Blocking Osa-miR1871 enhances rice resistance against Magnaporthe oryzae and yield

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Summary
MicroRNAs (miRNAs) play vital roles in plant development and defence responses against various stresses. Here, we show that blocking miR1871 improves rice resistance against Magnaporthe oryzae and enhances grain yield simultaneously. The transgenic lines overexpressing miR1871 (OX1871) exhibit compromised resistance, suppressed defence responses and reduced panicle number resulting in slightly decreased yield. In contrast, the transgenic lines blocking miR1871 (MIM1871) show improved resistance, enhanced defence responses and significantly increased panicle number leading to enhanced yield per plant. The RNA-seq assay and defence response assays reveal that blocking miR1871 resulted in the enhancement of PAMP-triggered immunity (PTI). Intriguingly, miR1871 suppresses the expression of LOC_Os06g22850, which encodes a microfibrillar-associated protein (MFAP1) locating nearby the cell wall and positively regulating PTI responses. The mutants of MFAP1 resemble the phenotype of OX1871. Conversely, the transgenic lines overexpressing MFAP1 (OXMFAP1) or overexpressing both MFAP1 and miR1871 (OXMFAP1/OX1871) resemble the resistance of MIM1871. The time-course experiment data reveal that the expression of miR1871 and MFAP1 in rice leaves, panicles and basal internode is dynamic during the whole growth period to manipulate the resistance and yield traits. Our results suggest that miR1871 regulates rice yield and immunity via MFAP1, and the miR8171-MFAP1 module could be used in rice breeding to improve both immunity and yield.

Keywords: miR1871, MIM1871, blast disease resistance, yield traits, MFAP1, PTI.

Introduction
Plants contain a two-perception-layer innate immune system to protect themselves from the invasion of pathogens. The first layer of immunity is the pathogen-associated molecular pattern (PAMP)- triggered immunity (PTI), which is activated following the recognition of pathogen-derived PAMPs by the receptors of plants. For example, the bacterium-derived PAMP flg22 could be recognized by Arabidopsis receptor flagellin sensing 2 (FLS2) (Felix et al., 1999; Gomez-Gomez and Boller, 2000) or by rice receptor OsFLS2 (Takai et al., 2008). The fungus-derived PAMP chitin could be recognized by Arabidopsis receptor chitin elicitor receptor kinase (CERK1) (Miya et al., 2007) or by rice receptors such as chitin elicitor binding protein (OsCEBIP), OsCERK1, lysin motif-containing proteins 4 (LYP4), and LYP6 (Liu et al., 2012; Shimizu et al., 2010). The second layer of immunity is effector-triggered immunity (ETI) triggered by the recognition of pathogen-derived avirulent effectors by the plant cognate resistance (R) genes (Jones and Dangl, 2006).

miRNAs are short non-coding RNAs that suppress gene expression by mediating DNA methylation, or mRNA cleavage, or translational inhibition via binding to the reverse complementary DNA/RNA sequences (Yu et al., 2017). Increasing evidence shows that miRNAs and their target genes participate in the regulation of plant PTI and ETI (Li et al., 2019b). miR393 is the first miRNA identified as an actor involving in both PTI and ETI against Pseudomonas syringae pv. tomato (Pst) DC3000. miR393 is up-regulated with the treatment of flg22 and suppresses the expression of TIR1, AFB2 and AFB3, which encodes the auxin receptors (Navarro et al., 2006). Moreover, miR393 is up-regulated during ETI triggered by the recognition of the avirulent effectors AvrRpt2 by the cognate R receptor RPS2 (Zhang et al., 2011). Subsequently, several miRNAs are characterized as the regulators in ETI. In Arabidopsis, miR472 is involved in ETI by targeting and suppressing the R genes encoding nucleotide-binding site leucine-rich repeat (NLR) receptors, such as RPS5, which recognizes the effectors AvrPhB to activate ETI against P. syringae (Bocca et al., 2014). In tomato, miR482 and miR2118 target the R genes encoding disease resistance proteins with a nucleotide-binding site (NBS) and leucine-rich repeat (LRR) motifs (Shivaprakash et al., 2012). In Medicago, miR2109 targets a large gene family containing conserved NB-LRR domains (Zhong et al., 2011).

