Identification and Characterization of Rice Circular RNAs Responding to Xanthomonas Oryzae pv. Oryzae Invasion

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Original article

Keywords: Rice, circRNAs, plant defense, host genes, miRNAs sponges

Posted Date: February 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-164815/v1

Version of Record: A version of this preprint was published at Phytopathology® on August 22nd, 2021.
See the published version at https://doi.org/10.1094/PHYTO-06-21-0235-R.

Abstract

Background

The emerging role of circular RNAs (circRNAs) in various biological processes have advanced our knowledge of transcriptional and post-transcriptional gene regulation. The number and expression of plant circRNAs vary with species and treatments. However, the expression profile and the potential role of circRNAs during plant response to pathogen invasion are still elusive.

Results

In this study, we identified 3517 circRNAs from PXO99^{A}-infected rice leaves using the ribosomal RNA (rRNA) depleted RNA-Sequencing technique coupled with the CIRI2 and CIRCexplorer2 pipeline. Among them, 2994 (85.13%) circRNAs arised from the exons of their parent genes, 1214 circRNAs were previously unknown and 276 circRNAs exhibited differential expression profiles upon PXO99^{A} infection over time. In addition, 31 differentially expressed circRNAs (DEcircRNAs) were predicted as the corresponding 121 miRNAs sponges. Functional analysis of both host genes and target mRNAs suggested that these identified circRNAs might play an important role in reprogramming rice responses to PXO99^{A} invasion, mainly by mediating photorespiration, chloroplast, peroxisome and diterpenoid biosynthesis associated pathways.

Conclusion

These results inferred a potential functional role of circRNAs in the regulation of rice immunity and provide novel clues for revealing the molecular mechanisms of rice-PXO99^{A} interaction.

Background

Rice bacterial blight is a highly destructive disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which is threatening rice production worldwide. However, rice plants have evolved multiple layers of innate immunity to prevent the attack of pathogens (Kawano and Shimamoto 2013). The first mode of defense responses called pathogen-associated molecular pattern-triggered immunity (PTI) occurs through recognizing pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) (Kawano and Shimamoto 2013). To evade the first line of defense, pathogens secrete effector proteins to suppress PTI. In turn, a second line of plant defense termed effector-triggered immunity (ETI) is activated by resistance (R) proteins recognizing specific microbial effectors (Kawano and Shimamoto 2013). Both PTI and ETI responses can be regulated at the transcriptional and post-transcriptional levels (Baldrich and San Segundo 2016). Many studies have traditionally been focused on identification and application of the related functional genes involved in rice immunity (Ke et al. 2017; Zhang and Wang 2013). Therefore, the exploitation of disease resistance (R) genes in rice breeding programs has been the most effective strategy for coping with the threat of pathogens (Moffat 2001). During the last years, advances in deep sequencing technologies coupled with high-efficiency

bioinformatics tools significantly facilitate the discovery of novel noncoding RNAs (ncRNAs) (Memczak et al. 2013; Ponting et al. 2009; Ravasi et al. 2006). Diverse types of ncRNAs with varied functions have been found in both unicellular and multicellular organisms, which have shifted the attention in the fields of molecular biology from protein-coding RNAs to ncRNAs (Morris and Mattick 2014).

As a novel class of ncRNAs, circular RNAs (circRNAs) has received increasing attention (Memczak et al. 2013). Structurally different from the linear RNA molecules, their 3' and 5' ends are covalently joined to form closed loops which arise from back-splicing of pre-mRNAs (Chen and Yang 2015) and can protect them from RNA exonuclease-mediated degradation (Cocquerelle et al. 1993; Jeck et al. 2013). Characterization analyses show that circRNAs are mainly divided into exonic, exon-intronic, intronic, and intergenic region (Salzman et al. 2012; Chu et al. 2018) and have various isoforms elicited from a circRNA locus (Zhao et al. 2019; Chu et al. 2018). Over the past few years, thousands of circRNAs have been identified in animals. Their significance is being addressed frequently in literature for their role in transcriptional and post-transcriptional regulation, such as functioning as miRNA sponges, modifiers of their host genes expression, and regulators of parental genes transcription and splicing (Ebbesen et al. 2016; Qu et al. 2015). However, their function remains elusive in plants, although they have been widely identified in both monocot and dicot plants (Lu et al. 2015; Sun et al. 2016; Wang et al. 2017; Zhou et al. 2018; Zuo et al. 2016). Recently, there has been an increasing focus on characterizing their relevance and function in plant stress responses. For example, in tomato fruit under chilling stress, 163 circRNAs were identified with chilling-responsive expression, of which 102 contain miRNA-binding sites and have the potential to act as miRNA sponges (Zuo et al. 2016). Likewise, 584 circRNAs were differentially expressed in kiwifruit plants during canker pathogen infection, and the expression of particular circRNAs is altered upon the stage of infection (Wang et al. 2017). A total of 2932 circRNAs were identified in rice leaf transcriptomes of blast-resistant and -susceptible accession, of which 636 were detected specifically subjected to *Magnaporthe oryzae* (*M. oryzae*) infection, responsible for circRNAs diversity in rice (Fan et al. 2020). These reports suggested that the number and the expression of plant circRNAs vary with treatments and plant species. However, the potential role of circRNAs in rice responding to *Xoo* invasion - at least to our knowledge - is largely missing. Our study primarily focused on identification and characterization of circRNAs involved in rice-*Xoo* interaction, and uncovered their potential regulation function in plant responses to bacterial pathogen invasion.

Results

Discovery and characteristic of circRNAs in rice leaves

OsPR1a and *OsPR1b* (pathogenesis-related proteins coding genes) were regarded as reliable marker genes of plant defense responses (Agrawal et al. 2001). To observe whether the transcriptional regulatory events occurred in rice-*Xoo* interactions, we checked the expression profiles of *OsPR1a* and *OsPR1b* by RT-qPCR using specific primers (Table S6) at different time points post inoculation (hpi) with PXO99^{A}. The levels of the *OsPR1a* and *OsPR1b* transcripts showed a similar induction profile (Figure S1), which is consistent with the defense-responsive expression in previous report (Jia et al. 2020). Their transcript

levels slightly induced at 2 hpi and 6 hpi or markedly increased at 12 hpi and decreased drastically at 24 hpi (Figure S1). Combining the infection strategy and time points chosen in the study of pathogen-responsive lncRNAs in rice (Yu et al. 2020), 0 h (control), 2, 6, 12 and 24 h were chosen as the ideal sampling time points for the identification of circRNAs responding to *Xoo* in rice.

