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Genome-Wide Identification and Expression Analysis of *RCC1* Gene Family under Abiotic Stresses in Rice (*Oryza sativa* L.)

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Abstract: In plants, the essential roles played by the regulator of chromosome condensation 1 (RCC1) in diverse biological processes, including UV-B (ultraviolet-B radiation) response, hormonal signal transduction, cold tolerance and phenotypic plasticity, have been identified. No comprehensive study on the evolution and function of *RCC1* gene family in rice has been carried out. A genome-wide analysis of this gene family is thus required. In this study, a total of 26 *OsRCC1s* unevenly distributed across 10 chromosomes were identified in rice. Based on their phylogenetic relationship and sequence composition, the *OsRCC1* family could be classified into six groups. Members within the same group share a similar gene structure and protein motif/domain composition. Gene duplication analysis revealed that segmental duplication might be the main contributor to the expansion of the *RCC1* gene family in rice. Several cis-regulatory elements (CREs) relevant to light, abscisic acid (ABA) and methyl jasmonate (MeJA) are abundant in the promoters of *OsRCC1s*. A large number of microRNA (miRNA) target sites were present in *OsRCC1* mRNAs. Additionally, we used data from gene microarray and qRT-PCR to analyze the expression of *OsRCC1* genes during various developmental stages and under abiotic stress conditions. *OsRCC1s* were found to be highly expressed in panicles and seeds, and most *OsRCC1s* were differentially expressed under abiotic stresses. Taken together, our study provides a systematic characterization of *OsRCC1s* and preliminarily explores their diversity as well as their biological functions. Evidence demonstrates that *OsRCC1s* may play vital roles in both development and abiotic stress response. The results presented here lay a foundation for further investigating the functions of *OsRCC1s*.

Keywords: rice; *RCC1*; evolution; gene expression; abiotic stress



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1. Introduction

RCC1 is known as a chromosomal protein that functions as a guanine nucleotide exchange factor (GEF) for the small GTPase protein Ran (a Ras-like nuclear protein) in the nucleus. RCC1 can convert Ran-GDP to Ran-GTP and establish a Ran-GTP gradient between the nucleus and cytoplasm [1,2], thereby regulating a variety of cellular physiological activities such as spindle formation, nuclear membrane assembly, nucleocytoplasmic transport and the G1/S phase transition in the cell cycle [3]. Furthermore, RCC1 is associated with DNA damage repair reaction [2]. RCC1 can be recruited to DNA damage repair sites and participate in the homologous recombination (HR) repair pathway. Overexpression of RCC1 can accelerate cell proliferation and DNA damage repair, as well as inhibit cell senescence caused by DNA damage [4].

Previous studies on RCC1s have primarily focused on mammals and yeast. Although few studies on RCC1s have been conducted in plants, recent studies in *Arabidopsis thaliana* have revealed that RCC1s are essential in abiotic stress signaling and responses. The first to be functionally characterized in *A. thaliana* was UVR8, a specific photoreceptor for UV-B.

UV-B absorption induces an instantaneous conversion of UVR8 from dimer to monomer. Interaction with COP1, the central regulator of light signaling, also regulates plant responses to UV-B [5,6]. RUG3 is another RCC1 family protein that is located in the mitochondria. It can interact with ATM to synergistically regulate alternative splicing of the *nad2* precursor mRNA, mitochondrial reactive oxygen species (ROS) accumulation, DNA damage response and cell cycle progression, so as to maintain mitochondrial function and redox homeostasis [7,8]. This function was confirmed by DEK47 (a functional homolog of AtRUG3 in maize). Furthermore, DEK47 was found to be closely related to seed development [9]. TCF1 could be rapidly induced and activate the expression of *BCB* during cold acclimation, thereby modulating lignin biosynthesis and subsequent cell wall remodeling. It regulates cold acclimation and freezing resistance through a chromatin-based regulatory mechanism (a CBF-independent pathway) [10]. SAB1 was discovered to be a key component in ABA signal transduction, negatively regulating ABI5 via a multi-dimensional mechanism [11]. RCC1 also regulates root development as well as phenotypic plasticity. For example, RLD regulates polar auxin transport by interacting with LZY. Gravity signals first appear in gravity-sensing cells in the form of LZY3 polarity distribution. It then regulates the flow of auxin via RLD1 polarity distribution and PIN3 repositioning to influence gravitropic set point angles of roots [12]. CmRCC1 from pumpkin, which is highly homologous to AtRLD1 and AtRLD4, also participates in root architecture control and *PIN* expression regulation. Moreover, overexpression of *CmRCC1* can enhance the cold tolerance of transgenic tobacco [13]. PROTON1 was identified to be involved in controlling rosette size plasticity in response to nitrogen during initial vegetative growth of *A. thaliana* [14]. Six previously reported RCC1s in *A. thaliana* were evaluated, and it was found that there was no GEF activity in UVR8, TCF1 and RUG3, suggesting that RCC1s may have distinct functions in plants and animals [8,10,15]. In plants, RCC1s could mediate multiple responses to environmental stimuli, participating in UV-B photomorphogenic response, ROS accumulation, cold signal transduction and hormone signaling pathways, etc., thereby, affecting plant morphogenesis and adaptation to the environment by influencing a variety of biological processes such as downstream gene transcription and epigenetic modifications.

Rice is an important crop that feeds more than half of the population in the world and serves as a model plant of monocot. However, the function of only one RCC1 (OsRLR4) has been clarified in rice so far. It directly binds to the promoter of *OsAUX1* and interacts with OsTrx1 to promote the accumulation of H3K4me3 on the *OsAUX1* promoter and positively modulate the *OsAUX1* expression, thus, regulating auxin accumulation in primary root (PR) tip and root apical meristem activity to control PR development [16]. Despite some progress that has been made in understanding the role of RCC1 family in *A. thaliana*, information about the rice RCC1 gene family is relatively rare. It is necessary to investigate the relationship between RCC1s and growth and development, as well as environmental stresses in rice.

In this paper, a total of 26 rice RCC1 family members were identified and characterized. Their phylogeny, physical-chemical properties, gene structures, motif patterns, domain compositions, CREs in promoter, chromosome distribution, gene duplication events, miRNAs targets and gene ontology (GO) enrichments were comprehensively determined. Finally, we examined the expression patterns of *OsRCC1s* in various organs/tissues and under different abiotic stresses by analyzing gene microarray and qRT-PCR data. Our study offers a theoretical basis for understanding the function of RCC1s in rice evolution, growth, development and survival, as well as a reference for the subsequent functional validation.

2. Materials and Methods

2.1. Identification of RCC1s in Rice

The Arabidopsis RCC1 protein sequences were downloaded from the TAIR database (<https://www.arabidopsis.org/>) (accessed on 30 April 2022) and used as queries for a BLASTP search in the rice genome of Ensembl Plants database (<http://plants.ensembl.org/Multi/Tools/Blast>) (accessed on 30 April 2022) to screen potential OsRCC1s with

E-value $\leq 1 \times 10^{-5}$ and identity $\geq 50\%$ as standards [17–19]. The Domain Search function of Rice Genome Annotation Project database (MSU-RGAP, http://rice.uga.edu/analyses_search_domain.shtml) (accessed on 30 April 2022) was used to search the proteins containing RCC1 domains (accession numbers: PF00415 and PF13540) in rice [20]. Verification was performed by the SMART website (<http://smart.embl-heidelberg.de/>) (accessed on 1 May 2022) as well as the Pfam database (<http://pfam.xfam.org/>) (accessed on 1 May 2022) to remove proteins without RCC1 domains [21–23].

