et al. 2011). *Heading Date 1* (*Hd1*), an *Arabidopsis CONSTANS* (*CO*) homolog, encodes a zinc finger domain protein (Yano et al. 2000) and predominantly regulates the *OsGI*-dependent photoperiodic pathway under either long or short days (Hayama et al. 2003). The *Arabidopsis CCA1/LHY* ortholog *OsLHY* controls flowering time through phosphorylation by CK2 (Ogiso et al. 2010); its expression is significantly decreased by the *OsGI* mutation (Izawa 2012). In *Arabidopsis*, an adaptation to salt stress that is mechanistically based on GI protein degradation under saline conditions can retard flowering, thereby indicating crosstalk between the abiotic stress response and circadian regulation (Kim et al. 2013). Drought is a major obstacle for crop production because it profoundly limits leaf and root growth (Farooq et al. 2009). To satisfy increasing demands for food, researchers have been working to develop rice plants with enhanced drought

tolerance. In response to water deficits, plants employ mechanisms such as ABA-independent and ABA-dependent pathways (Shinozaki and Yamaguchi-Shinozaki 2007). The *BASIC LEUCINE ZIPPER 23* transcription factor (*bZIP23*) and *bZIP46* are involved in drought-induced gene expression through the ABA signaling pathway (Bartels and Sunkar 2005; Fukao and Xiong 2013). *Responsive to Abscisic Acid 16* (*RAB16*) is another drought-responsive gene controlled by ABA (Oono et al. 2014). In rice, *Late Embryogenesis Abundant (LEA) 3-1* (*OsLEA3-1)* is required for conferring tolerance during the response to multiple abiotic stressors, including drought, salt, and cold (Goyal et al. 2005; Xiao et al. 2007). The FER pathway in *Arabidopsis* negatively regulates the ABA response that is mediated by activation of *Arabidopsis* ABSCISIC ACID-INSENSITIVE2 phosphatase (Yu et al. 2012). This also suggests that members of the CrRLK1L family modulate plant responses to abiotic stresses by means of the ABA signaling pathway.

In this systematic investigation, we used combined phylogenetics and meta-expression profiling analyses to obtain functional information about all of the CrRLK1L family members in rice. Phylogenomic sequences were compared between rice and *Arabidopsis* so that we could assign *Arabidopsis* orthologs with known functions to individual rice CrRLK1Ls. Data were acquired from public microarrays for both meta-anatomical and diurnal expression. These were integrated to develop a phylogenic tree for rice.

Materials and methods

Identification of rice *CrRLK1L* genes

Rice *CrRLK1L* genes were obtained from Greenphyl, a phylogenomics database for plant comparative genomics (http://greenphyl.cirad.fr/v2/cgi-bin/index.cgi) (Conte et al. 2008b), and from the Rice Genome Annotation Project database (RGAP; http://rice.plantbiology.msu.edu/). Our list of all 16 *CrRLK1L* genes in rice (Table S1) included Locus identifier (Locus id), RAP_id (http://www.rapdb.dna.affrc.go.jp/tools/dump), the protein, genomic and cDNA accession number from NCBI, the clone name from Knowledge-based Oryza Molecular biological Encyclopedia (KOME), and gene ontology (GO) terms from the Rice Oligonucleotide Array Database (http://www.ricearray.org/) (Cao et al. 2012; Chandran and Jung 2014). We also compiled the sequences for 17 *Arabidopsis CrRLK1L* genes (Lindner et al. 2012). Ortholog names were collected from The Arabidopsis Information Resource (TAIR; http://www.Arabidopsis.org/).

Gene mapping onto rice chromosomes

The distribution of 16 CrRLK1Ls on 7 rice chromosomes (Fig. S1) was determined and visualized using the map tool function installed in the Oryzabase database (http://www.shigen.nig.ac.jp/rice/oryzabaseV4/).

Phylogenetic analysis

To investigate the phylogenetic relationships among *CrRLK1L* genes from rice to *Arabidopsis thaliana*, we first conducted multiple alignments for 16 rice and 17 *Arabidopsis* protein sequences, using CLUSTAL X software (ver. 2). Those alignments were manually adjusted to retain the conserved RLK motifs. Phylogenetic analysis was then performed with the MEGA5 program based on the Neighbor-Joining (NJ) tree method, complete deletion, and bootstrapping with 500 replicates. These data were used to assign subgroups within the CrRLK1L family (Fig. 1).

Meta-expression analysis

Anatomical expression data for each *CrRLK1L* gene were collected using 1,150 Affymetrix arrays (GPL2025) and 143 Agilent 44 K arrays (GSE21494). Raw data were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database at the National Center for Biotechnology Information (NCBI) (Barrett et al. 2011). The Affymetrix microarray data were then normalized by the Mas 5.0 method implemented in the R package *affy*, and normalized values were converted to a $\log_2$ scale. For anatomical expression analysis, we organized the Affymetrix data into 18 groups of tissues/organs. For the Agilent arrays, we used the median Cy3 signal intensities in the Agilent 44 K microarray data sets (GSE21494) at $\log_2$ median intensities, then normalized the $\log_2$ intensities using a quantile normalization method (Bolstad et al. 2003; Cao et al. 2012). Data were then assigned to the 18 groups. In addition, we downloaded and analyzed diurnal expression data which were generated using leaves representing four developmental stages from wild-type (WT) japonica rice 'Norin 8' and the *osgi* mutant using Agilent array (GSE18685) from GEO. These were sampled each week between June 6 and June 27, in 2008. For transcriptome analysis (Itoh and Izawa 2011), field-grown plants were used from each developmental stage. Two leaves were harvested every 2 h for 2 days (13 time points over 24 h, beginning at 07:00 on 12 August). Heat maps were generated from the log-transformed data and average calculations using Multi Experiment Viewer (http://www.tm4.org/mev/). They were integrated into the phylogenetic tree context.