5 rRNA-depleted RNA libraries were constructed for 3-week-old seedlings of Nipponbare infected by PXO99^{A}. The deep-sequencing using Illumina Hiseq X-ten platform yielded 45057324, 47264368, 42590676, 41416705 and 51015354 raw reads from 0, 2, 6, 12 and 24 hpi, respectively, followed by a mapping to the rice reference genome (Table S1). After removing the redundant circRNAs by CD-HIT-EST, a total of 3517 circRNAs were identified using CIRCexplorer2 (589 circRNAs) and CIRI2 (2648 circRNAs) from all samples (Fig. 1A, Figure S2), only 280 circRNAs were shared by both algorithms (Figure S2). Among them, 512, 486, 622, 621 and 733 were respectively identified at 0, 2, 6, 12 and 24 hpi (Fig. 1A). According to their genomic loci, the identified 3517 circRNAs were grouped as exon, intergenic, antisense and intron regions. Most circRNAs (85.13%) were derived from exons, and a few from intergenic regions (13.48%) (Fig. 1B). Moreover, we compared the circRNAs detected in our study with the publicly available rice circRNAs from PlantcircBase. It revealed that 2303 (65.48%) of circRNAs in Nipponbare have already been identified, yet a lot of novel circRNAs (1214, 34.52%) were only detected in this study (Fig. 1C). Considering the length, most circRNAs ranged from 200 bp to 1000 bp (2780, accounting for 79.04 %), a few circRNAs (424, 12.06 %) distributed between 1000 and 1800 bp, and very few were longer than 2000 bp (313, 8.90%) (Fig. 1D). Regarding chromosome distribution, these circRNAs were distributed across the whole genome of rice, chromosome 1 contains the most while chromosome 11 contains the fewest (Fig. 1E). Alternative circularization analysis indicated that a substantial proportion of parental genes (1538, 80.86 %) had a tendency to produce one circRNA, 256 genes (13.62%) produced two different circRNA isoforms, and one region produced ten distinct circRNAs isoforms (Fig. 1F). Moreover, the splicing signals analysis of 3517 rice circRNAs revealed that a low proportion (349 of 3517) of circRNAs contain a canonical GT/AG (including CT/AC equivalent) splicing signal (Figure S3).

CircRNAs are differentially expressed in response to PXO99^{A} infection

To reveal circRNAs involved in rice-*Xoo* interaction, we investigated the expression profile of circRNAs and identified differentially expressed circRNAs (DEcircRNAs) from rice flag leaves infected by PXO99^{A} at different hpi. The expression profile revealed a total of 276 circRNAs were detected as significantly DEcircRNAs in response to PXO99^{A} infection (Fig. 2A). Among them, 91, 129, 129 and 138 DEcircRNAs were respectively detected at 2, 6, 12 and 24 hpi. The 112 circRNAs were shared by at least two post-infection time points and 30 circRNAs were overlapped among different time points (Fig. 2A). As to the up- and down-regulated DEcircRNAs, the down-regulated circRNAs were obviously more than the up-regulated genes at 2 hpi, and less than the up-regulated genes at other time points (Fig. 2B). Like all circRNAs in rice, exonic DEcircRNAs were predominant circRNAs (65.22%), followed by intergenic (31.88%) and antisense circRNAs (2.90%) (Fig. 2C), moreover, most DEcircRNAs were also shorter than 1000 bp (Fig. 2D).

Functional categorization of circRNA parental genes

Since circRNA biogenesis has close relationship with their functions in plants, GO and KEGG pathway analysis were performed for the host genes of the circRNAs collected from all the time points. The host genes are involved in three main functional categories, including cellular component, molecular function, and biological process (Fig. 3A). The major enrichments for the cellular component terms were ‘cell’, ‘organelle’ and ‘membrane’ while ‘catalytic activity’ and ‘binding’ were the most enrichments for molecular function terms with abundant circRNAs. The GO terms ‘cellular process’, ‘metabolic process’ and ‘response to stimulus’ were significantly enriched and accounted for the most of biological process, indicating the putative involvement of circRNAs in regulating rice response to pathogen infection. KEGG pathway analysis showed that the host genes were significantly enriched in carbon fixation in photosynthetic organisms and glyoxylate and dicarboxylate metabolism (p.adjust < 0.05), followed by photosynthesis proteins and photosynthesis (Figure S4, Table S2).

To uncover whether the DEcircRNAs-producing genes possess any bacterial blight resistance function, the parental genes of all the DEcircRNAs were also predicted and annotated. The photorespiration and chloroplast-related terms (like chlorophyll biosynthetic process, chloroplast membrane, glycolate oxidase activity, thylakoid) were significantly enriched by DEcircRNA-producing genes, indicating their vital role in rice-pathogen interaction (Fig. 3B, Table S2). In addition, we found involvement of DEcircRNA-producing genes in regulation of ‘glyoxylate and dicarboxylate metabolism’, ‘arginine biosynthesis’ ‘autophagy’, and ‘peroxisome’ (Fig. 3C, Table S2), inferring the relevance between circRNAs and rice response to pathogen infection.

circRNA-miRNA-mRNA regulating network

Given the functional regulatory evidence of some circRNAs with binding capabilities to miRNAs (Sablok et al. 2016), we constructed the circRNA-miRNA-mRNA network in this study to reveal the potential role of these DEcircRNAs in post-transcriptional regulation. As a result, 31 circRNAs out of the 276 DEcircRNAs harbored one or more miRNA binding sites for 121 miRNAs (Table S3). Furthermore, target prediction showed 4192 mRNAs to be the potential targets of 36 miRNAs, including several conserved miRNAs like miR398, miR444 and miR812 (Table S4). Functional enrichment analysis was subjected to uncover any resistance functions possessed by the targeted mRNAs. The GO enrichment analysis suggested that the targeted mRNAs were involved in various stress-responsive, such as pattern specification process and gibberellin metabolic process (Table S5). The KEGG pathways revealed significant enrichment of diterpenoid biosynthesis (p.adjust < 0.05), which was followed by starch and sucrose metabolism and transcription factors (TFs) (Fig. 4A, Table S5). To predict the functions of circRNAs more accurately, circRNA-miRNA-mRNA regulating network corresponding to the top 3 enriched pathways was further constructed using Cytoscape (v3.8.0) (Fig. 4B). In this study, the predicted miRNA target mRNAs included various TFs, like MYB, ABF, WRKY and TGA, which were predicted to participate in plant-pathogen interaction and plant hormone signal transduction (Table S5).

Experimental validation of the identified circRNAs

To confirm these circRNAs detected in this study, PCR and Sanger sequencing were performed to validate the back-splicing sites of 7 randomly selected circRNAs. 7 pairs of convergent primers were used to detect linear transcripts from gDNA and cDNA as positive controls (Fig. 5). In contrast, all 7 pairs of circRNAs can only be amplified in cDNA samples, but not in gDNA samples (Fig. 5), suggesting the presence of the head-tail splicing junctions. PCR products with expected length of divergent primers were further verified to span the back-splicing junction by Sanger sequencing and sequence mapping (Fig. 5). Moreover, the expression of four DEcircRNAs were detected by RT-qPCR using cDNA of total RNAs as templates.