2.2. Phylogenetic Analysis

To explore the evolutionary relationships of RCC1s among rice, *Arabidopsis* and maize (*Zea mays*), multiple sequence alignment of RCC1s from the three species was carried out using the ClustalW program in MEGA7 software [24]. Then, a phylogenetic tree of RCC1s was constructed by the Neighbor-joining (NJ) method using Poisson model. The bootstrap value was set to 1000, and the other parameters were all default values. Finally, Evolview (<http://www.evolgenius.info/evolview/#/>) (accessed on 5 May 2022) was used to display the evolutionary tree.

2.3. Physicochemical Properties of OsRCC1s

The computational physical and chemical properties of OsRCC1s were evaluated using the ProtParam website (<https://web.expasy.org/protparam/>) (accessed on 1 May 2022), including the number of amino acids, molecular weight (MW), theoretical isoelectric point (pI) and grand average of hydrophilicity (GRAVY) [25]. The subcellular localization was predicted using the Plant-mPLOC website (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>) (accessed on 1 May 2022) [26]. The detailed information of all OsRCC1s were listed in Table 1.

Table 1. Detailed information of the identified rice RCC1s.

Gene Name	Accession Number		Chromosome	Genomic Location	CDS Length (bp)	Protein				Subcellular Localization
	RAP-ID	MSU-ID				Size (aa)	MW (Da)	pI	GRAVY	
OsRCC1-1	Os01g0700200	LOC_Os01g50470	1	28978119–28983868	3135	1044	115003.703	8.54	−0.448	Nucleus
OsRCC1-2	Os01g0725600	LOC_Os01g52630	1	30251470–30254956	1674	557	60514.9883	5.05	−0.201	Nucleus
OsRCC1-3	Os01g0777800	LOC_Os01g56990	1	32932292–32929037	1206	401	43105.9492	5.65	−0.28	Nucleus
OsRCC1-4	Os01g0846800	LOC_Os01g62810	1	36379629–36385389	1533	510	52839.9297	5.37	−0.232	Cytoplasm. Nucleus
OsRCC1-5	Os01g0952300	LOC_Os01g72320	1	41931602–41925595	3261	1086	117823.773	9.02	−0.411	Nucleus
OsRCC1-6	Os01g0964800	LOC_Os01g73410	1	42541330–42547122	2733	910	98809.7266	8.76	−0.462	Nucleus
OsRCC1-7	Os02g0197500	LOC_Os02g10380	2	5468118–5465521	1335	444	46689.4883	5.68	−0.112	Nucleus
OsRCC1-8	Os02g0554100	LOC_Os02g34860	2	20913876–20919120	1362	453	48270.8086	5.4	−0.305	Nucleus
OsRCC1-9	Os02g0597000	LOC_Os02g38350	2	23186473–23183102	1665	554	59750.3789	5.59	−0.172	Nucleus
OsRCC1-10	Os02g0684900	LOC_Os02g45980	2	28014822–28020814	2883	960	106456.883	9.12	−0.389	Nucleus
OsRCC1-11	Os02g0599600	LOC_Os02g40260	3	22372924–22380193	1440	479	51732.4609	5.73	−0.186	Nucleus
OsRCC1-12	Os04g0435700	LOC_Os04g35570	4	21682409–21688306	1362	453	48172.6992	5.37	−0.334	Nucleus
OsRCC1-13	Os04g0583700	LOC_Os04g49430	4	29493928–29498951	3126	1041	114384.438	9.03	−0.359	Nucleus
OsRCC1-14	Os04g0662800	LOC_Os04g56720	4	33822411–33828795	1650	549	58408.1406	8.74	−0.654	Nucleus
OsRCC1-15	Os04g0686200	LOC_Os04g58960	4	35074912–35067281	3174	1057	115534.227	8.51	−0.543	Nucleus
OsRCC1-16	Os05g0106700	LOC_Os05g01610	5	362500–365961	2754	917	99334.1562	8.28	−0.401	Nucleus
OsRCC1-17	Os05g0384800	LOC_Os05g31920	5	18595443–18586998	3261	1086	117482.078	8.98	−0.462	Nucleus
OsRCC1-18	Os05g0456925	LOC_Os05g38270	5	22445332–22440563	1470	489	51368.3516	6.14	−0.259	Nucleus
OsRCC1-19	Os05g0545200	LOC_Os05g46740	5	27063217–27057383	3102	1033	112886.477	8.64	−0.448	Nucleus
OsRCC1-20	Os07g0165200	LOC_Os07g07080	7	3499332–3491976	3222	1073	116444.109	9.18	−0.433	Nucleus
OsRCC1-21	Os07g0459400	LOC_Os07g27490	7	16029455–16033425	1455	484	51691.5195	7.25	−0.067	Nucleus
OsRCC1-22	Os08g0109800	LOC_Os08g01830	8	496860–500021	2511	836	88439.2969	7.92	−0.12	Nucleus
OsRCC1-23	Os08g0430700	LOC_Os08g33390	8	20824910–20820888	1188	395	41731.5312	5.38	−0.341	Nucleus
OsRCC1-24	Os09g0560450	LOC_Os09g38755	9	22269102–22272680	1314	437	45397.1602	6.59	−0.055	Nucleus
OsRCC1-25	Os11g0545800	LOC_Os11g34320	11	20125144–20119442	1218	405	43902.8906	7.2	−0.129	Nucleus
OsRCC1-26	Os12g0284000	LOC_Os12g18650	12	10770978–10775430	1605	534	57538.0391	6.07	−0.117	Cell wall. Nucleus

2.4. Structural Analysis

Gene structures and conserved motifs of OsRCC1s were identified using the TBtools software [27]. The GFF3 file (Generic Feature Format version3 Data) of rice and protein sequences of OsRCC1s were used as input, respectively. Except for the number of motifs, which was set to 12, all other parameters were default values. Annotations of identified motifs were obtained from the InterPro database (<https://www.ebi.ac.uk/interpro/search/sequence/>) (accessed on 3 May 2022). Furthermore, conserved domains in OsRCC1s were identified using the HMMER database (<https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan>) (accessed on 5 May 2022) by submitting protein sequences of OsRCC1s. Gene structures, conserved motifs and domains of OsRCC1s were visualized using the TBtools software.

2.5. CREs Prediction in Promoter Regions of OsRCC1s

For each *OsRCC1*, 2000 bp sequence upstream of the start codon (ATG) was downloaded from RAP-DB (<https://rapdb.dna.affrc.go.jp/download/irgsp1.html>) (accessed on 10 May 2022) and submitted to the PlantCARE website (<http://bioinformatics.psb.u Gent.be/webtools/plantcare/html/>) (accessed on 10 May 2022) to extract CREs [28]. The obtained prediction information was categorized and summarized. Then, the CREs were displayed using TBtools software and Excel 2019 software.

2.6. Chromosome Distribution, Gene Duplication and Selective Pressure Analysis

Genome data for rice, *Arabidopsis*, tomato (*Solanum lycopersicum*), barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) were downloaded from the Ensembl Plants database (<http://plants.ensembl.org/info/data/ftp/index.html>) (accessed on 13 May 2022) and the Phytozome v13 database (<https://phytozome-next.jgi.doe.gov/>) (accessed on 13 May 2022) [29]. The TBtools software was used to analyze and visualize the chromosomal distribution, gene duplication events within the rice genome, as well as the gene level collinearity among plant genomes. The Simple Ka/Ks Calculator (NG) tool in TBtools software was used to calculate the ratio of non-synonymous to synonymous substitution (Ka/Ks) of duplicated gene pairs.