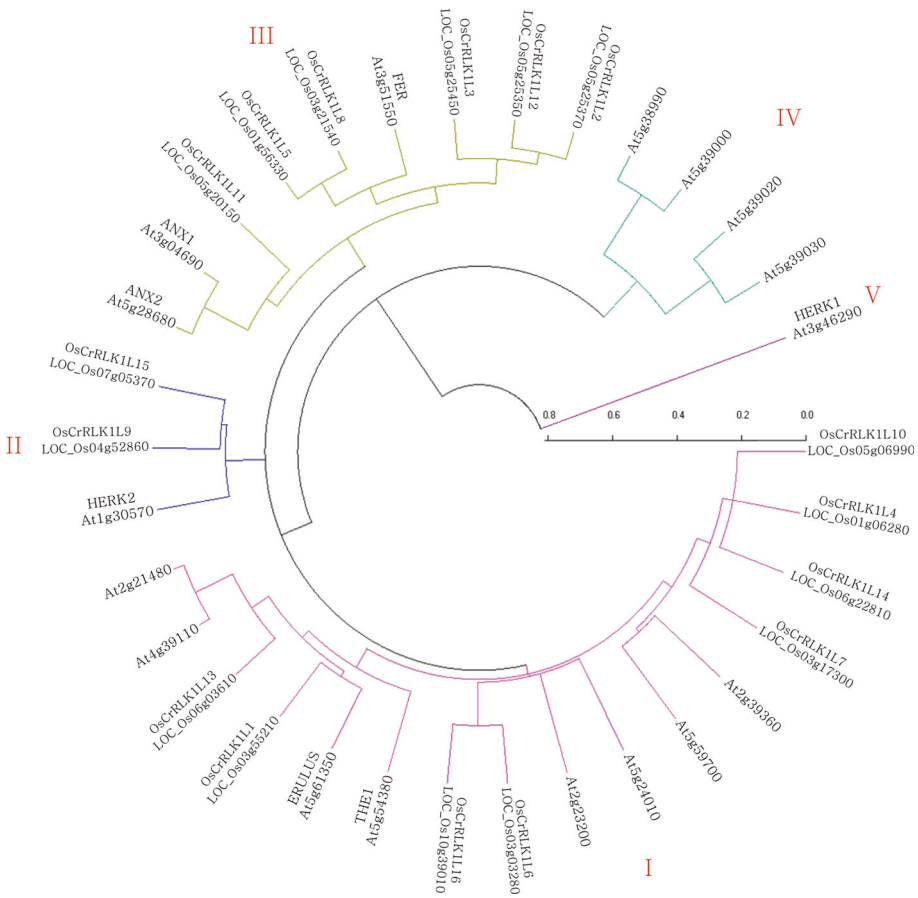

Fig. 1 Comparative phylogenetic analysis of rice and *Arabidopsis* CrRLK1L proteins. Unrooted phylogenetic tree was constructed using MEGA5 program and Neighbor-Joining method. Five colors indicate distinct CrRLK1L subgroups (I–V) in rice and *Arabidopsis*

Plant materials and growing conditions

Wild-type (WT) seeds of *Oryza sativa* L. ssp. Japonica, genotype Dongjin (Rural Development Administration of Korea) were germinated in a Murashige and Skoog (1962) agar medium for 7 days. The resultant seedlings were grown at 28 °C and 60 % humidity under a 12-h photoperiod. For evaluating anatomical expression, 7-day-old seedlings were transferred to water and incubated for 1 day under continuous light. Afterward, their roots and shoots were harvested, frozen in liquid nitrogen, and stored at −80 °C. For the flag leaves, tissues were collected after 3 months of growth in the field. To induce drought stress in a growth chamber (28 °C, 12-h photoperiod), we removed the water from the container having 30-day-old WT seedlings grown in watered plastic seed starting trays with 32 cells and examined them at 3 and 4 days after treatment (DAT). Non-stressed samples were maintained without water removal under the same environmental conditions. Roots were harvested simultaneously from both settings for RNA extraction. For examining the circadian influence, 10-day-old WT and *osgi* mutant seedlings harboring a T-photoperiod). Beginning at 30 DAT, their leaves were sampled every 4 h for 2 days, then frozen in liquid nitrogen and stored at −80 °C.

RNA isolation, RT-PCR, and real-time RT-PCR analyses

Total RNA was extracted with an RNAiso Plus Kit (Takara, Kyoto, Japan). For semi-quantitative reverse transcription-PCR (RT-PCR), 4 μg of total RNA was reverse-transcribed to cDNA using SuperScript II Reverse Transcriptase, according to the manufacturer's instructions. The same amount of cDNA was utilized for each PCR procedure. Two internal control genes—rice *UBIQUITIN 5* (*OsUBI5*, *LOC_Os01g22490*) and rice *ELONGATION FACTOR1 (OsEF1*, *LOC_Os03g08010*)—were used to balance the transcripts among samples.

