CRISPR-Cas9 mediated OsMIR168a knockout reveals its pleiotropy in rice

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Summary

MicroRNA168 (MIR168) is a key miRNA that targets the main RNA-induced silencing complex component Argonaute 1 (AGO1) to regulate plant growth and environmental stress responses. However, the regulatory functions of MIR168 need to be further elucidated in rice. In this paper, we generated clean OsMIR168a deletion mutants by CRISPR-Cas9 strategy. We then phenotypically and molecularly characterized these mutants. The rice OsMIR168a mutants grew rapidly at the seedling stage, produced more tillers and matured early. Compared to the wild-type plants, the mutants were shorter at maturity and produced smaller spikelets and seeds. Analysis of gene expression showed that the transcription levels of OsMIR168a’s target genes such as OsAGO1a, OsAGO1b and OsAGO1d were elevated significantly in the OsMIR168a mutants. Intriguingly, OsAGO18, a member of a new AGO clade that is conserved in monocots, was confirmed to be a target of OsMIR168a not only by informatic prediction but also by expression analysis and a cell-based cleavage assay in the OsMIR168a mutants. Many protein-coding genes and miRNAs showed differential expression in the OsMIR168a mutants, suggesting OsMIR168a exerts a major transcriptional regulatory role, likely through its potential target genes such as OsAGO1s and OsAGO18. KEGG enrichment analysis of these differentially expressed genes pointed to OsMIR168a’s involvement in important processes such as plant hormone signalling transduction and plant–pathogen interaction. These data collectively support that the complex regulation module of OsMIR168a-OsAGO1/OsAGO18-miRNAs-target genes contributes to agronomically important traits, which sheds light on miRNA-mediated crop breeding.

Introduction

MicroRNAs (miRNAs) are non-coding RNA of 19–24 nt in eukaryotes. They target messenger RNAs (mRNAs) through base-pairing, which result in mRNA degradation or translation inhibition (Voinnet, 2009). Plant miRNA genes are mainly present in intergenic and intronic regions. They are transcribed by RNA Polymerase II (Pol II) to primary miRNAs (pri-miRNAs), processed to precursor miRNAs (pre-miRNAs) by enzymes such as DCL1, HYL1 and SE. Pre-miRNAs are transported from nucleus to the cytoplasm by HASTY. In the cytoplasm, pre-miRNAs are cleaved near the loop into the small dsRNAs (one is guide and the other is passenger strand) by Dicer. Upon methylation by HEN1, the miRNA duplex is processed by RNA-induced silencing complex (RISC), and the resulting single guide stranded miRNA will complex with AGO for mRNA targeting. The passenger strand is destined for exit (Bartel, 2018; Voinnet, 2009). It has been well established that miRNAs are involved in nearly all aspects of plant biology such as growth and development, epigenic inheritance and responses to biotic and abiotic stress (Pan et al., 2018; Sunkar and Zhu, 2004; Tang and Chu, 2017; Wang et al., 2021c; Wu et al., 2017; Zhao et al., 2020b).

MIR168 is one conserved miRNA present in various plant species. It regulates plant growth and development (Vaucheret et al., 2004; Wang et al., 2021a; Xian et al., 2014; Zhou et al., 2020). It has also been found to participate in plant response to abiotic stress (Li et al., 2012; Liu et al., 2020b) and biotic stress such as virus (Iki et al., 2018; Varallyay et al., 2010) and fungus (Li et al., 2014; Wang et al., 2021a). MIR168’s broader roles in plants can be explained by the fact that it targets Argonaute 1 (AGO1) which encodes the core component of the RISC (Mallory et al., 2008; Vaucheret, 2008; Voinnet, 2009). AGO1 binds miRNAs and post-transcriptionally represses target genes in the cytoplasm. Previously, increased accumulation of miRNA target genes was found in ago1 mutants (Kurihara et al., 2009). More recently, AGO1 was found to regulate transcription of a small subset of miRNAs (Dolata et al., 2016). AGO1 can bind to the chromatin of actively transcribed genes to further promote their transcription in Arabidopsis (Liu et al., 2018). In addition, AGO1 is involved in the regulation of pathogen defence responses in Arabidopsis (Morel et al., 2002; Zhang et al., 2006). The rice AGO1 family contains four members: AGO1a, AGO1b, AGO1c and AGO1d (Kapoor et al., 2008). Overexpression of AGO1b induced adaxially rolled leaves by affecting leaf abaxial
sclerenchyma cell development in rice (Li et al., 2019). However, it remains unclear whether all these AGO family members and/or other AGO members are bona fide targets of MIR168 in rice.