2.7. miRNA Target Predictions in OsRCC1s

Potential *OsRCC1*-targeting miRNAs were predicted using the psRNATarget database (<https://www.zhaolab.org/psRNATarget/>) (accessed on 10 May 2022) [30]. The coding sequences of *OsRCC1* genes of rice were used as input in psRNATarget against the rice reference miRNAs. All the parameters were left as default values [30]. The Cytoscape v3.9.1 software was used to construct an interaction network between the identified miRNA and the corresponding targeted *OsRCC1s* mRNA [31].

2.8. Expression Profile and GO Annotation Analysis

For microarray analysis of *OsRCC1s*, Affymetrix GeneChip rice genome arrays (<http://www.ncbi.nlm.nih.gov/geo/>; Gene Expression Omnibus platform accession nos. GSE6893 and GSE6901) (accessed on 15 May 2022) were used. The Affymetrix values in 15 various organs/tissues of rice, including seedling roots, mature leaves, young leaves, stem apical meristems (SAM), panicles and seeds, as well as conditions of three different abiotic stresses (salt, drought and low temperature), can be queried through the Rice eFP Browser (<http://bar.utoronto.ca/efp Rice/cgi-bin/efpWeb.cgi>) (accessed on 15 May 2022). Heat maps that were sorted based on hierarchical clustering were created with the TBtools software.

GO enrichment analysis for *OsRCC1s* was performed by agriGO v2.0 (<http://systemsbiology.cau.edu.cn/agriGOv2/index.php>) (accessed on 15 May 2022) [32]. The histogram was generated using GraphPad Prism v8.3 software (GraphPad Software Inc., La Jolla, CA, USA).

2.9. Plant Material, Stress Treatment, RNA Extraction and Quantitative RT-PCR Analysis

Oryza sativa L. *japonica* cv. *Nipponbare* seeds were sterilized (2.5% NaClO) and germinated at 30 °C. Seedlings were cultivated in a greenhouse at 27 ± 2 °C with a 14 h light/10 h dark photoperiod. For stress treatments, the two-week-old seedlings grown in Yoshida medium (YM) were treated with YM supplemented with 150 mM NaCl, 20% (m/V) PEG6000, cold (4 °C), heat (40 °C) and 50 µM ABA, respectively. In each experiment, there were 3 replicates with 96 rice plants. The durations of all the stress treatments were 2, 4, 8 and 12 h, and untreated seedlings were taken as the experimental control. Rice leaf samples were collected randomly from more than 3 rice plants and quickly frozen in liquid nitrogen. Then, they were stored at −80 °C.

Total RNA was extracted by an OminiPlant RNA Kit (CWBIO, Beijing, China), and cDNA synthesis was performed using Hifair® II 1st Strand cDNA Synthesis SuperMix for

qPCR (YEASEN, Shanghai, China) according to the manufacturer's instructions. The Hieff® qPCR SYBR Green Master Mix (YEASEN, Shanghai, China) was used to conduct qRT-PCR amplification in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All primers were designed using the Primer Premier 5 software. Their specificity was then confirmed by using the Ensembl Plants database (<http://plants.ensembl.org/Multi/Tools/Blast>) (accessed on 20 May 2022) for BLASTN search in rice genome. *OsActin1* (LOC_Os03g50885) and *OsUBQ5* (LOC_Os01g22490) were used as the internal reference genes. The sequence information of all primers was listed in Supplementary Table S1. Three biological replicates were performed with three technical replicates for each sample. The relative expression level was calculated by the $2^{-\Delta\Delta C_t}$ method [33]. Gene expressions for selected *OsRCC1s* were visualized with heatmaps using the TBtools software.

3. Results

3.1. Identification of RCC1s in Rice

After removing redundant and incomplete sequences and verifying domains, a total of 26 *RCC1s* were identified in the whole rice genome (designated as *OsRCC1-1~OsRCC1-26* according to their chromosomal location). The physicochemical properties of *OsRCC1s* differ greatly. The average protein length of *OsRCC1s* is 699.5 aa (range from 395 to 1086 aa). The average MW is 75,527.4 Da (range from 41,731.5 to 117,823.8 Da). The theoretical pI ranges from 5.05 (*OsRCC1-2*) to 9.18 (*OsRCC1-20*), with an average pI value of 7.2. All of the *OsRCC1s* possess negative GRAVY values (range from −0.654 to −0.055), with an average value of −0.305, indicating the hydrophilic nature of *OsRCC1s*. Subcellular localization prediction showed that *OsRCC1-4* may be located in both cytoplasm and nucleus, *OsRCC1-26* in both cell wall and nucleus, and other *OsRCC1s* in nucleus. All the characteristics of *OsRCC1s* are shown in Table 1.

Phylogenetic relationships are crucial for understanding the structure and evolution of gene families. By comparing *RCC1* proteins among several plant species (rice, *Arabidopsis* and maize), a phylogenetic tree was constructed using the NJ method. As shown in Figure 1, *RCC1s* of rice and maize are more clustered together within the same branch than *Arabidopsis*, showing a closer genetic relationship. Based on phylogenetic analysis and motif composition, *OsRCC1s* could be divided into six groups (Group I~VI) of different sizes. Group VI is the largest, containing nine *OsRCC1s*, while Group I is the smallest, containing only one *OsRCC1* (*OsRCC1-22*).

3.2. Sequence Structure Analysis of *OsRCC1s*

To investigate the structural characteristics of *OsRCC1s*, we analyzed their exon–intron structures (Figure 2B). The results revealed that there is no non-coding region in *OsRCC1-22*, which is the only member of Group I. Three members in Group II contain 5~6 exons and 5 introns. A total of four members in Group III contain 11~12 exons and 10~11 introns. Five members in Group VI contain nine exons. The last four exons of all nine members in Group VI are similar in length. In general, *OsRCC1s* possess multiple exons and introns with the exception of *OsRCC1-22*, and members within the same group tend to share more similar gene structures and exon lengths.

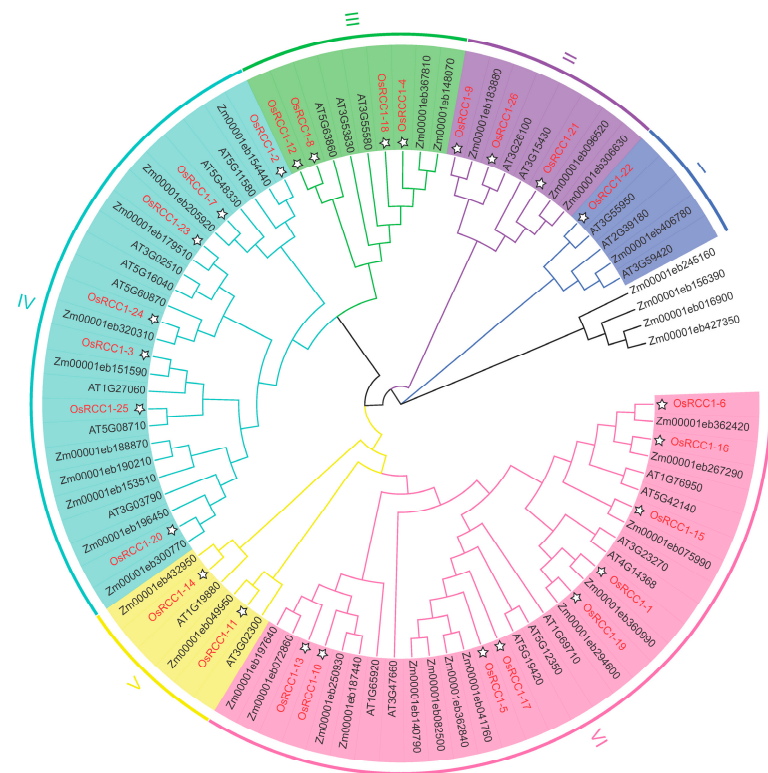


Figure 1. Phylogenetic analysis of OsRCC1s and their homologs from various organisms. AT represents *A. thaliana*, Zm represents *Z. mays*, and Os represents *O. sativa* L. The phylogenetic tree was generated using the NJ method by MEGA7. OsRCC1s are classified into 6 groups (Group I–VI) and distinguished by different colors.