For real-time RT-PCR analysis, primers were designed to amplify products between 150 and 200 bp (Wong and Medrano 2005). Each primer was searched using the NCBI database BLAST tool to check specificity by gene. The 2X SYBR green PCR premix Ex Taq (Takara) was used, and PCRs were run on a Rotor-Gene 6000 (Qiagen, Hilden, Germany). *OsUbi5* served as an internal control for normalization (Jung et al. 2013). Quantitative RT-PCR and the $2^{-\Delta Ct}$ comparative threshold cycle method were used to quantify gene expression (Wong and Medrano 2005), and PCRs were performed three times. All primers are listed in Table S2.

Results

CrRLK1L gene identification and chromosomal location in rice

We used the GreenphylDB database and obtained 25 models representing 16 loci annotated as "the CrRLK1L homolog". All 16 were verified by the RGAP database and a representative gene model was selected for each locus. These were denoted as *OsCrRLK1L1* through *OsCrRLK1L16*. Because all *OsCrRLK1L*s were annotated as expressed protein, we first tried to find orthologs in *Arabidopsis* from TAIR. We also identified GO terms to provide functional classification information about the category of 'cellular component' for each family member as previously preceded (Botstein et al. 2000; Nguyen et al. 2013). An annotation overview of all rice CrRLK1L proteins is provided in Fig. S2.

Family members were distributed on 7 of 12 rice chromosomes, with none found on chromosomes 2, 8, 9, 11, and 12. Five were located on chromosome 5, four on chromosome 3, two each on chromosomes 1 and 6, and one each on chromosomes 4, 7, and 10. We thought it is noteworthy that a tandem array of three *OsCrRLK1L*s occurred on chromosome 5, namely *OsCrRLK1L2, -3*, and *-12*. We compared the these protein sequences using blastp suite-2sequences tool in NCBI BLAST and identified that OsCrRLK1L2 has 67 % identity under 100 % query coverage with OsCrRLK1L3, and 69 % with OsCrRLK1L12. Therefore, we suspected that these were duplicated and might have redundant functions (Fig. S1).

Comparative phylogenetic analysis of CrRLK1L family between rice and *Arabidopsis*

Relying on RGAP and TAIR sequence information, we performed a phylogenetic analysis to examine the evolutionary relationship between *CrRLK1L* family genes in rice and *Arabidopsis*. A phylogenetic tree was constructed by aligning the full-length protein sequences for the 16 rice members and 17 *Arabidopsis* members. These genes were divided among Subgroups I through V. Subgroups IV and V contained only *Arabidopsis* CrRLK1L proteins (Fig. 1), evidence of unique evolutionary subgroups in that species. The organization for rice included eight members in Subgroup I (*OsCrRLK1L1*, *-4*, *-6*, *-7*, *-10*, *-13*, *-14*, and *-16*), two members in Group II (*OsCrRLK1L9* and *-15*), and six members in Group III (*OsCrRLK1L2*, *-3*, *-5*, *-8*, *-11*, and *-12*). *Arabidopsis* orthologs were assigned to 13 of those rice members (Fig. S2), five with known functions: *Arabidopsis HERK2*, an ortholog of *OsCrRLK1L9* and *-15*; *Arabidopsis ANX1* and *ANX2*, both orthologs of *OsCrRLK1L11*; *Arabidopsis FER*, an ortholog of both *OsCrRLK1L5* and *-8;* and *Arabidopsis ERULUS,* an ortholog of *OsCrRLK1L1*. We expected that those rice members would have functions similar to their *Arabidopsis* orthologs. However, it was more difficult to predict roles for the others due to the lack of functionally characterized *Arabidopsis* orthologs.

Integration of anatomical expression patterns with the CrRLK1L family phylogenic tree

In the next sections, the phylogenetic tree with this classification was integrated to the transcriptome data to represent expression levels of the *OsCrRLK1Ls*. For our anatomical work, the Affymetrix array data were organized into 18 types of tissues/organs, while the Agilent 44 K array data fell into 13 classifications. The expression data heat map was integrated into the phylogenic tree context (Fig. 2). For the Affymetrix meta-expression database, we analyzed all *CrRLK1L* expression data. However, in the case of Agilent 44 K, expression data were produced for all genes except *OsCrRLK1L1* and *-13* because probes for those genes had not been designed for that particular array.

The genes in the *OsCRLK1L* family were considered ubiquitously expressed, vegetative organ-preferential, or floral organ-preferential (Fig. 2). In the first category, *OsCrRLK1L8* was detected across all tissue types examined, suggesting a housekeeping role.