CRISPR-Cas based genome editing technology presents a powerful genetic knockout approach to study the function of coding genes and non-coding genes in plants (Bi et al., 2020; Cheng et al., 2021; Liu et al., 2020a; Miao et al., 2019, 2020; Ren et al., 2019, 2021; Tang et al., 2019; Wang et al., 2019; Zhong et al., 2019, 2020; Zhou et al., 2021). Previously, we successfully applied CRISPR-Cas9 to knock out multiple miRNAs in rice, which provided multiple insights in miRNA genesis and complex regulation (Zhou et al., 2017). A lesson learned from that study is that larger deletions, rather than 1 bp insertions and deletions (InDels), are required to robustly knock out miRNAs (Zhou et al., 2017). While a target mimic knockdown strategy was recently used for studying MIR168 function in rice (Wang et al., 2021a), it would be more desirable to create independent MIR168 deletion mutants for investigating the loss of function of this important miRNA. In this study, we used CRISPR-Cas9 to generate OsMIR168a deletion mutants in rice. Characterization of the resulting OsMIR168a mutants not only validated previous observations based on target mimic, but also shed new light into MIR168a-centred global transcription regulation that involves different pathways and underlines its pleiotropy.

Results

Creation of the OsMIR168a mutants with CRISPR-Cas9

There are two OsMIR168 members in rice: OsMIR168a and OsMIR168b. According to miBase (http://www.mirbase.org/), mature OsMIR168a-5p and OsMIR168b differ at four nucleotides (Figure 1a). More importantly, OsMIR168a is far more abundant than OsMIR168b (Figure 1b; Kozomara et al., 2019), suggesting OsMIR168a is likely to play a more important role than OsMIR168b. Furthermore, OsMIR168b is encoded by the reverse complementary sequence of the second exon of OsAGO1a (LOC_Os02g45070; Figure S1), making genetic knockout of OsMIR168b very complicated as it will also lead to OsAGO1a knockout. For these two reasons, we decided to focus our study on OsMIR168a in rice. Re-analysis of OsMIR168a expression data set (Gurjar et al., 2016) showed its relatively ubiquitous expression across different growth stages and tissue types (Figure S2). A recent study identified OsAGO1 as one major target of OsMIR168a (Wang et al., 2021a). Our in silico analysis suggested that OsAGO1b is also a putative target of OsMIR168a. We analysed the expression of five AGO genes (OsAGO1a, OsAGO1b, OsAGO1c, OsAGO1d and OsAGO1e) according to http://rice.plantbiology.msu.edu/ (Kawahara et al., 2013). All five genes are highly expressed in pistil tissue, while OsAGO1c and OsAGO1d are highly expressed in 25-day old embryo and pre-emergence inflorescences respectively (Kawahara et al., 2013; Figure S3). These expression patterns are negatively correlated with OsMIR168a expression, supporting that these OsAGO genes are targets of OsMIR168a.

To investigate the MIR168a-AGOs regulation module in rice, we decided to create OsMIR168a knockout mutants. Two sgRNAs were designed to create large deletions at the OsMIR168a locus (Figure 1c). A multiplexed CRISPR-Cas9 T-DNA vector (pZIP903) was generated (Figure 1d). In this vector, the Cas9 gene was expressed under a maize ubiquitin 1 promoter (pZmUbi1) and the two sgRNAs were each expressed under a rice U6 (pU6) promoter. We conducted Agrobacterium-mediated stable transformation of rice with this construct. Twelve independent T0 transgenic lines were analysed by PCR-SSCP (Zheng et al., 2016; Figure S4) and Sanger sequencing. Among them, ten (83.3%) lines carried mutations at the sgRNA01 target site, and nine (72%) lines carried biallelic mutations at this site; ten (83.3%) lines carried mutations at the sgRNA02 target site, and ten (83.3%) lines carried biallelic mutations at this site; three (25.0%) lines contained large chromosomal deletions due to the simultaneous cleavage by both sgRNAs (Figure 1e). We followed two biallelic lines (pZIP903-2-1 and pZIP903-5-1 were designated as osmir168a_M02 and osmir168a_M05 respectively; Figure S4) that contained large deletions to the T1 generation. Mutation segregation patterns all followed Mendelian inheritance (Figure 1f). Within the T1 population, we identified two independent OsMIR168a deletion lines (m02, m05) that carried homoyzogous chromosomal deletion alleles with deletion sizes of 71 and 33 bp respectively. Importantly, these lines were transgene-free as the CRISPR-Cas9 transgene was segregated out (Figure 1g). RNAfold prediction based on a web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) showed that the pre-miR168a stem loop was destroyed in these mutant lines (Figure S5), suggesting they are bona fide knockout mutants for OsMIR168a. These transgene-free T1 deletion mutants were subsequently propagated for producing sufficient T2 seeds that were used for all the following experiments.

Phenotypic analysis of the OsMIR168a mutants

We first conducted phenotypic analysis of the two OsMIR168a deletion mutants. Both mutants showed comparable germination rates to the wild-type (WT) plants (Figure S6a). However, the mutants grew much faster than the WT plants at the seedling stage, making them taller than the WT plants (Figures 2a,b and S6b). Coincident with early flowering, the growth of the mutants drastically slowed down upon plant maturation, resulting slightly shorter stature when compared to the WT plants (Figure 2c,d). The mutants produced 3–4 more panicles than the WT plants (Figure 2e), although these panicles were generally smaller than the WT (Figure 2f). Further analysis showed these mutants had reduced spike length (Figure 2g), grain number per spike (Figure 2h), seed length (Figure 2i,j) and seed width (Figure 2i, k). The smaller seed size for the mutants (Figure 2j,k) translated to reduced 1000-grain weight (Figure 2l). The OsMIR168a knockout phenotype except for faster growth at the seedling stage is similar with the phenotype reported recently with a target MiRNA mimic strategy to partially down-regulate the OsMIR168a’s expression (Wang et al., 2021a).