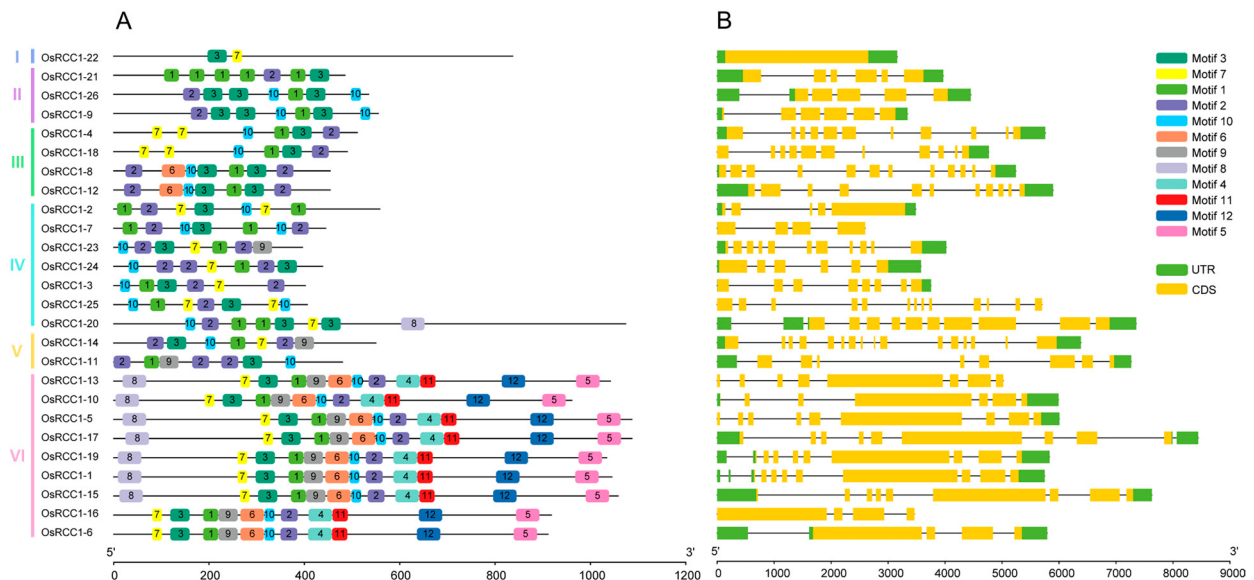


Figure 2. Conserved motifs and gene structures of OsRCC1s. (A) Motif patterns of OsRCC1s. Boxes with different colors represent various conserved motifs. (B) Exon/intron architectures of OsRCC1s. Introns, exons and untranslated regions are represented by black lines, yellow boxes and green boxes, respectively.

The conserved domains and motifs of OsRCC1s were also examined. The entire RCC1 family is characterized by the presence of at least one conserved RCC1 domains.

Some OsRCC1s also have other domains (Supplementary Figure S1). For instance, only OsRCC1-22 contains the protein kinase domain (PF00069) as well as the protein tyrosine and serine/threonine kinase domain (PF07714). Only OsRCC1-20 contains ankyrin-repeat (Ank) domain (PF00023, PF12796, PF13637), and only OsRCC1-15 contains the pleckstrin homology (PH) domain (PF16457). Moreover, nine members of Group VI all contain the FYVE zinc finger domain (PF01363) and BREVIS RADIX (BRX) domain (PF08381, PF13713, PF16627), demonstrating a close evolutionary relationship and potential functional similarity.

We identified 12 conserved motifs in OsRCC1s (Figure 2A), and motif annotations were predicted (Supplementary Table S3). Motifs 1, 2, 3, 6, 7, 9 and 10 belong to RCC1 domains. Motif 4 belongs to FYVE zinc finger domain, motif 5 belongs to the BRX domain, and motif 12 belongs to N-terminal BRX domain. Motifs 8 and 11 could not be annotated. Therefore, their functions remain unknown. Almost all OsRCC1s contain motifs 1, 2, 3, 7 and 10. Motifs 4, 5, 11 and 12 are only present in all members of Group VI; motif 6 is only present in 2 members of Group III and all members of Group VI; motif 8 is only in one member of Group IV and seven members of Group VI; and motif 9 is only present in one member of Group IV and all members of Group V and Group VI. These above results suggest that there are many group-specific motifs in OsRCC1s, which may be related to specific functions.

3.3. CREs Analysis in Promoter of OsRCC1s

As a crop that is sensitive to various biotic and abiotic stresses, rice needs to rapidly adapt to the constantly changing environment [34]. Altering gene transcription via CREs in the promoter region remains a crucial mechanism of gene expression regulation. CREs within promoters (2000 bp upstream of initiation codon “ATG”) of *OsRCC1s* were predicted, so as to speculate on the possible factors affecting *OsRCC1* gene expression and the regulatory pathways in which *OsRCC1s* may be involved. As shown in Figure 3, in addition to the basic CREs (such as CAAT-box and TATA-box), the other functional CREs found in the promoters of *OsRCC1s* could be grouped into four categories: (1) hormone-responsive elements, such as ABA-responsive element (ABRE), auxin-responsive elements (AuxRR-core, TGA-element), salicylic acid-responsive element (TCA-element), MeJA-responsive element (CGTCA motif/TGACG motif), etc.; (2) light-responsive elements, such as Box 4, G-box, GT1-motif, GATA-motif, etc.; (3) stress-responsive elements, such as drought-responsive element (MBS), low temperature-responsive element (LTR), anaerobic-responsive element (ARE), etc.; (4) growth and development regulation elements, such as zein metabolism regulation-related element (O2-site), meristem expression-related element (CAT-box), etc. *OsRCC1s* have the most light-responsive elements distributed within promoter regions (309), and they all contain G-box elements (135). Furthermore, 121 ABRE, 65 CGTCA-motif/TGACG-motif, 51 ARE, 27 MBS and 21 O2-sites were found in the promoter regions of *OsRCC1s*. CREs that response to phytohormone, light and environmental stresses were detected in all of the 26 *OsRCC1s*, whereas only 19 *OsRCC1s* possess growth and development-related CREs. The above results imply that transcriptional profiles of *OsRCC1s* may vary depending on environmental, hormonal and developmental factors.

3.4. Chromosome Mapping and Gene Duplication Analysis of OsRCC1s

OsRCC1s are unevenly distributed on 10 of the 12 rice chromosomes (Figure 4). The number of *OsRCC1s* distributed on chromosome 1 is the largest, with a total of six. Chromosomes 2, 4 and 5 both have four *OsRCC1s*. Chromosomes 7 and 8 both have two *OsRCC1s*. The number of *OsRCC1s* distributed on chromosomes 3, 9, 11 and 12 is the least, with only one each. *OsRCC1s* are not found on chromosome 6 or 10.