Another eight genes showed higher expression in vegetative organs than in reproductive tissues. Of these, *OsCrRLK1L4* and *-7*; *OsCrRLK1L6* and *-16*; and *OsCrRLK1L2*, *-3*, and *-12* were clustered together in the phylogenic tree. The eighth gene—*OsCrRLK1L1*—was the outlier in that second category. Both *OsCrRLK1L4* and *-7* were more highly expressed in the leaves than in the roots. However, the latter gene also demonstrated strong expression in callus tissue and embryos, indicating that it has a unique function. Whereas *OsCrRLK1L6* showed higher expression in the roots than in the leaves, the opposite was true for *OsCrRLK1L16*, which possibly meant functional divergence. Levels of expression in the roots and leaves were similar between *OsCrRLK1L3* and *-12*, while *OsCrRLK1L2* tended to be leaf-preferential, suggesting a unique feature when compared with its paralogs. In addition to being expressed in the roots, transcripts in the stigma/ovary and seed were higher for *OsCrRLK1L3* and *-12* than for *OsCrRLK1L2*. Expression patterns also differed, however, between *OsCrRLK1L3* and *-12*. Because transcript levels of *OsCrRLK1L12* are generally very low in indica rice samples, our functional characterization might explain the evolutionary difference between japonica and indica cultivars. Predominant expression was most obvious in *OsCrRLK1L2,* suggesting major role among those three clustered members. The root-preferential

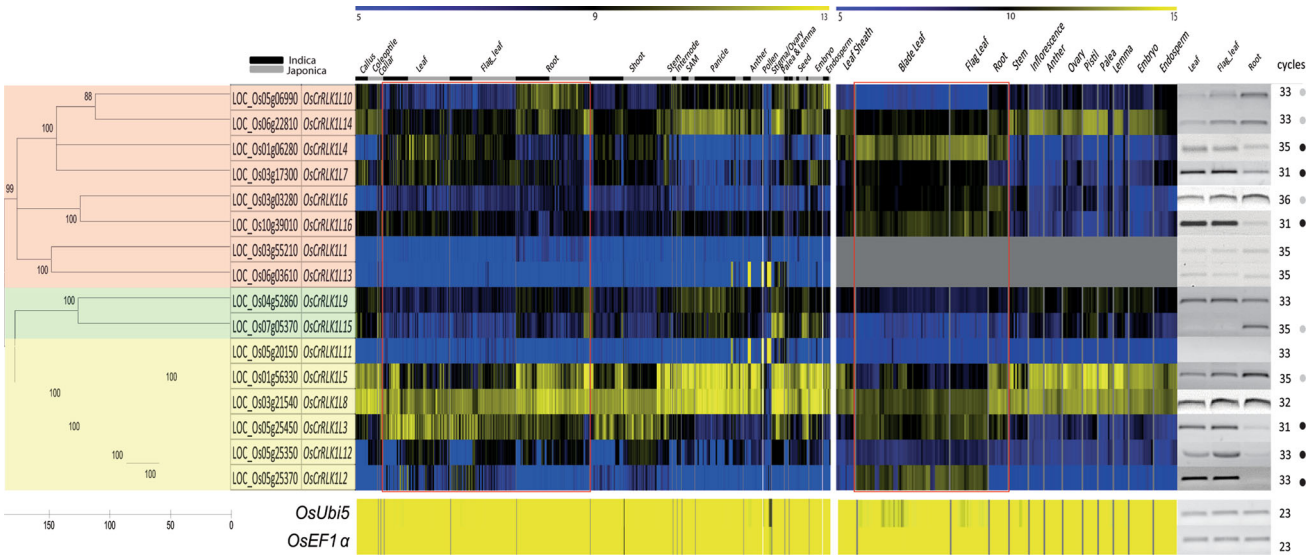

Fig. 2 Anatomical expression patterns integrated with CrRLK1L phylogenetic tree. Data for 18 organs/tissues were classified from 1,150 Affymetrix arrays and 209 Agilent 44 K arrays. *Yellow* high expression; *blue* low expression. RT-PCR was performed to confirm patterns for 16 *OsCrRLK1L*s in leaves, flag leaves, and roots (*red box*). *OsUbi5* and *OsEF1α* were used as internal controls for reactions. *Gray dots* on right-hand side, high expression in roots; *black dots*, high expression in leaves and flag leaves, based on RT-PCR results

expression of *OsCrRLK1L1* implied that it has a unique role in the development of that organ.

Transcripts of six *OsCrRLK1L* genes were more abundant in reproductive organs than in vegetative organs. Both *OsCrRLK1L5* and *-14* showed fairly high expression in all floral organs analyzed, although that of the latter was mild in the endosperm. *OsCrRLK1L11* and *-13* were preferentially expressed in anthers and pollen. Those two pairs of genes were also separately located in the phylogenic tree, indicating that each might have had unique functions during evolution of that species. In contrast, *OsCrRLK1L9* and *-15* were clustered together in the tree, and showed similar patterns overall, although expression was higher in the pistils and embryos for *OsCrRLK1L15* than for *OsCrRLK1L9*. This implied functional redundancy between the two in most anatomical functions except those pertaining to the pistils and embryos.

As the sixteenth gene in this family, *OsCrRLK1L10* had higher expression in the roots, developing seeds, and endosperm than in other organs. This pattern closely overlapped that of *OsCrRLK1L14,* although its levels were inconsistent in the roots and seeds, suggesting antagonistic regulation between them in these organs. Expression by *OsCrRLK1L10* was also higher in the endosperm when compared with *OsCrRLK1L14,* suggesting a unique role in endosperm development.