Expression profiling of the OsMIR168a mutants

The pleiotropic phenotype that we observed in the OsMIR168a mutants suggests a critical role for MIR168a in plant growth and development. To get a better picture of the transcriptional regulation landscape shaped by OsMIR168a, we conducted RNA sequencing (RNA-seq) and small RNA-seq experiments, which were tailored for profiling mRNAs and small RNAs respectively. For RNA-seq, we obtained over 38 million clean reads for each sample, which mapped to about 30,000 genes (Table S1, S2), indicating high coverage. We identified 763 genes (2.54% of total protein-coding genes) that showed significant differential gene expression (P-value < 0.01) in the OsMIR168a mutants when compared to the WT (Table S3). For small RNA-seq, we obtained...
over 5 million reads per sample. About 900 small RNAs were identified in each sample, including ~450 known miRNAs and ~420 novel miRNAs (Tables S4 and S5). A total of 119 miRNAs (~13.3% of total miRNAs) showed differential gene expression between the mutants and the WT plants (P-value < 0.01; Table S6). These results suggest loss of function for OsMIR168a affects the expression of many protein-coding and miRNA genes in rice.

Based on small RNA-seq, about 3500 transcripts per million (TPM) were detected for OsMIR168a (OsMIR168a-5p) in the WT

Figure 1 Creation of OsMIR168a deletion mutants in rice with CRISPR-Cas9. (a) Stem-loop structures of OsMIR168a and OsMIR168b. The mature miRNAs are highlighted in blue and pink. The different nucleotides between mature OsMIR168a-5p and OsMIR168b are indicated in red. (b) The expression level of mature OsMIR168 according to miRbase, http://www.mirbase.org/. (c) The sgRNA01 and sgRNA02 were designed to target the pre-miR168a stem-loop sites. (d) Schematics for the multiplexed CRISPR-Cas9 T-DNA vector (pZJP093) for targeted deletion of the mature OsMIR168a. The Cas9 gene is expressed under a maize ubiquitin 1 promoter (pZmUbi1), and the sgRNAs are each expressed under a rice U6 promoter (pU6). (e) Summary of mutation frequencies at the OsMIR168a locus in the T0 lines. (f) Heredity test of the mutations at the OsMIR168a locus in the T1 generation. (g) The genotypes of two OsMIR168a deletion mutants (m02 and m05) which are transgene-free.
plants, but none in both OsMIR168a mutants (Figure 3a). In addition, a high level of OsMIR168a* (OsMIR168a-3p) was also detected in the WT plants, but not in the OsMIR168a mutants (Figure 3a; Table S6). It is surprising that the expression level of pri-miR168a (also annotated as a putative protein-coding gene, LOC_Os02g03700) was significantly up-regulated in the OsMIR168a deletion mutants (Figure S7). The reason for this interesting phenomenon is unclear and needs to be studied in the

Figure 2 Phenotypic analysis of the OsMIR168a mutants. (a) The OsMIR168a mutants grew faster than WT at the seedling stage. Bar = 5 cm. (b) Seedling length of the OsMIR168a mutants. Bar = 20 cm. (c) Quantification of panicle number. (d–g) Spike length of the OsMIR168a mutants, Bar = 5 cm. (h) Grain number per spike. (i left, j) Seed length of the OsMIR168a mutants. (i right, k) Seed width of the OsMIR168a mutants. Bar = 1 cm. (l) 1000-grain weight analysis. Statistical significance is indicated by asterisks (** indicative of a P-value < 0.01 by the student’s t-test).

Figure 3 Elevated expression of OsMIR168a target genes in the rice OsMIR168a mutants. (a) Transcript levels of OsMIR168a-5p and OsMIR168a-3p in the WT and the OsMIR168a mutants according to the small RNAseq experiment. (b–f) The expression profiles of OsAGO1a, OsAGO1b, OsAGO1c, OsAGO1d and OsAGO18, respectively, based on the mRNA transcriptome data and qPCR. Statistical significance is indicated by asterisks (** indicative of a P-value < 0.01 by the student’s t-test).
future. These data suggest that CRISPR-Cas9 induced large deletions in both mutants indeed eliminated OsMIR168a from the transcriptome, confirming that they are null alleles. We could not detect OsMIR168b in both the mutants and the WT plants, further supporting that OsMIR168b is unlikely to play any important role in rice.