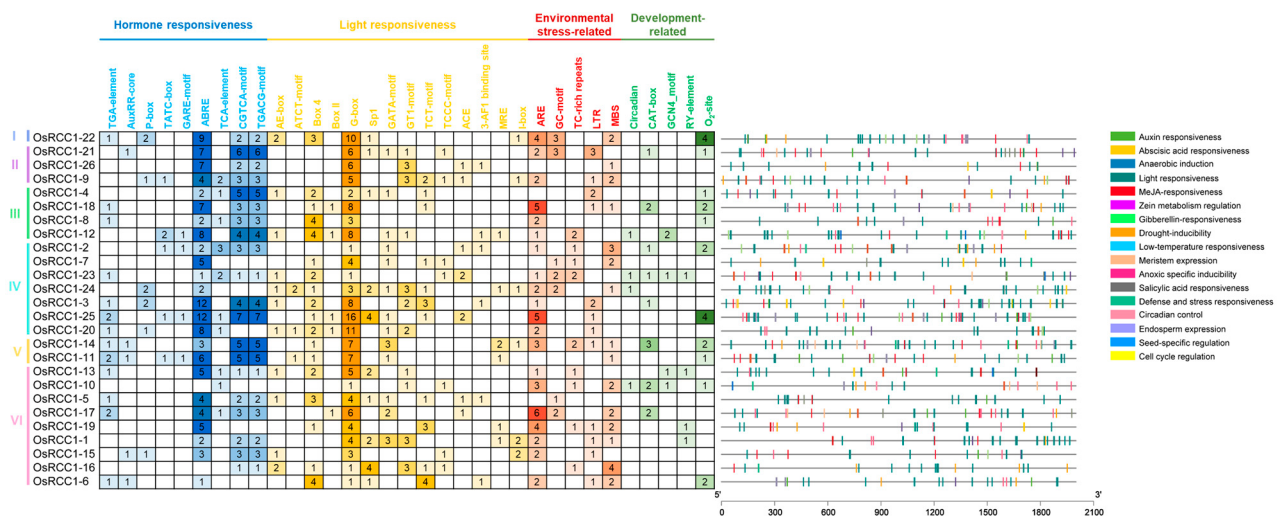


Figure 3. CREs distribution in the promoter regions of *OsRCC1s*. CREs in the promoter of each *OsRCC1* gene are classified based upon the putative functions, including hormone-responsive CREs, light-responsive CREs, environmental stress-responsive CREs and growth and development-responsive CREs. The positional distribution of various CREs on promoters is shown as vertical bars with different colors.

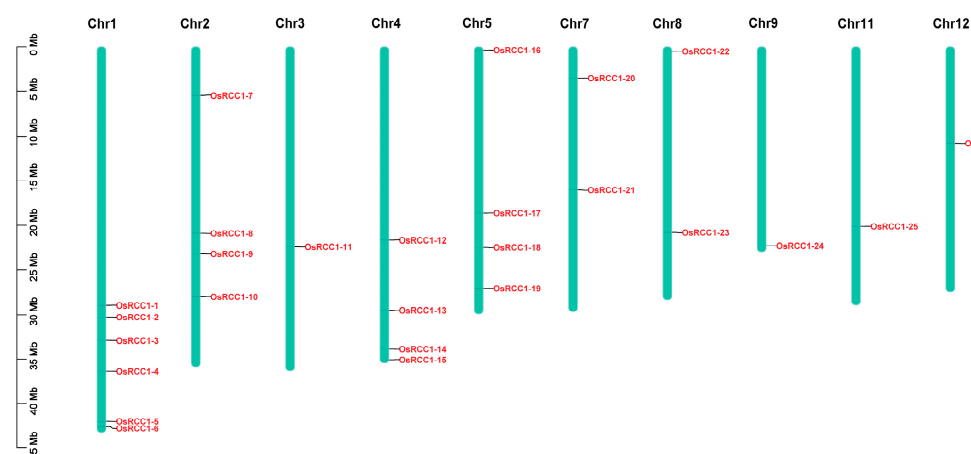


Figure 4. Chromosome distribution of *OsRCC1s*. Chromosome numbers are shown at the top of each chromosome, and the name of each *OsRCC1* is labeled at the right side of each chromosome. Scale bar on the left side indicates the chromosome lengths (Mb).

Gene duplication events, such as tandem and segmental duplication, provide major forces that drive the expansion of gene families and the evolution of the entire genome [35]. We used the TBtools software to examine the duplication events of *OsRCC1s* within the rice genome. Gene family members generated by tandem duplication are typically closely aligned on the same chromosome, forming a cluster of genes with similar sequences and functions. According to Holub's description, a tandem duplication event is defined as three or more gene family members clustering within a 200-kb chromosome region, which means that duplicated genes are contiguous to each other [36]. However, members of *OsRCC1* gene family are distant from each other on chromosomes, and tandem duplication events did not occur. Segmental duplication can be defined as the copies of whole blocks of genes from one chromosome to another. This event would result in gene duplication to unlinked sites, even when a segment is translocated to the same chromosome [37]. There are six pairs of segmental duplication genes in the *OsRCC1* gene family (Figure 5). These

genes are distributed on four chromosomes (chromosome 1, 2, 4 and 5), belonging to Group III (*OsRCC1-4* & *OsRCC1-18*, *OsRCC1-8* & *OsRCC1-12*) and Group VI (*OsRCC1-1* & *OsRCC1-19*, *OsRCC1-5* & *OsRCC1-17*, *OsRCC1-6* & *OsRCC1-16*, *OsRCC1-10* & *OsRCC1-13*). Hence, segmental duplication events may be a vital driving force for the expansion of *OsRCC1* gene family. The K_a/K_s ratio is an important indicator reflecting the type and strength of selection pressure in evolution, with $K_a/K_s < 1$ indicating negative selection pressure. The K_a/K_s ratios for segmental duplicated *OsRCC1* gene pairs within the rice genome vary from 0.099 to 0.277, with an average value of $0.159 < 1$. It is presumed that the *OsRCC1* gene family had mainly been subjected to strong purifying selection during evolution, which was crucial for the functional conservation of *OsRCC1* gene family (Supplementary Table S4).

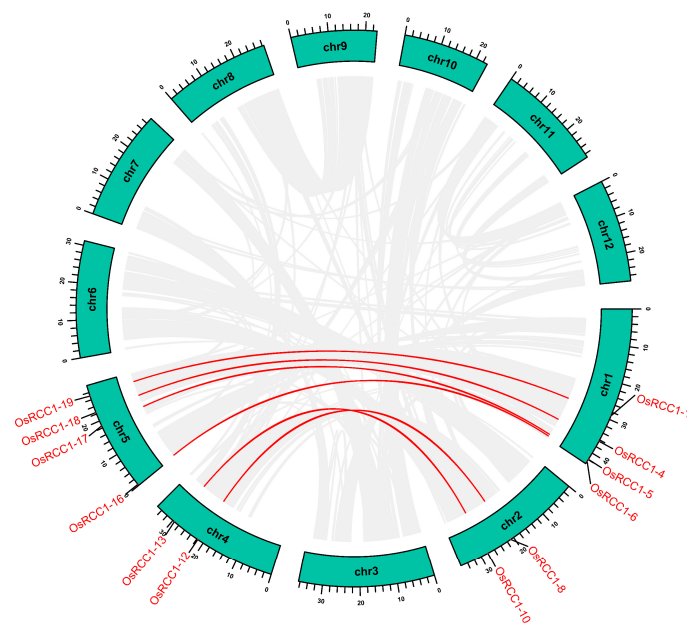


Figure 5. Segmental duplication events of *OsRCC1s*. Segmental duplicated *OsRCC1* gene pairs are linked by the red lines between chromosomes. Segmental duplicated gene pairs within the rice genome are linked by the gray lines. The chromosome numbers are shown at the center of each chromosome. The scale bar marked on the chromosome indicates chromosome lengths (Mb).