Obviously, in comparison with 0 h, the expression of all the four circRNAs were significantly up-regulated at different hpi (Fig. 6), which is basically consistent with the deep sequencing results, indicating that the circRNAs identified in our study are reliable (Fig. 6). The above results revealed that those identified circRNAs may participate in rice innate immunity or the regulation of rice bacterial leaf streak disease development.

Discussion

As an emerging potential class of regulatory RNAs, circRNAs have attracted the attention of many researchers. Widespread and various circRNAs have been detected in both animals and plants with the progress in the development of sequencing and bioinformatics technology (Kristensen et al. 2019; Zhao et al. 2019). Recent studies also highlighted the tight correlation between circRNAs and plant development and stress-induced responses (Li et al. 2017; Litholdo and da Fonseca 2018). In case of rice, current evidence has pointed out the potential biological roles of circRNAs in response to fertility transition (Wang et al. 2019), phosphate starvation (Ye et al. 2015) and fungus infection (Fan et al. 2020). However, a little is known regarding the regulation roles of circRNAs in rice defense responses against pathogens.

To obtain comprehensive and reliable circRNA predictions, two state-of-the-art tools, CIRCexplorer2 (Zhang et al. 2016) and CIRI2 (Gao et al. 2018), were adopted for circRNAs characterization in this study. Obviously, there are quite different in terms of the number of detected circRNAs and little overlap between the two prediction algorithms when they were adopted for rice circRNA libraries. Such differences are largely due to different algorithms possessing different advantages and shortcomings regarding the prediction accuracy and sensitivity of detecting circRNAs (Hansen et al. 2016), no relation to animal or plant species. For example, CIRCexplorer identified the most grape circRNAs, followed by find_circ and CIRI (Gao et al. 2019). While in *Arabidopsis thaliana*, find_circ identified the most circRNAs among the three methods (Zhang et al. 2020b), however, CIRI identified the most circRNAs when they were applied to human circRNA libraries (Zeng et al. 2017). Thus, it is necessary to combine several algorithms to achieve reliable and comprehensive predictions in both animal and plant species (Gao and Zhao 2018; Gao et al. 2019; Hansen et al. 2016). Several rice circRNA back-splicing sites and expression pattern were experimentally validated by divergent PCR combined with

Sanger sequencing and RT-qPCR, respectively. All these data further confirmed the reliability of prediction results in this study.

With respect to the characteristics of circRNAs identified in this study, 2303 (about 65.5 %) were homologous to the rice circRNA sequences in PlantcircBase database and 1214 were novel in rice. Consistent with previous studies, all these circRNAs derived from diverse genomic regions, and the exon circRNAs accounted for the largest proportion (Fan et al. 2020; Wang et al. 2019; Ye et al. 2015; Gao et al. 2019; Tong et al. 2018), which can be explained as the exon skipping events contributing to the most circRNA formation in these plants (Conn et al. 2017). However, most circRNAs were intergenic in kiwifruit (Wang et al. 2017) and potato (Zhou et al. 2017). In soybean, the highest proportion of intronic circRNAs occurred in root, while exonic in the stem and leaf (W. Zhao et al. 2017). Integrating these findings showed that plant circRNAs biogenesis are tissue- and species-specific. A recent study of cucumber also found that the circRNA numbers are significantly positive in correlation with chromosome length (Zhu et al. 2019), which is also confirmed by our results and previous study in rice (Wang et al. 2019), the longest chromosome in rice genome (chromosome 1) producing more circRNAs than others. Moreover, diverse non-GT/AG splicing signals, except the canonical GT/AG, were also used by the most circRNAs in our study, which is consistent with the results in rice (Ye et al. 2015) and cucumber (Zhu et al. 2019), and inconsistent with those in grape (Gao et al. 2019) and cotton (T. Zhao et al. 2017), inferring a circular preference of circRNAs processing in most plants and highlighting the complexity of back-splicing in rice.

Plant stress responses are very complex processes and involve many specific-inducible genes and signaling transduction pathways. We observed that PXO99^{A} infection could specifically induce the formation of some circRNAs in rice, of which the host genes were significantly related to various primary metabolic pathways, mainly involving photorespiration, chloroplast, and peroxisome-associated pathways (Table S2). Similar results were also indicated in the GO enrichment analysis for protein-coding genes in kiwifruit induced by bacterial canker pathogen invasion (Wang et al. 2017). More importantly, evidences from the literature have confirmed the involvement of these metabolic pathways in plant defense responses (Kangasjärvi et al. 2012; Rojas et al. 2014; Hofius et al. 2017; Mammarella et al. 2015). For instance, glycolate oxidase (GOX), the first enzymes in the photorespiratory pathway, could be induced in response to fungal pathogen in *Brassica napus*, *Arabidopsis* (Bohman et al. 2002) and *Hordeum vulgare* (Schäfer et al. 2004). While *gox* mutants of *Arabidopsis* and *Nicotiana benthamiana* showed susceptibility to non-host pathogens (Rojas et al. 2012). Chloroplast Cu/Zn superoxide dismutase (CSD2) participates in controlling ROS levels in infected tissues (Mateo et al. 2004). Peroxidases PRX33 and PRX34 play important roles in *Arabidopsis* basal resistance via regulating apoplastic ROS production (Daudi et al. 2012). As outlined above, the positive correlations between circRNAs expression and that of their cognate linear genes have been observed in many plant species, such as *Arabidopsis* (Ye et al. 2015), rice (Ye et al. 2015), kiwifruit (Wang et al. 2017), grape (Gao et al. 2019), etc. These findings confirmed our hypothesis that the DEcircRNAs have valuable functions in the

synthesis of metabolic processes through regulation of their parent genes expression during rice-PXO99^{A} interaction.

Besides cis-regulating parent genes, circRNAs have been proposed to sponge to specific miRNAs and to further prohibit them from regulating their target mRNAs (Sablok et al. 2016). Here, we found 31 DEcircRNAs containing one or more miRNA binding sites in circRNA-miRNA-mRNA network. Unlike the various functions of circRNAs producing genes, mRNAs predicted in this network were only significantly involved in diterpenoid biosynthesis, which has been confirmed to be relevant to rice plant defense against bacterial pathogen *Xoo* recently (Lu et al. 2018). One possible explanation was that miRNA sponges might not be the main mechanism for plant circRNAs (Chu et al. 2018). In addition, several conserved miRNAs were predicted to be the targets of certain rice DEcircRNAs, including miR156, miR398, miR414, miR444, and miR812, which are well known to regulate a wide range of developmental and physiological processes (Baldrich and San Segundo 2016; Kansal et al. 2015; Kruszka et al. 2012; Li et al. 2014; Wang et al. 2016). Particularly, rice plants overexpression miR398b showed enhanced resistance to *M. oryzae* by influencing H_2O_2 accumulation at the infection site and defense gene expression (Li et al. 2014). These results further implied that these circRNAs might make a regulatory contribution to rice immunity, whether control rice immunity via acting as miRNA sponges need experimental evidence in the future.