We next investigated the target genes and putative target genes of OsMIR168a by analysing the RNA-seq data. In both OsMIR168a mutants, the expression levels of most OsAGO1 genes (OsAGO1a, OsAGO1b and OsAGO1d) were significant elevated, except for OsAGO1c where the elevation was not statistically significant (Figure 3b–e; Tables S7 and S8). These results are generally consistent with the recent report that used miR168a mimic (Wang et al., 2021a). In addition, we found that the expression level of OsAGO18 was also higher in both mutants than in the WT (Figure 3f; Tables S7 and S8), supporting that OsAGO18 is also a target of OsMIR168a. We further validated these results by quantitative reverse transcription PCR (qRT-PCR; Figure 3b–f). By contrast, analysis of RNA-seq data on the additional 14 OsAGO or OsAGO-like genes did not reveal any altered expression in the OsMIR168a mutants when compared to the WT plants (Figure S8; Table S8), suggesting that they are not targets of OsMIR168a. Together, these results support OsMIR168a-OsAGO1 and OsMIR168a-OsAGO18 regulation modules in rice. Basically, OsMIR168a negatively regulates OsAGO1a, OsAGO1b, OsAGO1d and OsAGO18, presumably through mRNA cleavage.

To further validate the target genes of OsMIR168a, we conducted a cell-based assay using a GFP reporter. In this reporter system, the OsMIR168a binding site in the OsAGO genes (OsAGO1a, OsAGO1b, OsAGO1c, OsAGO1d and OsAGO18) was fused to a GFP sequence (Figure 4a). Expression of this reporter alone in rice protoplasts resulted in GFP signals (Figure 4b). As with the GFP control construct (Figure 4g), however, such GFP signals disappeared when the GFP reporter was co-expressed with an OsMIR168a over expression construct (Figure 4b–f). All these data provide another line of evidence supporting that these OsAGO genes including OsAGO1c are targeted by OsMIR168a in rice cells.

Expression analysis of putative rice miRNAs targeting OsAGO1 family and OsAGO18 in the OsMIR168a mutants

To see whether there are additional miRNAs that may target OsAGO1 family genes and OsAGO18, we explored an online prediction tool (Lin et al., 2020). Using a parameter with interaction energy < –16 kcal/mol, we identified 40, 39, 66, 71 and 70 miRNAs that putatively target OsAGO1a, OsAGO1b, OsAGO1c, OsAGO1d and OsAGO18 in rice respectively (Tables S9–S14). Many of these miRNAs were differentially expressed in the OsMIR168a mutants. For instance, for the miRNAs putatively targeting OsAGO1a (Tables S9 and S10), one (OsMIR6251) was up-regulated (Figure S9A) and two were down-regulated including OsMIR166g-5p (Figure S9B; Table S10). For the miRNAs putatively targeting OsAGO1b (Tables S9 and S11), three were up-regulated (one of them is OsMIR2111b; Figure S9C; Table S11) and four were down-regulated (including OsMIR167e-3p and OsMIR167i-3p; Figure S9D; Table S11). For the miRNAs putatively targeting OsAGO1c (Tables S9 and S12), three were up-regulated (including OsMIR3966-5p; Figure S9E; Table S12) and four were down-regulated (including OsMIR166d-5p; Figure S9F; Table S12). For miRNAs putatively targeting OsAGO1d (Tables S9 and S13), two were up-regulated (including OsMIR2111b; Figure S9C; Table S13) and four were down-regulated (Table S13). For miRNAs putatively targeting OsAGO18 (Tables S9 and S14), two were up-regulated (including OsMIR29867-3p; Figure S9G; Table S14) and three were down-regulated (including OsMIR529a; Figure S9H; Table S14). For these miRNAs whose expression levels were up-regulated in the OsMIR168a mutants, it is less likely that the predicted OsAGO genes tested here are their true targets. After all, expression of the corresponding OsAGO1 genes and OsAGO18 was elevated in the OsMIR168a mutants. For the miRNAs whose expression levels were down-regulated in the OsMIR168a mutants, it is possible that they may target the corresponding OsAGO genes. Compared to these miRNAs, OsMIR168a may simply have larger effect on regulating the OsAGO1 and OsAGO18 expression. Hence, it remains to be investigated whether such additional miRNA-AGO1/AGO18 regulation modules exist and exert some functional relevance for the phenotype that we observed in the OsMIR168a mutants.

Differential expression of miRNAs and their target genes with implications for the OsMIR168a mutant phenotype

To further investigate the molecular basis for the growth and development phenotypes of the rice OsMIR168a mutants, we examined additional differentially expressed miRNAs in the OsMIR168a mutants that do not appear to target OsAGO1 genes or OsAGO18 (Tables S6 and S7). Interestingly, we identified 13 such miRNAs whose expression was negatively correlated with their predicted target genes (Table S7). Among them is OsMIR172d-5p, whose expression was decreased in both OsMIR168a mutants (Figure 5a). Correspondingly, three predicted target genes of OsMIR172d-5p, namely OsNF-YA8 (LOC_Os10g25850), LOC_Os10g08530 and LOC_Os04g12430, were up-regulated (Figure 5a). In Arabidopsis, NF-YA8 is involved in the transition from juvenile to adult (Zhao et al., 2020a). It is yet to be tested whether OsNF-YA8 plays a similar role in rice. By contrast, the expression level of OsMIR396e-5p was elevated in the OsMIR168a mutants (Figure 5b). Its target gene, OsGRF6, was down-regulated (Figure 5b). The OsMIR396-OsGRF6 regulation module was previously reported to impact inflorescence architecture in rice (Gao et al., 2015; Tang et al., 2018b) and hence may partly explain the phenotypes of the OsMIR168a mutants (Figure 2). Additionally, the expression level of OsMIR162a was up-regulated in the OsMIR168a mutants (Figure 5c), and its target genes, OsMET1b and OsCIPK28, were down-regulated (Figure 5c). As MET1b encodes a DNA methyltransferase that is mainly responsible for maintaining CG methylation after DNA replication (Lei et al., 2014; Ning et al., 2020), it is possible that DNA methylation is affected in the OsMIR168a mutants.