Syntenic relationships of *RCC1s* between rice and three other representative species, including two dicots (*A. thaliana* and tomato) and two monocots (wheat and barley), were also investigated (Figure 6). There are 57 collinear gene pairs between rice and wheat, 20 pairs between rice and barley, and 4 pairs between rice and both *Arabidopsis* and tomato (Supplementary Table S5). There is greater collinearity between *OsRCC1s* and *TaRCC1s*. Compared with dicots, *OsRCC1s* display a high level of identity with orthologs in other monocots with fewer evolutionary separation events. *OsRCC1-4* and *OsRCC1-18* exhibit multi-collinearity among species, suggesting that they are more conservative in evolution.

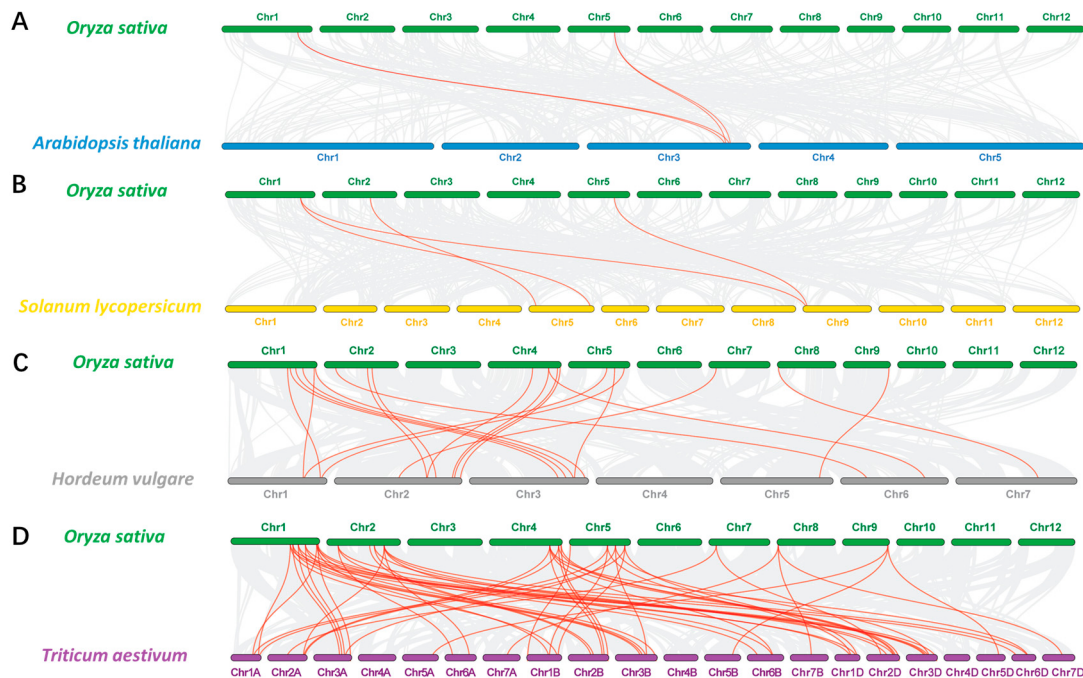


Figure 6. Collinearity relationship of the *RCC1* genes in rice and other four plant species, including *Arabidopsis* (A), tomato (B), barley (C) and wheat (D). Gray lines in the background indicate the syntenic blocks between rice and other plant genomes, while the red lines highlight the syntenic *RCC1* gene pairs.

3.5. Putative miRNA Targeting *OsRCC1s* Prediction

miRNAs are a class of small non-coding single-stranded RNA that recognize target mRNAs via complementary base pairing. They then cause mRNA cleavage and/or translational inhibition to regulate target gene expression at the post-transcriptional level, thereby affecting plant growth and development, crop yield, seed quality, stress response, etc. [38]. The CDS of *OsRCC1s* was employed to distinguish target miRNAs by using the psRNATarget database. The results showed that a total of 336 mature miRNAs (19–24 nt) were identified (Supplementary Table S6). All of the *OsRCC1s* contain target miRNAs, but no major target miRNAs (Supplementary Figure S2). The number of target miRNAs ranges from a minimum of 3 (*OsRCC1-8*, *OsRCC1-21*) to a maximum of 44 (*OsRCC1-1*). A number of 274 miRNAs regulate the expression of *OsRCC1s* through the cleavage effect, while only 62 miRNAs through translational inhibition. It could be speculated that the cleavage effect, as the main function of miRNAs, plays a vital role in regulating the expression of *OsRCC1s*.

3.6. Expression Profiles of *OsRCC1s* in Various Tissues/Organs

The analysis of tissue-/stage-specific expression profiles could help to further unveil the potential functions of *OsRCC1s* in rice. The expression data of 25 *OsRCC1s* (*OsRCC1-24* was not found) in different organs/tissues of *indica* rice variety IR64 were collected from the Rice eFP Browser database to generate a clustering heat map (Figure 7A). The results revealed that *OsRCC1s* mainly display two expression patterns among various tissues/organs. For instance, *OsRCC1-1*, *OsRCC1-10* and *OsRCC1-12~OsRCC1-17* exhibit similar expression patterns, which are highly expressed in SAM and panicles, but lower levels in other tissues/organs. Due to their obvious tissue-specific characteristics, these genes may be necessary for panicle morphogenesis. Other *OsRCC1s* (*OsRCC1-2~OsRCC1-9*, *OsRCC1-11*, *OsRCC1-18~OsRCC1-23*, *OsRCC1-25~OsRCC1-26*) were found to be predominantly expressed in seeds and at least one other tissue/organ. The above results suggest that *OsRCC1s* are differentially expressed in various tissues/organs and may participate in panicle and seed development.

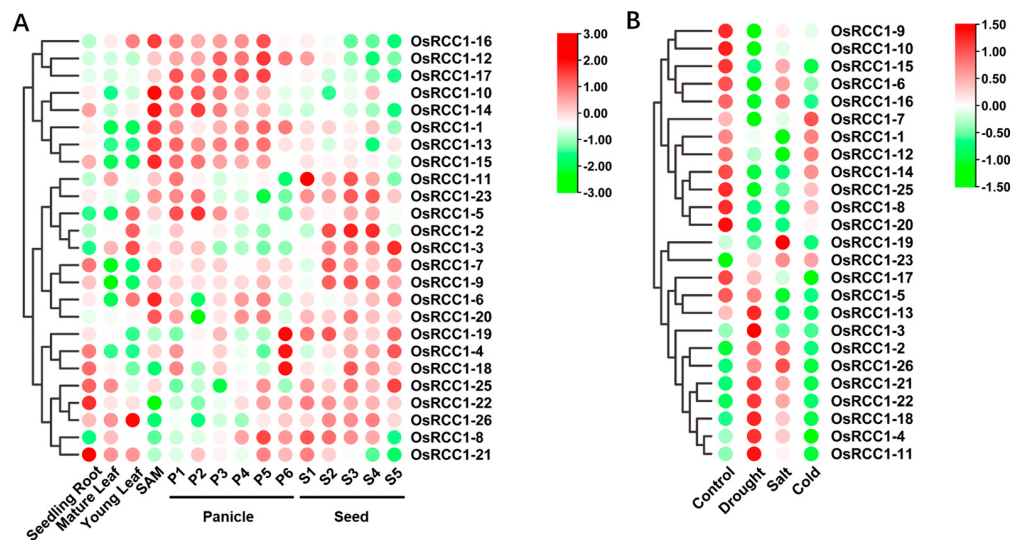


Figure 7. Expression patterns of *OsRCC1s* from microarray data. **(A)** Expression patterns of *OsRCC1s* in diverse tissues and across various developmental stages. The sample sources are as follows: roots of 7-day-old seedling, mature leaf (collected before pollination), young leaf, shoot apical meristem (SAM), P1 (0–3 cm panicle), P2 (3–5 cm panicle), P3 (5–10 cm panicle), P4 (10–15 cm panicle), P5 (15–22 cm panicle), P6 (22–30 cm panicle), S1 (0–2 DAP), S2 (3–4 DAP), S3 (5–10 DAP), S4 (11–20 DAP) and S5 (21–29 DAP). Different stages of panicle and seed development have been categorized according to panicle length and days after pollination, respectively. **(B)** Expression patterns of *OsRCC1s* under three different abiotic stress conditions. Seven-day-old rice seedlings were subjected to salinity (200 mM NaCl), dehydration (dried between folds of tissue paper) and cold (4 °C) treatments for 3 h. The seedlings kept in water for 3 h at 28 ± 1 °C served as control. The color scale represents $\log_2$ of average signal values. The red color shows the higher and the green color shows the lower expression levels in expression bar.