Conclusions

In this study, all these findings collectively confirmed rice circRNAs may also regulate rice defense against pathogens at multiple levels. Functional characterization of rice circRNAs and discovery of their interaction machinery between host genes or miRNAs will aid to develop appropriate strategies for rice protection.

Methods

Plant materials and pathogen inoculation

The rice cultivar Nipponbare (*Oryza sativa* L. ssp. *japonica*) seeds were soaked in water and placed in an incubator (28 °C) for 2 days. The germinated seeds were sown in pots containing nursery soil, placed in an illuminated growth chamber with a 14 h light/10 h dark cycle at 70% RH and 28-32 °C. One-month-old rice seedlings were inoculated with wild *Xoo* strain PXO99^{A} (laboratory collection) at 10^9 cfu ml^{-1} by the leaf-clipping method (Kauffman 1973). Briefly, the tips of rice leaves are cut with scissors previously dipped in bacterial suspension. Approximately 2 cm-length leaf fragments next to the inoculated sites were harvested from two stages: before inoculation (0 h, used as a control) and after inoculation (2, 6, 12 and 24 h), respectively. At each time point, samples were collected from at least eighteen different plants and immediately frozen in liquid nitrogen.

CircRNA-seq library preparation and sequencing

A total of 5 samples from 5 time points were harvested for library construction. The RNA-Seq library construction and sequencing were performed by the Novogene Corporation (Beijing). RNA purity and concentration were assayed by using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). The integrity of total RNAs was further evaluated by gel electrophoresis and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

A total amount of 5 μg RNA per sample was used for circRNA-seq library preparation. Ribosomal RNAs were depleted using an Epicentre Ribo-ZeroTM Kit (Epicentre Technologies Corp., Chicago, IL), whereas linear RNAs were digested with RNase R (Epicentre, USA). The remaining RNAs were broken into small fragments under elevated temperature and converted into double stranded DNAs. The cDNA fragments (250 ~ 300 bp in length) were purified and used for PCR amplification. Finally, PCR products were purified and libraries were quality-controlled using the BioAnalyzer 2100 system. The resulting libraries were sequenced using an Illumina HiSeq 2000 system and 150 bp paired-end reads were generated.

Identification of circRNAs

Low-quality reads ($Q \leq 20$) and adapters were removed from the sequencing data by Trim galore (https://github.com/FelixKrueger/TrimGalore) before circRNA identification. The remaining clean reads were mapped to the rice reference genome (IRGSP-1.0), generating a sequence alignment map file by bwa (v0.7.17, mem-T 19) with default parameters (Li and Durbin 2009). The output of BWA was used to identify the circRNAs by CIRIquant (V1.1) (Zhang et al. 2020a) . CIRI2 and CIRCexplorer2 are the two tools that were used in the pipeline (Gao et al. 2018; Zhang et al. 2016). One or more base differences might be present between the results of different prediction software programs. Then, repeated circRNAs in the prediction results were removed by CD-HIT-EST following a method proposed in a previous report (Fu et al. 2012; Zhang et al. 2020b): (1) a length difference between the two sequences less than 10 bp and (2) an alignment sequence exceeding 99.7% of the shorter sequence. The other CD-HIT-EST parameters were the default parameters.

Differential expression and annotation analysis

All the differentially expressed circRNAs (DEcircRNAs) were examined in the comparison after PXO99^{A} infection (2, 6, 12 and 24 h) versus 0 h using the CIRIquant (V1.1) with default parameters. In addition, the circRNAs that DE_score value equal to zero were removed by host python script. The functions of some DEcircRNAs may depend on their host genes; thus, the function of DEcircRNAs host genes were annotated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Gene enrichment analysis of these host genes were performed using clusterProfiler R package (Yu et al. 2012). All detected DEcircRNAs were compared against the PlantcircBase database with BLASTN. We did BLAST (with a threshold of E-value < 1E-5) for finding the conserved circRNAs using our identified DEcircRNAs as the query.

Prediction for circRNA-miRNA-mRNA relationships

The functions of some circRNAs may be independent of their host genes and may act as miRNA decoys to achieve their effects (Hansen et al. 2013). To construct the circRNA-miRNA-mRNA network, miRNA sequences were obtained from miRBase (http://mirbase.org/) and the plant microRNA database (PMRD: http://bioinformatics.cau.edu.cn/PMRD/) (Kozomara et al. 2019; Zhang et al. 2010), while mRNA sequences were downloaded from the rice annotation project database (https://rapdb.dna.affrc.go.jp/index.html). The DEcircRNAs sequences were extracted with an in-house python script. Then, the miRNAs identical alignment regions in circRNAs and mRNAs were predicted by GSTAr.pl (https://github.com/MikeAxtell/GSTAr), and the minimum free energy (MFE) of miRNA-circRNA or miRNA-mRNA duplexes was calculated with the RNAhybrid program. Then, the miRNA-targeted mRNA and miRNA-decoyed circRNA were predicted following a method proposed in a previous report (Tang et al. 2018; Ivashuta et al. 2011). The general criteria used to define a miRNA decoy were as follows: no more than six mismatched or inserted bases present between the 9^{th} to 20^{th} nucleotides of the miRNA 5' end, perfect matching of the 2^{nd} to 8^{th} bases of the miRNA 5' end sequence, and no more than four mismatches or indels in other regions. The circRNA-miRNA-mRNA regulating network was generated by Cytoscape (v3.8.0) (Shannon et al. 2003).

Validation of circRNAs and quantitative real-time PCR (RT-qPCR)

Genomic DNA was extracted from fresh rice leaves using Dzup (plant) Genomic DNA Isolation Reagent (Sangon Biotech, Shanghai). In accordance with the manufacturer's protocol, total RNA of all samples was isolated using EZ-10 Total RNA Mini-Preps Kit (Sangon Biotech, Shanghai) and reverse-transcribed into cDNA with a combination of random and oligo (dT) primers using the Hifair® III 1st Strand cDNA Synthesis Kit (gDNA digester plus) (YEASEN) in accordance with manufacturer's protocol.

To validate the identified circRNAs in this study, a pair of divergent primers and convergent primers (Table S6) were designed for each circRNA using Primer5 software, and both cDNA and gDNA (negative control) were used as template for polymerase chain reactions (PCRs). For each PCR amplification, 100 ng of cDNA or genomic DNA was used with TaKaRa Ex Taq ® DNA Polymerase. The PCR results were confirmed by agarose gel electrophoresis and Sanger sequencing.