KEGG enrichment analysis of differentially expressed genes in the OsMIR168a mutants

Our above analyses suggest a large-scale transcription alteration caused by OsMIR168a knockout in rice. To understand the potential phenotypical consequences of these transcription profile changes, we did a KEGG enrichment analysis of differentially expressed miRNAs in the OsMIR168a mutants (Figure 6a; Table S15). The top five most affected processes are plant–pathogen interaction (30 genes affected), starch and sucrose metabolism (19 genes affected), plant hormone signalling transduction (16 genes affected), MAPK signalling pathway (15 genes affected), and 70 miRNAs that putatively target OsAGO1c (Tables S9 and S10), one (OsMIR6251) was up-regulated (Figure S9A) and two were down-regulated including OsMIR166g-5p (Figure S9B; Table S10). For the miRNAs putatively targeting OsAGO1b (Tables S9 and S11), three were up-regulated (one of them is OsMIR2111b; Figure S9C; Table S11) and four were down-regulated (including OsMIR167e-3p and OsMIR167i-3p; Figure S9D; Table S11). For the miRNAs putatively targeting OsAGO1c (Tables S9 and S12), three were up-regulated (including OsMIR3966-5p; Figure S9E; Table S12) and four were down-regulated (including OsMIR166d-5p; Figure S9F; Table S12). For
affected) and DNA replication (14 genes affected; Figure 6a; Table S15). For hormone pathways, we found multiple auxin pathway genes were down-regulated, while some genes involved in the cytokinin and ABA pathways were up-regulated (Figures 6b,c and S10). For the genes annotated to be involved in plant-pathogen interaction, there were more genes down-regulated than those up-regulated (Figures 6d,e and S11).

Discussion

A deep understanding of miRNAs will shed light on their complex regulatory networks and miRNA-based crop breeding. Suppression the function in miRNAs can be achieved by the short tandem target mimic (STTM) strategy (Franco-Zorrilla et al., 2007; Yan et al., 2012), which has been widely applied for studying miRNA function in plants (Liu et al., 2019; Tang et al., 2012; Teotia and Tang, 2017; Teotia et al., 2017; Yan et al., 2016; Zhang et al., 2017). In this study, we investigated the function of a conserved and important miRNA, OsMIR168a, in rice, through CRISPR-Cas9 mediated miRNA locus deletion. In the OsMIR168a deletion mutants, the expression of OsAGO1a, OsAGO1b and OsAGO1d was up-regulated (Figure 3b–e). A protoplast cell-based reporter assay further confirmed that these OsAGO1 family genes including OsAGO1c are targeted for mRNA cleavage by OsMIR168a. Our conclusion is consistent with previous studies in other plant species (Iki et al., 2018; Li et al., 2012; Liu et al., 2020b; Xian et al., 2014) and a recent study in rice (Wang et al., 2021a). The phenotypes of the mature plants and seeds of the OsMIR168a rice mutants are also similar with the transgenic rice lines that carried the MIM168a construct (Wang et al., 2021a).
Interestingly, we found that seedling growth of the OsMIR168a mutants is faster than the WT plants, which probably contributed to the early flowering phenotype as we observed. While our genetic knockout approach generated similar phenotypes to the transgenic MiM168a lines, it can be argued that our genetic knockout approach has two advantages. First, it completely removed OsMIR168a expression in the mutants (Figure 3a), providing true knockout rather than knockdown, a suppression effect achieved by using the miRNA mimic or sponge strategy. Second, because OsMIR168a suppression improves yield and immunity in rice (Wang et al., 2021a), our transgene-free OsMIR168a knockout lines can be directly translated into the breeding practice that may face less regulatory burden in many countries.

Using these OsMIR168a knockout mutants, we investigated transcription regulation networks that is controlled by OsMIR168a, a master regulator in plants. It is not surprising that many genes are differentially expressed in the OsMIR168a mutant background. Among the up-regulated genes, some may be targets of OsMIR168a that have not been previously identified. One of them is OsAGO18, which was confirmed to be a target of OsMIR168a not only by informatic prediction but also by expression analysis (Figure 3f) and a cell-based cleavage assay (Figure 4f). Previously, OsAGO18 was reported to be involved in plant–virus interaction (Wu et al., 2015, 2017). In the future, it would be interesting to see whether elevated expression of OsAGO18 as documented in this study would contribute to enhanced resistance to Magnaporthe oryzae, the causal pathogen for rice blast disease (Wang et al., 2021a).