3.7. Expression Profiles of *OsRCC1s* under Abiotic Stresses

To investigate the potential role of *OsRCC1s* in response to abiotic stresses, microarray data of 7-day-old rice seedlings (*indica* variety IR64) under three abiotic stresses (salt, drought and cold) and control conditions for 3 h were obtained from the public database, Rice eFP Browser, and analyzed (*OsRCC1-24* was not found). As shown in Figure 7B, all *OsRCC1s*, except for *OsRCC1-24*, were differentially expressed under different abiotic stress conditions, indicating that *OsRCC1s* play a role in the plant response to abiotic stress. The response patterns of *RCC1s* were different under varying types of stress conditions. For salt and drought stresses, 16 *OsRCC1s* (*OsRCC1-1*, *OsRCC1-5*–*OsRCC1-10*, *OsRCC1-12*–*OsRCC1-17*, *OsRCC1-19*–*OsRCC1-20*, *OsRCC1-25*) were down-regulated under at least one stress factor. Notably, the expression of most *OsRCC1s* were suppressed by chilling stress. Meanwhile, 2-week-old rice seedlings (*Japonica* variety Nipponbare) were treated with salt (150 mM NaCl), drought (20% (m/V) PEG6000), heat (40 °C), cold (4 °C) and ABA (50 μ M) for 0, 2, 4, 8 and 12 h, respectively. A total of 11 *OsRCC1s* with higher expression levels in leaves at seedling stage were chosen from each group (according to the results shown in Figure 7A). Expression profiles of these genes in leaves under these five stresses were detected by qRT-PCR. The results showed that all selected *OsRCC1s* were differentially expressed over time, but with distinct response patterns under different stresses (Figure 8). After salt, drought, ABA and heat treatments, the expression levels of most *OsRCC1s* were down-regulated compared with the control at most time points. However, the expression of most *OsRCC1s* was up-regulated by chilling stress, reaching the peak value at 8 or 12 h. It was noted that *OsRCC1-2* was significantly induced by all five stresses. All of the above findings pointed toward a potential involvement of *OsRCC1s* in the response to environmental stresses. Interestingly, the trends in expression of *OsRCC1s* before and after

stresses derived by qRT-PCR were not consistent with the microarray data, which could be attributed to the differences in genetic background, seedling age and stress conditions.

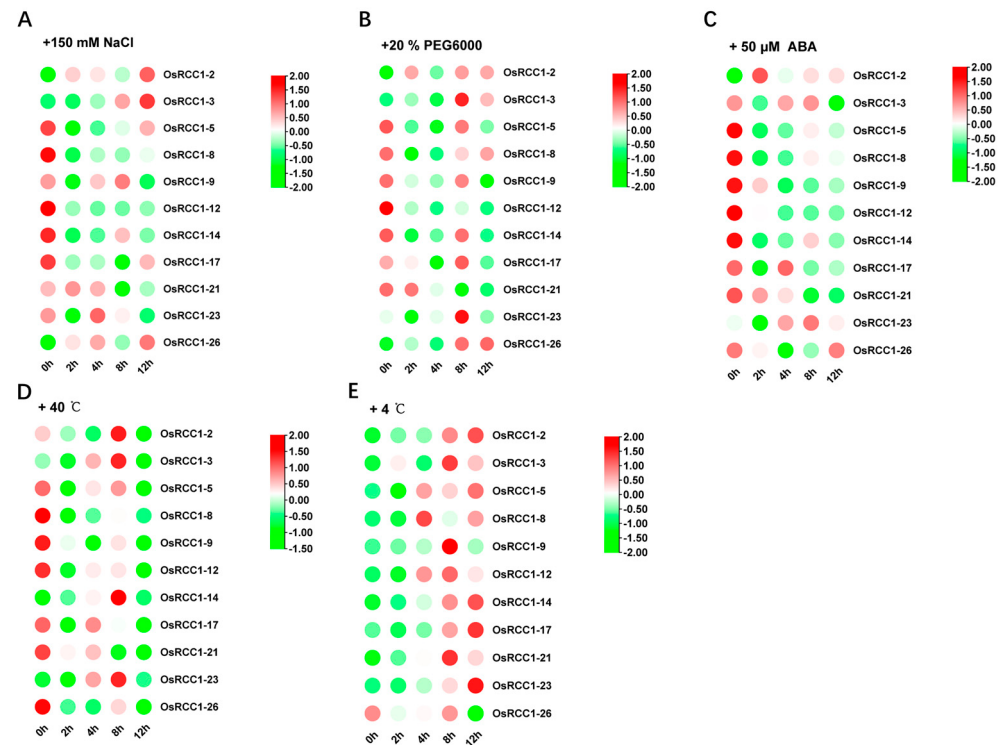


Figure 8. qRT-PCR based expression analysis of 11 *OsRCC1s* under abiotic stresses, viz salt (A), drought (B), ABA (C), heat (D) and cold (E) stresses. The red color shows the higher and the green color shows the lower expression levels in expression bar.

4. Discussion

To date, the research on *RCC1* gene family in plants is limited to the functional research on a particular *RCC1* family member in *A. thaliana* [10,39], with sporadic reports in other plants like rice [16], wheat [40], maize [9], alfalfa (*Medicago truncatula*) [41], poplar (*Populus euphratica*) [6] and *Spartina alterniflora* [42]. Therefore, it is critical to identify and characterize the *OsRCC1* family at a genome-wide level, which would help to elucidate their functions and evolutionary relationships in important crops. In this study, by using bioinformatics and experimental methods, we conducted a comprehensive analysis of *OsRCC1s* and investigated their potential functions in development and abiotic stress response.

The size of gene families is variable across species, which may be a functional result of adaptation or speciation [43,44]. Herein, the number of *RCC1s* in rice (26) is more than those identified in cacao trees (21) and grape (21), comparable to those in *Arabidopsis* (27), but lower than those in maize (35), soybean (55) and upland cotton (56) [45,46]. Hence, it could be inferred that *RCC1* genes are more numerous in species with larger genome sizes.

Based on the phylogenetic analysis and structural characteristics, *OsRCC1s* can be divided into six groups (Group I–VI). The largest group is Group VI, which contains nine members. Most *OsRCC1s* within the same group are less variable in exon–intron structures, motif and domain distributions. Except for *OsRCC1-22* in Group I (no intron), other *OsRCC1s* are composed of several exons and introns, which is consistent with *RCC1s* in upland cotton [46]. All the *OsRCC1s* have more than two motifs associated with the *RCC1* domain (motifs 1, 2, 3, 6, 7, 9 and 10). Motif 4 (associated with FYVE zinc finger domain), 5 (associated with BRX domain), 11 (function is unknown) and 12 (associated with BRX N-terminal domain) only exist in Group VI. Motif 8 (function is unknown) exists in one member of Group IV and seven members of Group VI. In addition to *RCC1* domains,

Group VI also contains FYVE domains, BRX domains and PH domains, which are peculiar to the PRAF subfamily [47,48]. In terms of structural characteristics, Group VI differs significantly from other groups.