The expression level of circRNA candidates in rice leaves were experimentally detected by RT-qPCR with Magic SYBR Green qPCR Mix (Magic Biotech, Hangzhou, China) and the ABI 7500 quantitative PCR system (Applied Biosystems, Foster City, CA). Data were calculated using the $2^{-\Delta\Delta CT}$ method and were normalized to the expression of the reference gene *OsActin*. Each assay was performed three times in triplicate and the results are displayed as the means ± SD of three biological replicates.

Statistical Analysis

Statistical analysis was done by GraphPad Prism 8.0, significant values are marked with asterisks (*P < 0.05, **P < 0.01, and ***P < 0.001).

Abbreviations

Xoo: *Xanthomonas oryzae* pv. *oryzae*; CircRNAs: Circular RNAs; PTI: Pattern-triggered immunity; PAMPs: Pathogen-associated molecular patterns; PRRs: Pattern recognition receptors; ETI: Effector-triggered immunity; ncRNAs: Non-coding RNAs; DEcircRNAs: Differentially expressed circRNAs; RNA-seq: RNA sequencing; Hpi: Hour post inoculation; KEGG: Kyoto encyclopedia of genes and genomes; GO: Gene Ontology; RT-qPCR: Real-time quantitative PCR.

Declarations

Acknowledgements

Not applicable.

Authors' Contributions

Conceived and designed the research: CGY, ZB, WPH, WS. Performed the experiments: WPH, WY, NWH, Ayizekeranmu YM, LJL. Analyzed the data and transcriptomes: WPH, WS. Contributed reagents/materials/analysis tools: ZB. Drafted the manuscript: WPH. Revised the paper: WPH, Iftikhar A, FLY, GLB. All authors read and approved the final manuscript.

Funding

This work was supported by National Key R&D Program of China (2018YFD0201202, 2017YFD0201108), the Agri-X Interdisciplinary Fund of Shanghai Jiao Tong University (Agri-X2017010), the State Key Laboratory for Biology of Plant Diseases and Insect Pests (SKLOF201802) and Shanghai Committee of Science and Technology (19390743300). The funders had no role in study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

Availability of Data and Materials

All data supporting the conclusions of this article are provided within the

article (and its additional files). RNA-seq data generated in this study were deposited in NCBI with bioproject number (PRJNA674954).

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Conflict of Interests

The authors declare no conflict of interests.

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Figures

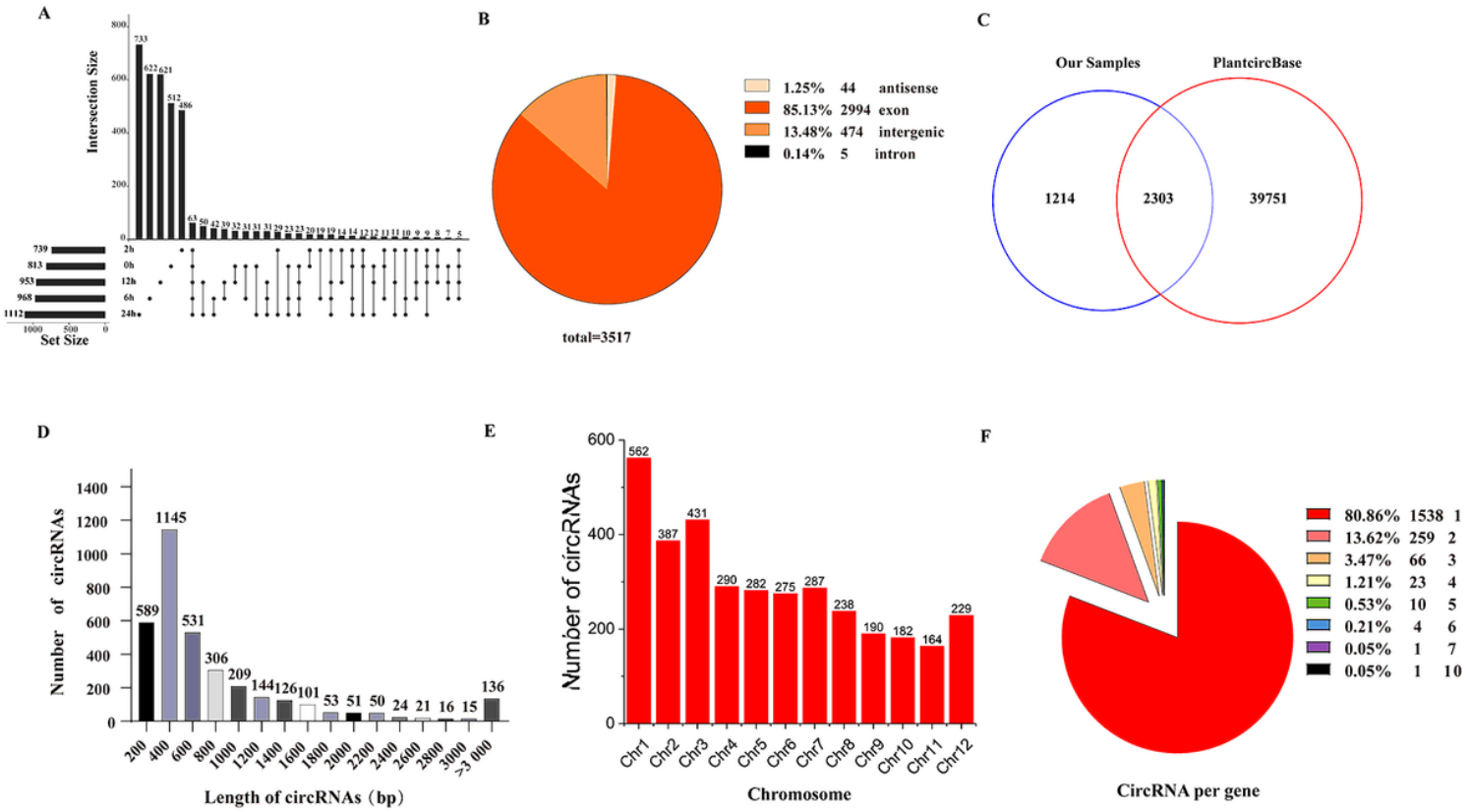

Figure 1

Genome-wide analysis of circular RNAs (circRNAs) in Xoo-infected rice leaves. A. UpSet showing the number of detected circRNAs for the 5 samples. The combination matrix identifies the intersections, while the bars above it encode the size of each intersection (number). B. Classification of the identified circRNAs. C. Venn diagrams of the circRNAs detected in this study and all rice circRNAs collected in the database “PlantcircBase”. D. The length distribution of identified circRNAs. E. The distribution of detected circRNAs in each chromosome. F. Number of circRNAs produced from one gene.