Expression patterns for the 16 *OsCrRLK1L*s were analyzed in the vegetative organs via RT-PCR. Transcript levels for *OsCrRLK1L5*, *-6*, *-10*, *-14*, and *-15* were higher in roots than in leaves and flag leaves, while *OsCrRLK1L2*, *-3*, *-4*, *-7*, *-12*, and *-16* showed stronger expression in the leaves and flag leaves. For most genes, expression patterns were similar to those revealed by the meta-anatomical expression data (Fig. 2). Preferential expression by *OsCrRLK1L1*, *-8*, *-9*, and *-13* was unclear for individual tissues or organs and no transcripts were detected for *OsCrRLK1L11* (Fig. 2). Overall, our findings demonstrate that 11 of these genes can be used to study functions related to early root or leaf development in seedlings.

Circadian regulation associated with the rice CrRLK1L family

The circadian clock regulates physiological events such as flowering time, hypocotyl elongation, and leaf movement (Sugiyama et al. 2001). We analyzed the publicly available microarray data related to circadian rhythms for 17 *Arabidopsis* CrRLK1Ls, using Affymetrix *Arabidopsis* ATH1 genome array data sets (GSE3416) that contained information about gene expression from three biological replicates of a diurnal time series spanning 24 h (sampling every 4 h). Based on those data, *THE1* (*At5g54380*) showed a clear pattern of diurnal expression while the other 16 did not, suggesting that, in the *Arabidopsis* family, *THE1* was likely to be under the control of the circadian clock (Fig. S3). To elucidate the role of *OsCrRLK1L*s in circadian-guided physiological events, we used expression data from leaves of the 'Norin 8' WT and the *osgi* mutant collected through four developmental stages in the Agilent 44 K microarray from NCBI GEO. Microarray and RT-

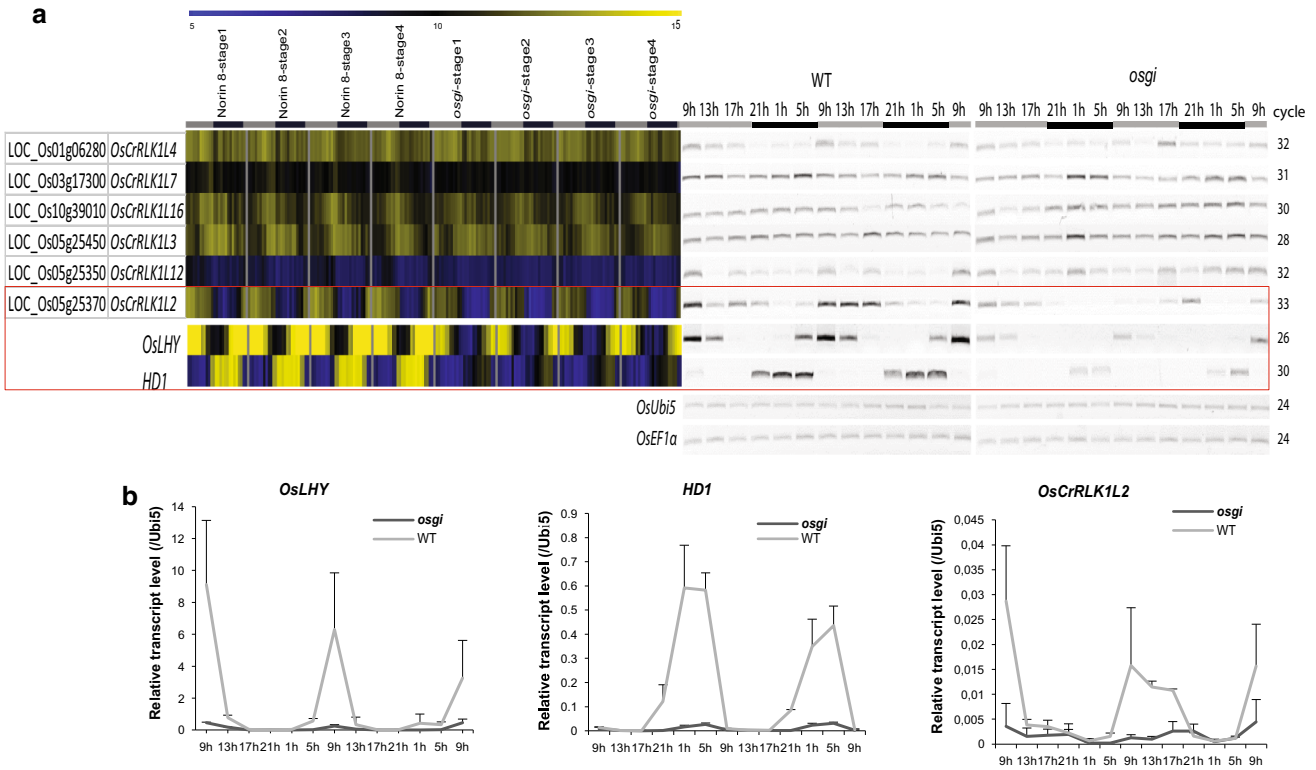

Fig. 3 Circadian regulation associated with 6 CrRLK1Ls in rice. **a** Heat map was generated using Agilent 44 K microarray data sets (GSE18685) for regulation in WT and *osgi* mutant confirmed by RT-PCR. *Yellow* high expression, *blue* low expression. For RNA expression analysis after 30 days, leaves of WT and *GIGANTEA* (GI) mutant were harvested every 4 h for 2 days. *Gray bar* daytime, *black bar* night time. *OsUbi5* and *OsEFα1* were used as internal controls. *Red box* indicates genes that were differentially expressed between WT and mutant. **b** Expression of *OsLHY*, *Hd1*, and *OsCrRLK1L2* in WT and *OsGI*. *OsUbi5* was used as internal control for real-time PCR. Error bars represent standard error of three replicates