In the promoter regions of *OsRCC1s*, we found numerous CREs related to hormone response, light response, stress regulation and development. The number of light-responsive elements is the largest (309), followed by MeJA-responsive elements (CGTCA-motif/TGACG-motif, 65) and ABA-responsive elements (ABRE, 121). Several studies have shown that UVR8 is involved in the regulation of plant photomorphogenesis [5,49–51]. It can be speculated that there may be other photoreceptors in *OsRCC1s*. ABA and MeJA are crucial phytohormones that have been reported to regulate the stress resistance in rice [52,53]. Thus, *OsRCC1s* may participate in regulation of rice against environmental stress.

The gene duplication events may help to illuminate the mechanism about the expansion of gene family. Furthermore, gene duplication in plants can facilitate the generation of new genes, which contributes to the diversity of gene functions; thus, the ability of plants to adapt to a variety of adverse survival environments, such as drought, disease and extreme temperature is improved, and their reproductive development is ensured [54,55]. Our results showed that 46.15% (12/26) of *OsRCC1s* have segmental duplication, but there are no tandem duplication events, indicating that segmental duplication may be the predominant driving force for the expansion of the *OsRCC1* gene family. Ka/Ks ratios of segmental duplicated *OsRCC1* gene pairs are less than one, so we could infer that the *OsRCC1* gene family has undergone strong purification selection during the evolutionary processes. The results of collinearity analysis among different species revealed that *OsRCC1s* show higher homology with counterparts from monocots, but less homology with dicots, reflecting the functional divergence with dicots. *OsRCC1-4* and *OsRCC1-18*, which belong to Group III, formed the most colinear gene pairs, providing potential clues to the origin and evolution of *OsRCC1s*.

Numerous studies have previously demonstrated the role of miRNAs in regulating development and stress response in rice [56–58]. Mul-miR482a-5p may reduce the resistance of mulberry to biotic stress by inhibiting the expression of *RCC1* [59]. In the present study, we identified 252 miRNAs that target *OsRCC1s*. Many *OsRCC1s* are targeted by multiple miRNAs, with up to 44 miRNAs targeting *OsRCC1-1*, suggesting that *OsRCC1s* may affect transcription regulation by miRNA target, with cleavage as a major function in miRNA–*OsRCC1s* interaction.

In order to better comprehend the possible functions of *OsRCC1s*, we examined the transcription patterns of *OsRCC1s* from various organs/tissues. According to gene microarray data, *OsRCC1-1*, *OsRCC1-10* and *OsRCC1-12~OsRCC1-17* are preferentially expressed in SAM and panicles, whereas other *OsRCC1s* are preferentially expressed in seeds and at least one other organ/tissue. These findings suggest that *OsRCC1s* may play regulatory roles in rice growth and development, especially in the reproductive developmental stage. Notably, several *RCC1s*, including MtZR1 [41], AtRUG3 [8], AtRLD [12], AtUVR8 [60] and OsRLR4 (*OsRCC1-15*) [16], are reported to be closely related to root architectural traits.

Previous studies have shown that *RCC1s* can function as regulators to mediate stress resistance in plants. For instance, AtTCF1 affects the cold tolerance by regulating lignin homeostasis in the cytoplasm [10]. Ectopic expression of GmTCF1a (homologous to AtTCF1 in soybean) in *Arabidopsis* confers enhanced chilling tolerance [45]. Overexpression of *SaRCC1* in *Arabidopsis* resulted in a salt-sensitive phenotype, indicating that it negatively modulates salt tolerance [42]. The T-DNA insertion mutant of AtUVR8, as well as the VIGS lines of Gh_A05G3028 and Gh_D10G2310 (homologous to AtTCF1 and AtUVR8 in upland cotton), both exhibited salt-sensitive phenotypes [46]. The expression profiles of *OsRCC1s* under abiotic stresses were obtained from microarray data, and we also detected the transcript abundance of 11 *OsRCC1s* selected from each Group (except Group I) in leaves under abiotic stresses by qRT-PCR. qRT-PCR analysis revealed that the transcriptional expression of all 11 *OsRCC1s* was regulated by salt, drought, ABA, heat and chilling

stresses. Most *OsRCC1s* were inhibited by salt, drought, heat and ABA, but induced by chilling stress. *OsRCC1-2* was strongly induced by all five stresses, and *OsRCC1-3* was induced by the other four stresses except for the ABA treatment. Furthermore, many stress-related elements (such as MBS, LTR and TC-rich repeats) were observed in the promoter regions of *OsRCC1s*. The above results reveal that *OsRCC1s* may be involved in rice response and resistance to abiotic stress.

We also conducted GO enrichment analysis of *OsRCC1s* using agriGO (Supplementary Figure S3). According to the annotation, there are no significant GO terms in the categories of “biological processes” and “cellular components”. In terms of molecular function, 10 *OsRCC1s* (*OsRCC1-1*, *OsRCC1-5*, *OsRCC1-6*, *OsRCC1-10*, *OsRCC1-13*, *OsRCC1-15*, *OsRCC1-16*, *OsRCC1-17*, *OsRCC1-19*, *OsRCC1-22*) are significantly enriched in GO terms related to binding (GO:0005488). Nine genes (other than *OsRCC1-22*) are involved in metal ion binding (GO:0046872), ion binding (GO:0043167), cation binding (GO:0043169), transition metal ion binding (GO:0046914) and zinc ion binding (GO:0008270) (Supplementary Table S7), which are not uncovered in previous studies. Taken together, our study systematically analyzed the characteristics and expression patterns of *OsRCC1s*, but the regulatory networks between *OsRCC1s* and development as well as stress response in rice remain unclear and require further exploration.

5. Conclusions

In this study, 26 *OsRCC1s* were identified from the rice genome. According to phylogenetic and structural analysis, *OsRCC1s* can be divided into six groups (Group I–VI). Members within the same group share similar structural characteristics. Gene duplication detection showed that segmental duplication and purifying selection contribute to the amplification and evolution of *OsRCC1* gene family. In addition, the synteny analysis among rice, *A. thaliana*, barley, wheat and tomato showed different degrees of correlation. The expression of *OsRCC1s* may be influenced by various factors, including hormones, light, stress and miRNAs. *OsRCC1s* are highly expressed during panicle and seed development, implying that they may be involved in the regulation of reproductive growth in rice. Furthermore, several *OsRCC1s* exhibited differential expression patterns under different abiotic stresses, suggesting that *OsRCC1s* may be associated with the response to abiotic stresses. Overall, genome-wide identification and molecular characterization of *OsRCC1s* could provide novel insights into their evolutionary history, as well as their functional roles.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy13030703/s1>, Figure S1: Domain distribution of *OsRCC1s*; Figure S2: Network illustration of predicted miRNA-targeted *OsRCC1s*; Figure S3: GO analysis of *OsRCC1s*. (A) GO analysis of *OsRCC1s* based on molecular functions. (B) Bar chart of enriched GO terms in *OsRCC1s* based on molecular functions; Table S1: Primers used in this study; Table S2: Validation of the RCC1 domains using the HMMER Search tool; Table S3: Motif sequences of RCC1s in rice; Table S4: Ka/Ks ratios of segmental duplicated *OsRCC1* gene pairs; Table S5: List of orthologous proteins of *OsRCC1s* in Arabidopsis, tomato, barley and wheat; Table S6: Predicted miRNA targeting *OsRCC1* mRNAs; Table S7: The GO annotation and enrichment results of *OsRCC1s*.

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