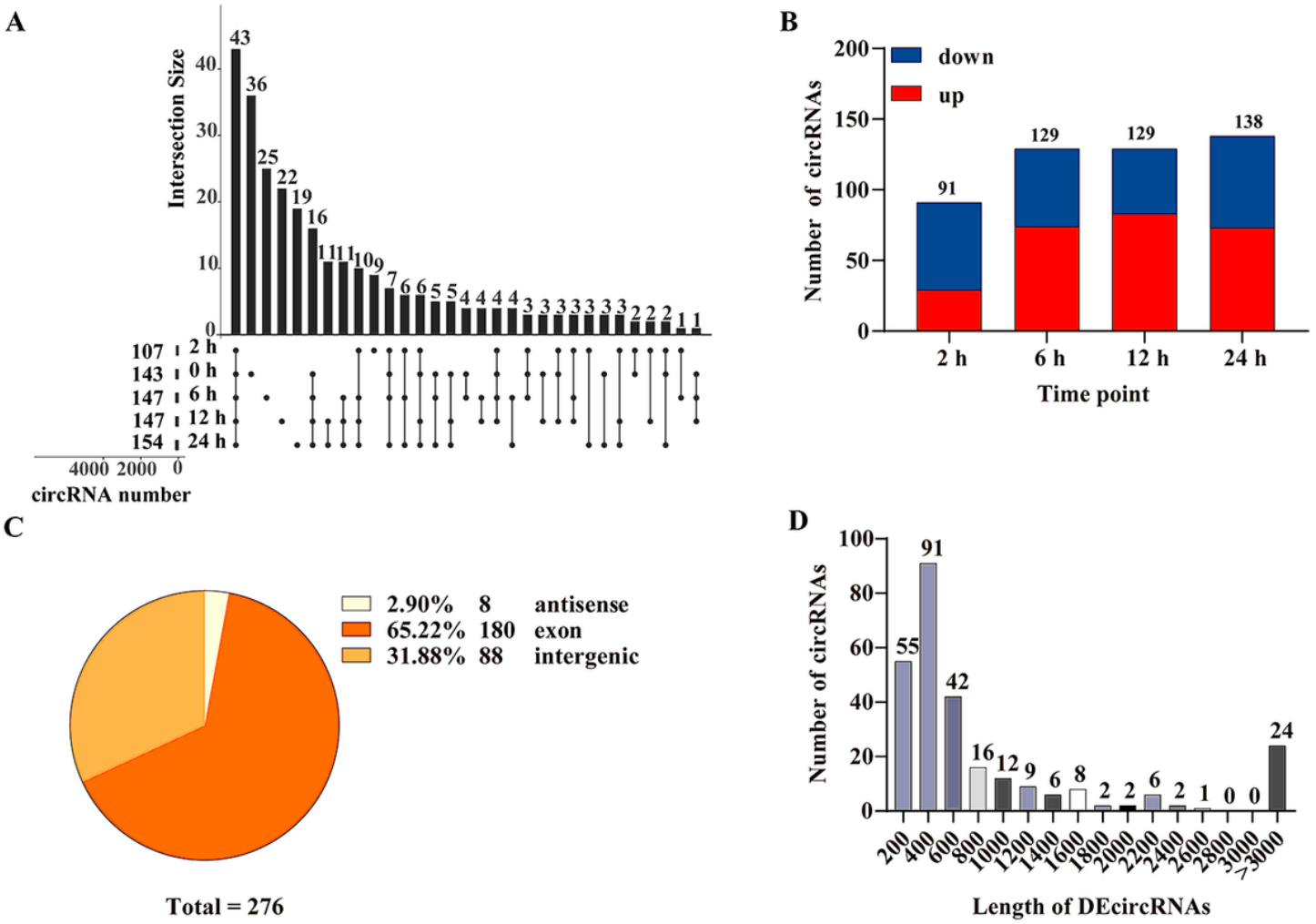

Figure 2

Expression analysis of DEcircRNAs. A. UpSet showing the number of DEcircRNAs at 5 time points. The combination matrix identifies the intersections, while the bars above it encode the size of each intersection (number). B. Number of differentially expressed circRNAs. C. Classification of the identified DEcircRNAs. D. Length distribution of rice DEcircRNAs.

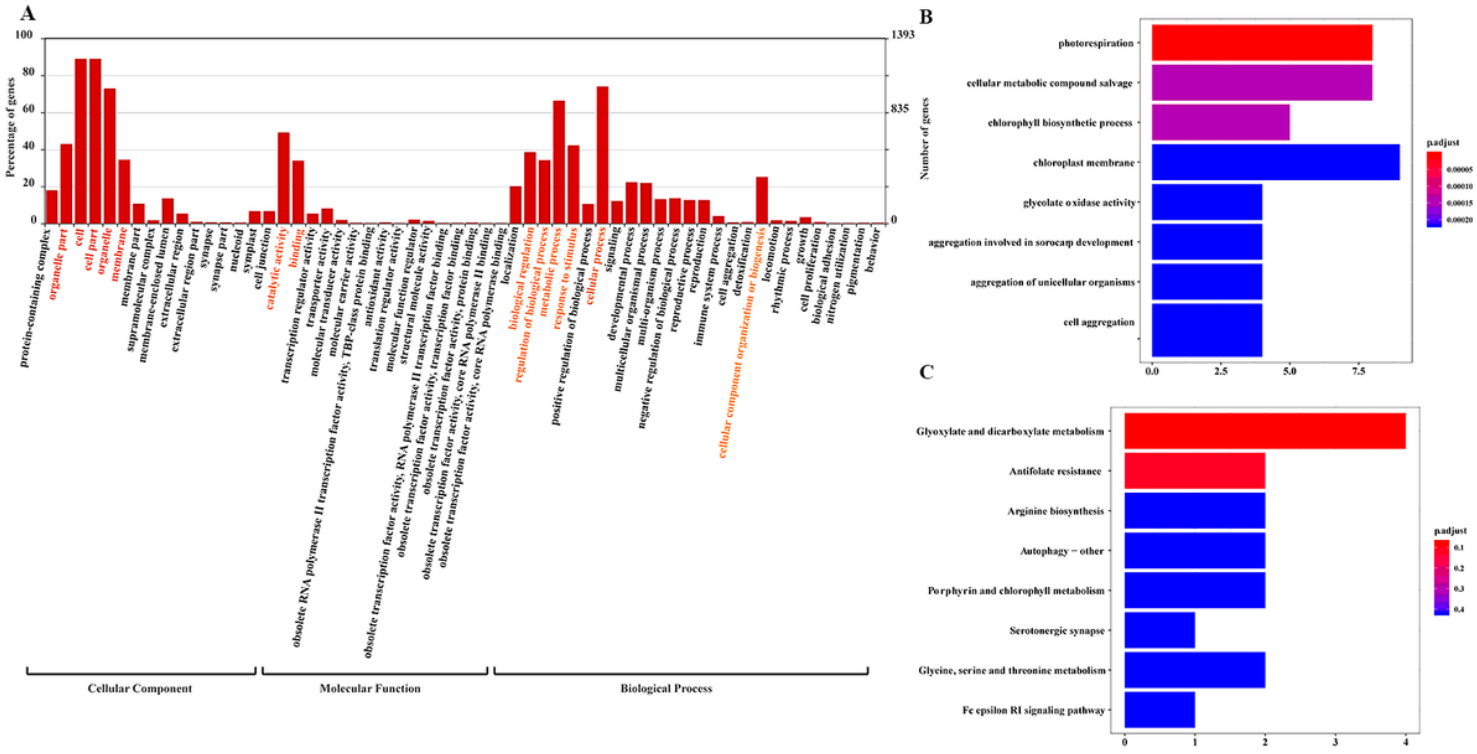

Figure 3

GO enrichment and KEGG pathway analysis for the host genes producing circRNAs. A. GO terms of all host genes producing circRNAs. B and C. GO and KEGG pathways analysis of host genes producing DEcircRNAs, respectively.