Our transcriptional analysis also revealed many differentially expressed miRNAs in the OsMIR168a mutants. Interestingly, some of the differentially expressed miRNAs (Figure S9) were predicted to target OsAGO1 family members and OsAGO18 as well, though the putative targeting relationship between these miRNAs and the OsAGO genes requires further investigation. A perplexing question is why some of these miRNAs were up-regulated, and some were down-regulated (Figure S9). It at least suggests different regulation modes underlining these two groups of miRNAs. The functions of these up-regulated miRNAs (e.g. OsMIR5291, OsMIR2118o, OsMIR166d-5p and OsMIR2867-3p) are largely unknown. It remains to be determined whether up-regulation of these genes is due to a feedback regulation to counter the elevated expression of OsAGO1 family members and OsAGO18. However, such a plausible regulation mechanism may be partly cancelled out due to down-regulation of another group of miRNAs (OsMIR166g-5p, OsMIR167e-3p, OsMIR166d-5p and OsMIR529a; Figure S9B, D, F) that might also target these OsAGO genes. Nevertheless, up-regulation of these miRNAs may partly explain the OsMIR168a mutant phenotypes. For example, OsMIR166g-5p and OsMIR166d-5p were significantly down-regulated in both OsMIR168a mutants (Figure S9B, F). This observation is consistent with a previous report that STTMM-mediated OsMIR166 suppression in rice resulted in decreased plant height and altered seed size (Zhang et al., 2018). Also, OsMIR167 was reported to down-regulate four AUXIN RESPONSE FACTOR genes that are associated with growth and development in rice (Liu et al., 2012). In addition, it was previously shown that MIR156, MIR529 and MIR535 target SQUAMOSA promoter binding protein-like (SPL14) (also named as IPAT), which positively regulates panicle development (Jiao et al., 2010; Sun et al., 2019; Wang et al., 2015, 2021b). OsMIR529a was undetectable in both OsMIR168a mutants (Figure S9H). Interestingly, OsMIR529a was
neither detectable in the MIM168a lines nor in the OsMIR168a overexpression lines (Wang et al., 2021a). However, we detected down-regulation of OsSPL17, a target of OsMIR529a (Figure S12). Unlike the finding in the recent study (Wang et al., 2021a) with MIM168a lines, we found OsMIR156 and OsMIR535 expression remained the same level in the OsMIR168a mutants (Table S16). This discrepancy could be explained by different tissues and growth stages investigated in both studies and/or by the fact that true knockout OsMIR168a mutants were used in our study.

Figure 6 KEGG enrichment of hormone and biotic response pathway genes affected by the OsMIR168a mutations. (a) KEGG enrichment of the differentially expressed mRNAs listed in 20 pathways. (b) KEGG enrichment of the differentially expressed mRNAs in plant hormone signal transduction (including Auxin, CK, ABA) pathways. Green highlight indicates down-regulated genes. Red highlight indicates up-regulated genes. (c) Heat map clustering of mRNA KEGG enrichment to plant hormone signal transduction pathway. (d) KEGG enrichment of the differentially expressed mRNAs to the plant–pathogen interaction pathway. Green highlight indicates down-regulated genes. Red highlight indicates up-regulated genes. Blue highlight indicates the occurrence of both down-regulation and up-regulation for the sector. (e) Heat map clustering of mRNA KEGG enrichment to the plant–pathogen interaction pathway.

Aside from these putative OsAGO-targeting miRNAs, additional miRNAs were found to be differentially expressed in the OsMIR168a mutants (Figure 5). They include up-regulated miRNAs such as OsMIR396e-5p (Figure 5b) and OsMIR162a (Figure 5c) and down-regulated miRNAs such as OsMIR172d-5p (Figure 5a). Consequently, their target genes were regulated in the opposite directions (Figure 5). These altered miRNA regulation modules may have implications for the phenotypes of the OsMIR168a mutants. For example, OsGRF6, a target of OsMIR396e-5p, was down-regulated in the OsMIR168a mutants.
(Figure 5b). GRF6 was reported to bind to the promoters of TAWAWA1 and MADS34 to positively regulate auxin biosynthesis and transduction in rice, which subsequently promotes flower development and increases grain numbers (Gao et al., 2015). Furthermore, GRF6 is also involved in the Gibberellin biosynthesis and transduction pathways, shaping plant height (Tang et al., 2018b). The down-regulation of OsMIR172d-5p (Figure 5a) was well correlated with the up-regulation of its target genes such as OsNF-YA8 (Figure 5a). In Arabidopsis, NF-YA8 inhibits the juvenile-to-adult transition through MIR156 activation (Zhao et al., 2020a). However, we have observed an accelerated juvenile-to-adult transition in the rice OsMIR168a mutants, despite the OsNF-YA8 up-regulation. Further, OsMIR156 expression was not altered by the OsMIR168a mutations in rice (Table S16). Hence, the function of OsNF-YA8 in rice may be different from Arabidopsis. This hypothesis warrants further investigations. Interestingly, OsMIR1320-3p was significantly up-regulated in both OsMIR168a mutants (Figure S13; Table S16). This observation is consistent with a previous report that suppression OsMIR168a in rice resulted in enhancing immunity (Wang et al., 2021a). At the same time, we conducted qRT-PCR analysis for the expression levels of two target genes each for OsMIR396 (OsGRF4 and OsGRF6), OsMIR259/156 (OsSPL14 and OsSPL17), OsMIR172 (OsSNB and OsDS1) and OsMIR167/160 (OsARF8 and OsARF18; Figure S14). The results are consistent with the high expression profile of transcriptome by RNA-seq.