PCR analyses indicated that 6 of the 16 genes (*OsCrRLK1L2*, *-3*, *-4, -7*, *-12*, and *-16*) demonstrated leaf-preferential expression during the early seedling stages. All of them except *OsCrRLK1L4* also showed diurnal expression in the meta-analysis. Transcript levels were reduced in the *osgi* mutant (Fig. 3). Meta-expression analysis of the two marker genes, *OsLHY* and *HD1*, indicated that, as expected, expression of *OsLHY* peaked during the day, but then declined at the onset of darkness before increasing as daylight returned. By contrast, *HD1* showed peak expression under darkness; its regulatory patterns were antagonistic to those of *OsLHY*. Patterns for *OsCrRLK1L3*, *-7,* and *-16* were similar to that of *HD1* whereas *OsCrRLK1L2* and *-12* resembled the pattern of *OsLHY*. Diurnal expression was most obvious for *OsCrRLK12* based on both microarray and quantitative real-time RT-PCR analyses, and its expression was significantly reduced in the *osgi* mutant (Fig. 3). Therefore, these results suggested that *OsCrRLK1L2* functions downstream of *OsGI*. Data were rather inconclusive for the others, leading us to recommend that further examination is required to confirm these changes in gene expression as presented by the meta-expression data.

Effect of *hd1* mutant on *OsCrRLK1L2* expression

To investigate further how the circadian clock regulates the expression of *OsCrRLK1L2*, we used the mutant (*hd1*) of the circadian clock-controlling gene, *HD1*, which is part of a short day-dependent flowering pathway (Izawa et al. 2003). Expression in the mutant was compared with that of the field-grown WT as well as that of plants exposed to short days (12-h light/12-h dark cycle). Transcripts of *OsCrRLK1L2* were up-regulated in the mutant under both environmental scenarios (Fig. 4), indicating that *HD1* represses *OsCrRLK1L2* expression. This finding suggested a possible molecular link between *OsGI*-mediating circadian rhythms or flowering pathway and *OsCrRLK1L2* (Fig. 4).

Drought-stress response associated with CrRLK1L family

Anatomical roles were revealed via meta-expression analysis and RT-PCR for *OsCrRLK1L5*, *-6*, *-10*, *-14*, and *-15*, all of which were preferentially expressed in the roots during the early stage of seedling development

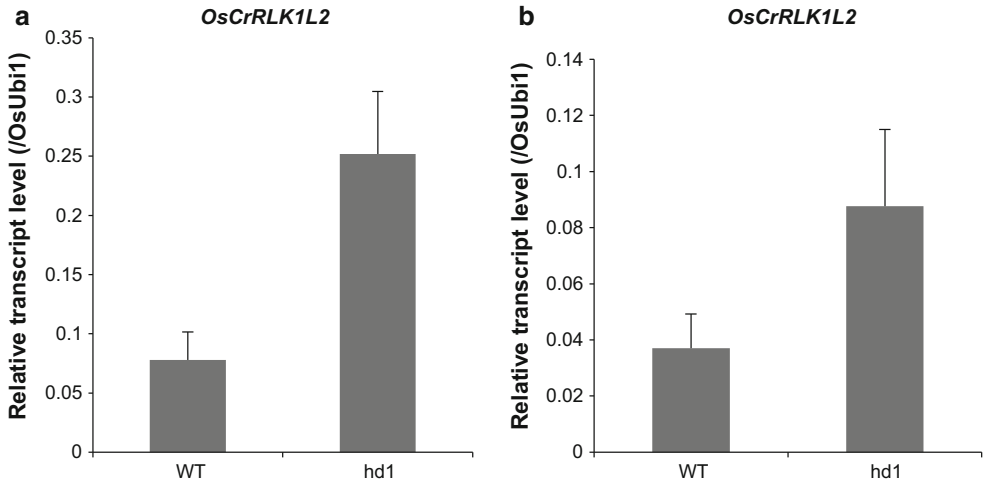

Fig. 4 Quantitative RT-PCR analyses for *OsCrRLK1L2* in WT and *hd1* mutant. **a** Expression in field-grown plants. **b** Expression under short days (12-h photoperiod). *OsUbi1* was used as internal control