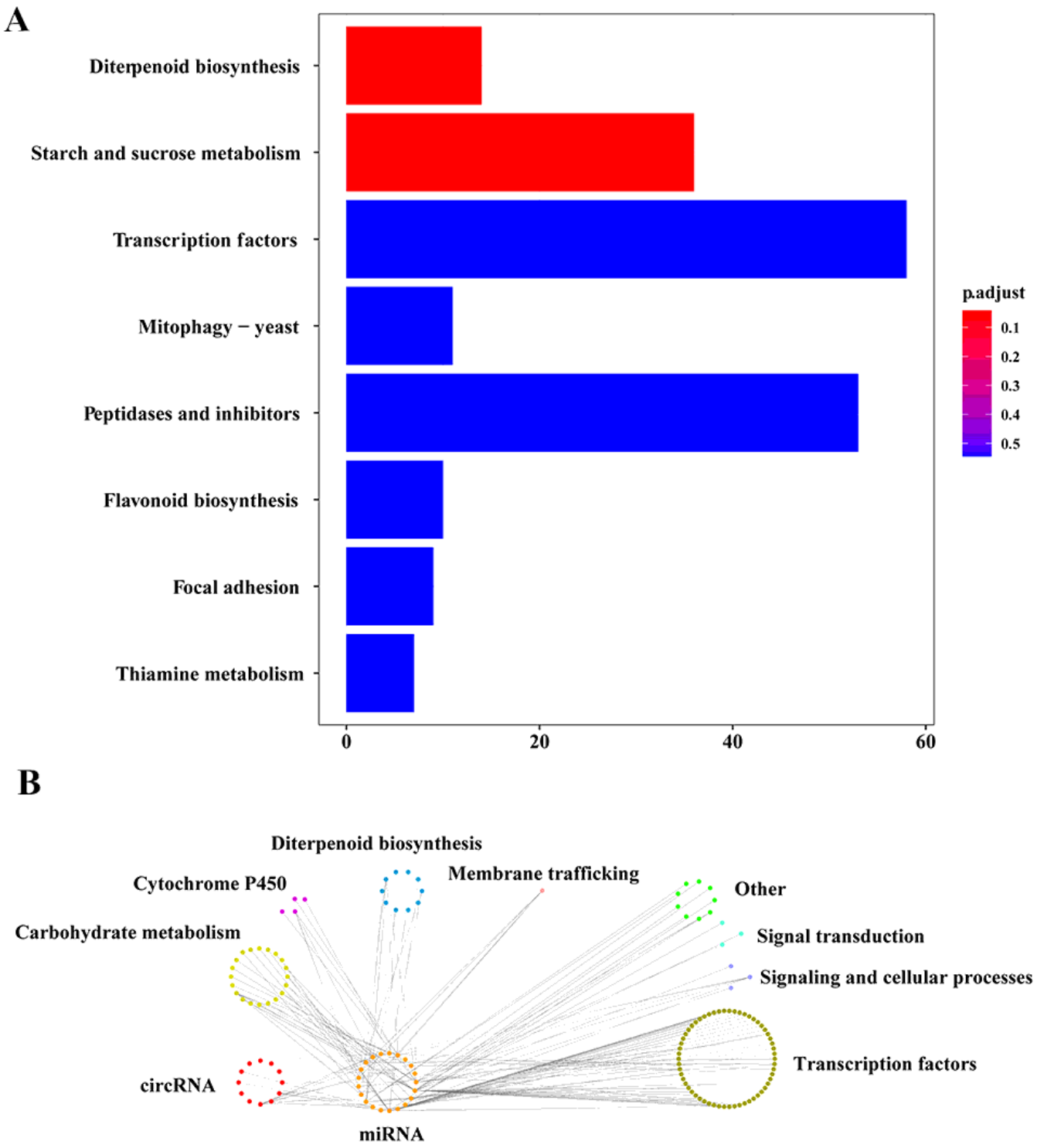

Figure 4

Possible regulatory networks involved in rice-Xoo interaction. A. The top 8 enriched pathways of target mRNAs corresponding to miRNAs sponged by DEcircRNAs. B. The networks include differentially expressed circRNAs and their target genes. Red nodes represent the circRNAs, orange nodes represent the miRNAs, and other colored nodes represent mRNAs, respectively. Detailed information referring to relationship among circRNAs, miRNAs, and mRNAs, and annotation of mRNAs were listed in Table S5.