The global changes of the transcriptome due to loss of function of OsMIR168a in rice can be further categorized with the KEGG enrichment of differentially expressed genes. Our KEGG enrichment analysis (Figures 6, S10 and S11) identified critical roles of enrichment mechanisms leading to such global transcriptome changes in and our observations, there are at least three molecular regulative roles of OsMIR168a, OsMIR1320-3p and transduction pathways, shaping plant height (Tang et al., 2020a). However, we have observed an accelerated juvenile-to-adult transition in the rice OsMIR168a mutants, despite the OsMIR156 expression was not altered by the OsMIR168a mutations in rice (Table S16). Hence, the function of OsNF-YA8 in rice may be different from Arabidopsis. This hypothesis warrants further investigations. Interestingly, OsMIR1320-3p was significantly up-regulated in both OsMIR168a mutants (Figure S13; Table S16). This observation is consistent with a previous report that suppression OsMIR168a in rice resulted in enhancing immunity (Wang et al., 2021a). At the same time, we conducted qRT-PCR analysis for the expression levels of two target genes each for OsMIR396 (OsGRF4 and OsGRF6), OsMIR259/156 (OsSPL14 and OsSPL17), OsMIR172 (OsSNB and OsDS1) and OsMIR167/160 (OsARF8 and OsARF18; Figure S14). The results are consistent with the high expression profile of transcriptome by RNA-seq.

The global changes of the transcriptome due to loss of function of OsMIR168a in rice can be further categorized with the KEGG enrichment of differentially expressed genes. Our KEGG enrichment analysis (Figures 6, S10 and S11) identified critical roles of OsMIR168a in plant growth and development as well as in plant–pathogen interaction, which resonates with previous findings for this conserved miRNA in these biological processes (Li et al., 2012; Wang et al., 2021a; Xian et al., 2014). Based on previous studies and our observations, there are at least three molecular regulation mechanisms leading to such global transcriptome changes in the absence of OsMIR168a in rice. First, OsMIR168a specifically targets nearly all OsAGO1 family members and OsAGO1B. AGO1 is a key component of the miRNA-mediated gene silencing machinery (Iki et al., 2018; Vaucheret et al., 2004, 2006; Voinnet, 2009). Up-regulation of the AGO1 genes would result in global expression changes for many miRNAs, as we indeed observed in this study. The same could be said for AGO1B. As a result, altered miRNA expression will lead to altered expression of their target genes and additional genes regulated by such genes. Second, aside from binding miRNAs to exert post-transcriptional regulation of gene expression, AGO1 in Arabidopsis was recently found to also bind chromatin to promote gene transcription in response to hormones and stresses (Liu et al., 2018). This suggests that AGO1 can play a more direct role in regulation of transcription landscape in a genome-wide scale. Given that AGO1 orthologs were found to play a similar role in C. elegans (Conine et al., 2013), drosophila (Cernilogar et al., 2011) and human cells (Huang et al., 2013), it is anticipated such a conserved function of AGO1 is also retained in rice. Hence, some of the transcriptome changes in the rice OsMIR168a mutants might result from the action of enhanced OsAGO1 expression and hence their binding to chromatin. Third, the large-scale transcription profile changes in the rice OsMIR168a mutants might be partly attributed to DNA methylation changes. DNA methylation is a conserved epigenetic mark in many higher eukaryotes, which is important for the gene silencing (Law and Jacobsen, 2010; Lei et al., 2014). Plant DNA methyltransferase 1 (MET1), an ortholog of mammalian DNMT1, is mainly responsible for maintaining CG methylation after DNA replication (Saize et al., 2003). In the OsMIR168a mutants, OsMIR162a expression was up-regulated (Figure 5c) and its target gene, OsMET1b (LOC_Os07g08500), was down-regulated (Figure 5c). Thus, it is likely that DNA methylation has been altered in the OsMIR168a mutants, which could subsequently lead to transcriptome changes.

In conclusion, we used CRISPR-Cas9 to generate clean OsMIR168a knockout mutants in rice. Our transcriptome-focussed molecular analysis revealed large-scale transcription profile changes due to the loss of OsMIR168a. Based on our data and earlier studies, we came up with a working model that can explain the pleiotropy observed for OsMIR168a (Figure 7), which provides a framework for future investigations. Additionally, it remains to be tested whether OsMIR168a knockout represents a new breeding approach for rice germplasm improvement.

**Experimental procedures**

**Plant materials**

The rice cultivar Nipponbare (Oryza sativa L. japonica) was used as the WT control and transformation host. The T2 generation of homozygote mutants without the transgene was used for phenotypic and molecular characterization.