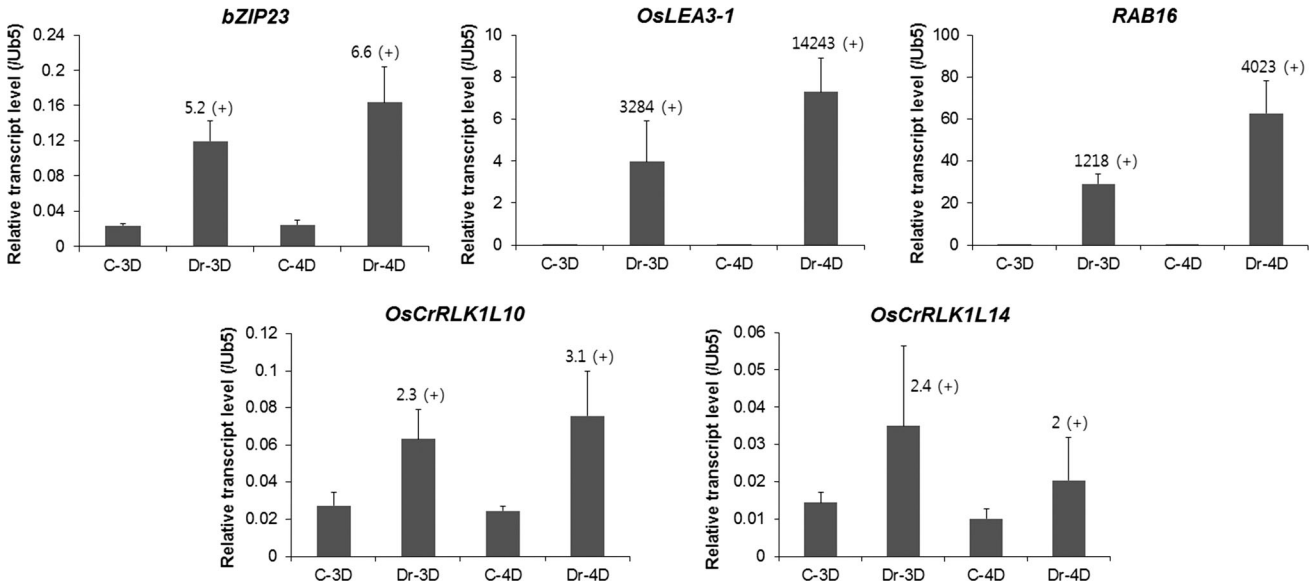

Fig. 5 Quantitative RT-PCR analyses for two *OsCrRLK1Ls* isolated in drought-stressed roots. Relative transcript levels were normalized with respect to internal control *OsUbi5*. Error bars represent standard error of three replicates. *C* control, *Dr* drought-stressed (water withheld). 3D and 4D indicate sampling at 3 and 4 DAT. Numbers at top of each bar are fold-changes for treatment over control value; *plus* indicates upregulation

(Fig. 2). As we were also interested in understanding their levels of activity under drought stress, we investigated their responses by quantitating their expression. After 3 days of stress, leaf morphology was severely damaged, as manifested by rolling, drying, and wilting when compared with leaves from well-watered control plants (Fig. S4). The expression patterns of several well-characterized drought stress-responsive genes were monitored, including *bZIP23* (*LOC_Os02g52780*), *LEA3-1* (*LOC_Os05g46480*), and *RAB16* (*LOC_Os11g26790*). As expected, all three were strongly up-regulated in the roots at 3 and 4 days after stress treatment (DAT) (Fig. 5). Moreover, of the five root-preferential genes, a milder response was found with *OsCrRLK1L10* and *-14*, which were induced by 3.1-fold and 2.0-fold at 4 DAT, respectively (Fig. 5). These results suggested that *OsCrRLK1L10* and *-14* have roles in both root development and the response to drought stress.

Discussion

Members of the *Arabidopsis* CrRLK1L family have diverse functions in regulating cell wall integrity (Hématy et al. 2007; Boisson-Dernier et al. 2013), cell-to-cell communications (Escobar-Restrepo et al. 2007; Ngo et al. 2014), and responses to biotic and abiotic stresses (Kessler et al. 2010; Yu et al. 2012). However, their biological and genetic roles are still completely unknown in crop plants such as rice. All *Arabidopsis* and rice CrRLK1L family have one or two extracellular carbohydrate-binding malectin-like domains and a kinase domain (Fig. S5), indicating that all rice CrRLK1L family members in this study retain the structural features of the CrRLK1L family. Our comparative phylogenetic analysis indicated that genes in Subgroups IV and V are specific to the *Arabidopsis* family, suggesting unique evolutionary roles. Of the known *Arabidopsis CrRLK1Ls*, *HERK1* and *THE1* do not have assigned orthologs in rice. Likewise, *OsCrRLK1L2*, *-3*, and *-12* from rice do not have assigned orthologs in *Arabidopsis* (Fig. S2), again implying unique roles. However, absolute functional conservation between rice and *Arabidopsis* CrRLK1L families must still be determined in future experiments.

Chromosomal mapping of rice members revealed an array of tandem-duplicated genes on chr 5, including *OsCrRLK1L2/LOC_Os05g25370*, *OsCrRLK1L3/LOC_Os05g25450*, and *OsCrRLK1L12/LOC_Os05g25350*. This suggested functional redundancy among them. When their anatomical expression was integrated into our phylogenetic tree, *OsCrRLK1L2*, *-3*, and *-12* in Subgroup III showed conserved expression in the leaves and flag leaves, possibly because of redundancy in those organs. However, we found it interesting that the diurnal expression data integrated to the phylogenetic tree suggested functional divergence between *OsCrRLK1L2* and *-3* because expression by the former was increased at sunrise. Furthermore, expression of *OsCrRLK1L3* was stimulated when *OsCrRLK1L2* expression was repressed. This implied that the two genes function antagonistically by phases in the circadian rhythm. Genes with redundant functions are a potentially important source of evolutionary modifications (Krakauer and Nowak 1999). Several examples exist among *CrRLK1Ls* in *Arabidopsis*, such as between *THE1* and either *HERK1* or *HERK2* with regard to cell expansion (Guo et al. 2009a); or between *ANX1* and *ANX2* during pollen tube growth (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Thus, future genetic evaluations of functional redundancy or divergence are necessary for rice CrRLK1L family members.