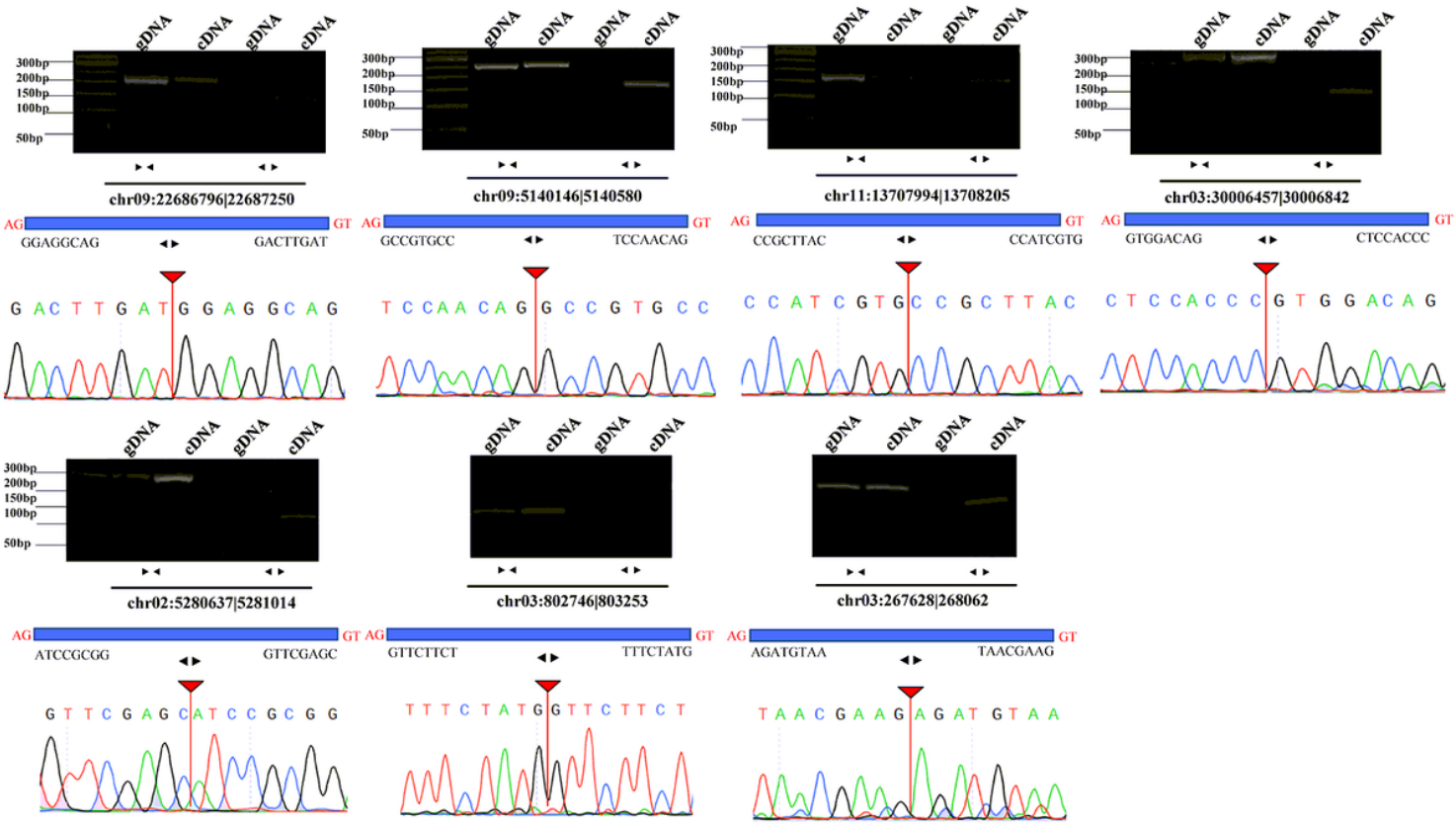

Figure 5

Seven representative circRNAs validated in rice leaves by PCR amplification and Sanger sequencing. For each circRNA, (Top) gel electrophoresis of PCR products using divergent primers and convergent primers in gDNA and cDNA samples, (Bottom) sanger sequence validation using divergent primers.

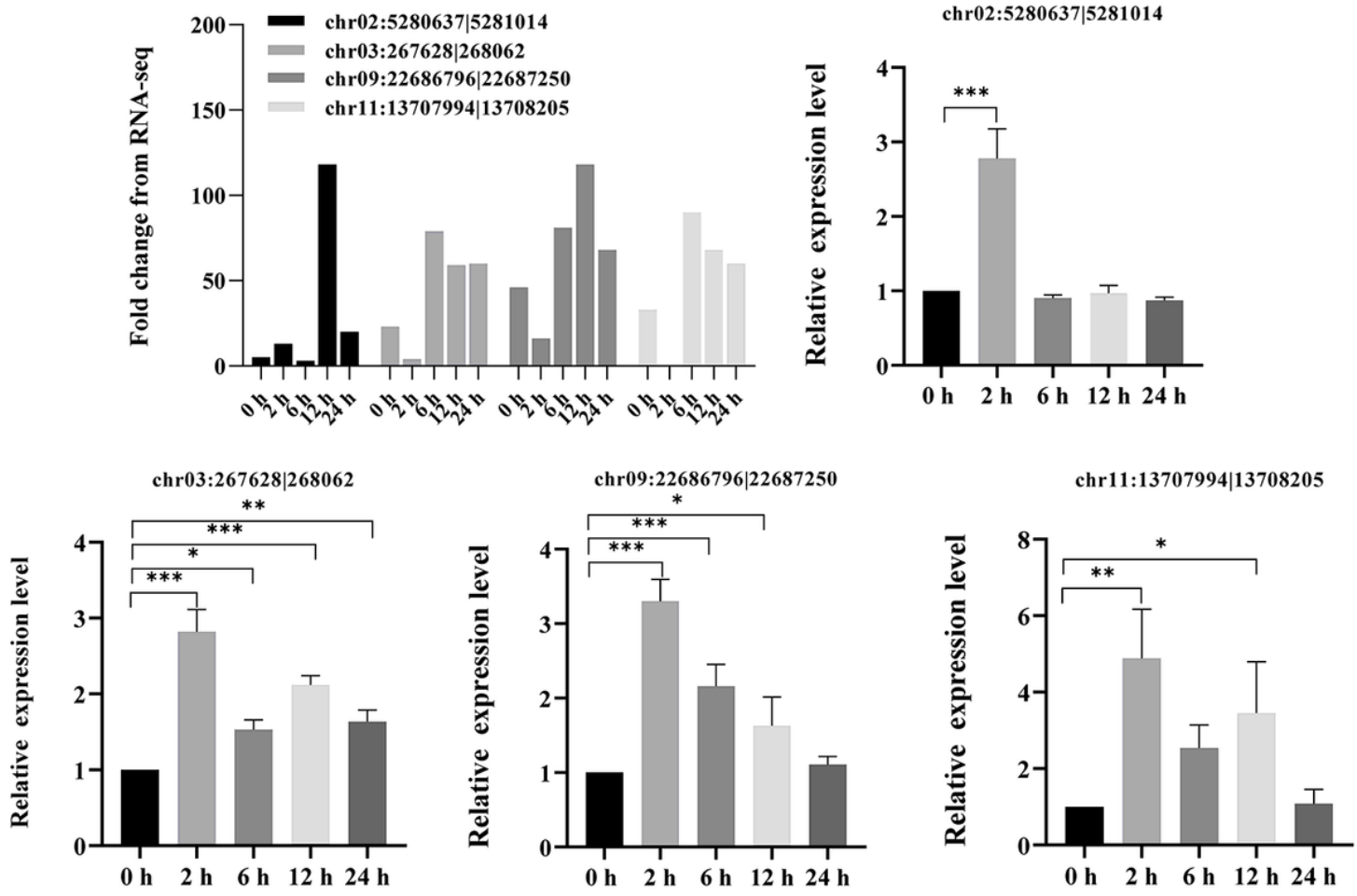

Figure 6

Expression patterns of the different genes between the RNA-seq and RT-qPCR. For RNA-seq, no expression signal of chr11:13707994|13708205 was detected at 2 hpi. For RT-qPCR, OsActin2 was used as an internal reference gene and the expression levels of 0 h were set to 1 (as the control). Results are expressed as the mean ± SD of three biological replicates. Error bars indicate SD (n = 3). Significant values are marked with asterisks (*P < 0.05, **P < 0.01, and ***P < 0.001).

Supplementary Files

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