**Construction of the vectors**

For vector pZIP093, we used a Cas9 expression backbone vector pGEL026 (pZmUB1::Cas9::Hsp-T-PosU6::sgRNA-::pt). To make a dual-sgRNAs vector, two expression cassettes of OsU6 promoter-sgRNA scaffolds-OsU6 terminator were cloned into pGEL026 using fusion PCR followed by ligation. All the primers are listed in Table S17. The DNA fragment was obtained from PCR products using OsMIR168a-sgRNA1-P1F and OsMIR168a-sgRNA2-P2R using pZIP025 as a template (Zhou et al., 2017). The PCR fragment was cut by Bsa I and then cloned into Bsa I-digested pGEL026.

For overexpression vector MIR168a-OE, the pri-miR168a DNA sequence was cloned to the backbone vector pZH2113 (pZmUB1::HspT). For overexpression vectors OsAGO1a-eYFP, OsAGO1b-eYFP, OsAGO1c-eYFP, OsAGO1d-eYFP and OsAGO18-eYFP, the fragments of OsAGO1a, OsAGO1b, OsAG-Go1c, OsAGO1d and OsAGO18 (including code start code ATG and the OsMIR168 cleavage site) fused with eYFP were cloned to the backbone vector pZH2669 (pZmUB1::eYFP::HspT). All the oligos are listed in Table S17.

**Rice protoplast and stable transformation**

Rice protoplast transformation was performed as described previously (Tang et al., 2016, 2020; Zhang et al., 2013; Zhong et al., 2018). After transformation, rice protoplasts were incubated at 28 °C for 2 days before DNA extraction or imaging. The T-DNA constructs were introduced into Agrobacterium EHA105 by the freeze-thaw method. Rice stable transformation was carried out according to a previously published protocol (Tang et al., 2016, 2018a; Zhou et al., 2019).

**Detection of targeted mutations by CRISPR-Cas9**

Genomic DNA was extracted from protoplasts or transgenic plants by using the CTAB method. Genomic regions of targeted new breeding approach for rice germplasm improvement.
analysed by PCR-SSCP (Zheng et al., 2016). T0 and T1 mutant lines were further genotyped by Sanger sequencing.

**Small RNA sequencing and mRNA transcriptome sequencing**

The mutants and WT plants were chosen for small RNA sequencing and mRNA transcriptome sequencing. Whole plants of 40-day old grown in the growth chamber under long-day conditions (16-h light at 28 °C and 8-h dark at 22 °C) were collected. Three independent plants for each mutant were chosen for library construction, sequencing and analysis. Small RNA and mRNA transcriptome sequencing were done using Illumina HiSeq 2500 planform at Biomarker Technologies Co. Ltd. Data processing and analysis were carried out with the BMKCloud service (http://www.biocloud.net/).

**RNA extraction and qRT-PCR**

Total RNA was extracted using TRIzol Universal Reagent (Tiangen, China), treated with DNase I and then used for cDNA synthesis. Reverse transcription (RT) was carried out using HiScript III RT SuperMix for qPCR (Vazyme, China), and qRT-PCR was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) according to the manufacturer’s instructions. Actin mRNA was used as an internal control. The relative levels of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates (three independent mutant seedlings) were examined to ensure reproducibility. The experiments were performed 3 times independently with similar results.

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**Conflict of interest**

The authors declare no competing interests.

**Authors’ contributions**

Y.Z. proposed the project. Y.Z., Q.Q. and J.Z. designed the experiments. J.Z., X.J. and H.Y. designed sgRNAs and constructed all the vectors. H.Y. and Z.X. did the rice stable transformation. J.Z., R.Z., X. J., X.T. and Y.G. did the rice mutants identification and analysis. J.Z., X.J. and Y.G. performed RNA-Seq experiments. Y.Z., Y.Q., Q.Q. and J.Z. analysed the data and wrote the manuscript. All authors participated in discussion and revision of the manuscript. All authors read and approved the final manuscript.

**Data availability statement**

All data generated or analysed during this study are included in this article and in its Supplementary Information files.
materials are available upon request. Source data are provided with this paper.

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Figure S12 Reduced expression of OsSPL17 in the OsMIR168a mutants.

Figure S13 Elevated expression of OsMIR1320-3p in the OsMIR168a mutants.

Figure S14 qPCR analysis of some miRNA target genes.

Table S1 Information of mRNA-seq

Table S2 The mapped genes via mRNA-Seq

Table S3 The differential expression of mRNAs via mRNA-Seq

Table S4 Information of miRNA-seq

Table S5 The detected miRNAs via small RNA-seq

Table S6 The differential expression miRNAs via small RNA-seq

Table S7 The differential expression miRNAs and their differentially expressed potential targets

Table S8 The expression profiling of the core components Argonauts

Table S9 Bioinformatics analysis rice miRNAs potentially target OsAGO1 families and OsAGO1B

Table S10 The miRNAs potentially target OsAGO1a

Table S11 The miRNAs potentially target OsAGO1b

Table S12 The miRNAs potentially target OsAGO1c

Table S13 The miRNAs potentially target OsAGO1d

Table S14 The miRNAs potentially target OsAGO18

Table S15 KEGG enrichment of mRNAs

Table S16 The expression profile of some miRNA listed in Wang et al. (2021a)

Table S17 Oligos used in this paper

Supinfo S1 Figure and Table legends