Identification of orthologs is a common method used for genome-wide assignment of conserved functions between different plant species (Conte et al. 2008a). Therefore, orthologs found in *Arabidopsis* are very useful for predicting functions by rice CrRLK1L family members. Our orthology search showed that six of 16 OsCrRLK1Ls have *Arabidopsis* orthologs with known functions. Namely, *OsCrRLK1L9* and *-15* is an ortholog of HERK2; *OsCrRLK1L11*, ANX1 and ANX2; *OsCrRLK1L5* and *-8*, FER; and *OsCrRLK1L5, ERULUS*. If expression patterns of orthologous pairs are conserved, we would expect to have higher certainty of their functional orthology. For example, *OsCrRLK1L11* with anther- and pollen-preferential expression is a functional ortholog of tissue-specific *ANX1* and *ANX2*, which control pollen tube growth and integrity (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Likewise, *FER* regulates pollen tube reception (Rotman et al. 2003) as well as root hair growth and integrity (Duan et al. 2010). The rice orthologs *OsCrRLK1L5* and *-8* showed high levels of expression in the ovary and root, both of which are locations for *FER* transcripts in *Arabidopsis*.

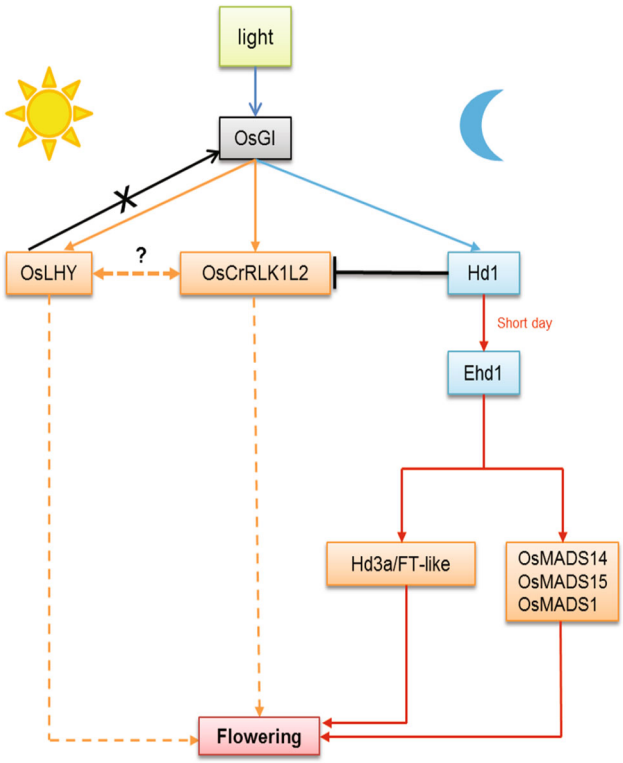

Fig. 6 Functional network construction associated with *OsCrRLK1L2* and simplified model for regulating flowering time and circadian rhythms. Circadian clock includes gene series downstream of *OsGI*: *OsLHY*, *HD1*, *EHDd1*, *OsMADS1*, *OsMADS14*, *OsMADS15*, and *HD3a/FT-LIKE*. *Brown boxes* genes associated with daytime, *blue boxes* genes associated with night time, *brown arrows* day signaling pathway, *blue arrow* night signaling pathway, *red arrow* short day signaling pathway, *T line* negative regulation. *Dotted arrows* indicate pathways less characterized; *solid arrows* show well-characterized pathways. *Question marks* represent unconfirmed data

Some *OsCrRLK1Ls* may have roles in regulating circadian rhythms, as proposed by meta-expression analysis. We also showed that expression of *OsCrRLK1L2* was regulated positively by *OsGI* but negatively by *HD1*. Previous studies have indicated that *OsGI* controls *HD1* in a manner similar to the way that *CO* is regulated by GI in *Arabidopsis* during the daytime (Fowler et al. 1999). In addition, expression of *Early Heading Daye 1 (EHD1)*, which is involved in the short day-promoted flowering pathway in rice, is induced by *HD1* under short days (Doi et al. 2004) as well as by blue light in an *OsGI*-dependent manner (Izawa 2012). The downstream pathway of *EHD1* includes *HD3/FT-LIKE* genes in rice and three *Mads Box* genes (*OsMADS1*, *OsMADS14*, and *OsMADS15*) (Doi et al. 2004). During the day, *OsGI* controls *OsLHY* expression, but *OsLHY* does not control *OsGI* (Izawa 2012). Moreover, the *oslhy* mutant is associated with late flowering (Ogiso et al. 2010). Therefore, our observations for *OsCrRLK1L2* suggest it plays a novel role in regulating the flowering time signaling pathway or circadian rhythms. Future genetic analysis using gain-of-function or loss-of-function for *OsCrRLK1L2* will clarify our model illustrated in Fig. 6.

In conclusion, basing phylogenomics and meta-expression profiling analyses on a large set of microarray data are a useful means for obtaining functional annotation of rice CrRLK1L family members. Integrating the meta-expression data within the context of a phylogenetic tree provides more precise estimates of the functional redundancy among closely linked rice CrRLK1Ls. Moreover, the involvement of *OsCrRLK1L2* in the downstream pathway of *OsGI* hints at the existence of a novel pathway for flowering or circadian rhythm-mediated regulation.

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