Rice is the most important crop supporting half of the world population. Rice blast disease is one of the most serious diseases threatening global rice production. This disease is caused by the fungal pathogen Magnaporthe oryzae (M. oryzae) and can occur during the whole rice growth period. Research over the recent years has revealed that miRNAs play key role in rice resistance against M. oryzae (Li et al., 2019b). For example, several miRNAs have been identified as positive regulators in rice blast disease resistance, including miR159, miR160, miR162, miR166k/h, miR7695, miR398b and miR812w (Campos et al., 2013, 2021; Chen et al., 2021; Li et al., 2014, 2019a, 2020; Salvador-Guirao...
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et al., 2018). In contrast, some other miRNAs negatively regulate rice resistance to blast fungus, such as miR156, miR164a, miR167, miR168, miR169, miR319b and miR1873 (Li et al., 2017; Wang et al., 2018c, 2021; Zhang et al., 2018; Zhao et al., 2020) (Zhang et al., 2020; Zhou et al., 2020).

miRNAs not only regulate rice immunity but also participate in the regulation of rice development and yield. For example, miR172 regulates rice maturity and yield traits by controlling flowering time and floral organ development (Lee et al., 2014; Wang et al., 2015; Zhu et al., 2009). miR393 is involved in rice development by controlling tiller development and flowering (Xia et al., 2012). miR397 regulates grain size, grain number and grain yield (Zhang et al., 2013).

Moreover, some miRNAs and their target genes control both yield traits and immunity. In Brassica, miR1885 directly targets a defence-related R gene BraTNL1 and a photosynthesis-related gene BraCP24, to maintain a trade-off between development and immunity (Cui et al., 2020). In rice, miR162 enhances the resistance against M. oryzae but compromises grain yield with reduced filled grain numbers per plant, whereas blocking miR162 enhanced grain yield but compromises the blast disease resistance (Li et al., 2020; Zhang et al., 2015). miR156 penalizes yield and suppresses blast disease resistance by suppressing Ideal Plant Architecture 1 (IPA1), whereas the expression of a miR156-insensitive IPA1 enhances both rice yield and resistance significantly (Jiao et al., 2010a; Miura et al., 2010; Wang et al., 2018a, Zhang et al., 2020). Blocking miR168 enhances yield and rice blast disease resistance by manipulating the whole miRNA network via AGO1 (Wang et al., 2021). Blocking miR396 improves rice blast disease resistance and yield with increased panicle branching and grain size, as well as enhances the expression of the target genes growth-regulating factors (GRFs) (Chandran et al., 2019; Che et al., 2015; Duan et al., 2015). Consistently, overexpression of these GRFs enhances blast disease resistance and leads to a different change in yield traits (Chandran et al., 2019; Che et al., 2015; Duan et al., 2015). The transgenic lines overexpressing miR444 showed reduced tillers and decreased resistance, whereas overexpressing a target gene, OsMADS57, results in increased tillers and enhanced resistance (Guo et al., 2013; Xiao et al., 2017). Therefore, some miRNAs could be used in rice breeding to improve both yield and resistance or improve resistance without penalty of yield.

miR1871 is a rice-specific 24-nt miRNA and responsive to abiotic or biotic stresses. During drought stress, the accumulation of miR1871 is enhanced in the flag-leaves of two tolerant cultivars, Nagina 22 and Vandana, whereas decreased in the two sensitive cultivars, Pusa Basmati 1 and IR64, suggesting that miR1871 acting as a positive regulator in rice drought responses (Balyan et al., 2017). Besides, the accumulation of miR1871 is fluctuated following the infection of M. oryzae (Li et al., 2014), indicating that miR1871 is involved in rice blast disease resistance. However, whether miR1871 regulates rice resistance against M. oryzae, and the roles of miR1871 in the regulation of rice yield traits, is still unknown.

In this study, we constructed the transgenic lines overexpressing MiR1871, the transgenic lines blocking miR1871, the mutants of a target gene of miR1871, mfaP1, the transgenic lines overexpressing MFAp1 and the transgenic lines overexpressing both MFAp1 and MiR1871. We examined the resistance and yield traits of these lines. Our results showed that blocking miR1871 improved the expression of MFAp1, and enhanced rice blast disease resistance and yield traits. We carried out RNA-seq to explore miR1871-regulated signalling pathways and examined the miR1871-MFAp1 module-regulated PTI responses. Our results indicate that the miR1871-MFAp1 module could be used as regulators in rice breeding to improve both resistance and yield simultaneously.

Results

Overexpression of miR1871 in rice compromises blast disease resistance

In a previous study, we demonstrated that miR1871 is differentially responsive to M. oryzae in a susceptible accession Lijiang xin Tuan Heigu (LTH) and a resistance accession Pyricularia-Kanto51-m-Tsuyuake (IRBLkm-Ts). LTH is a susceptible cultivar sensitive to over 1300 regional isolates of M. oryzae worldwide (Lin et al., 2001). IRBLkm-Ts is a resistant cultivar carrying a resistance (R) locus Pi-km (Tsumematsu et al., 2000). In this study, we further examined the expression pattern of miR1871 following the inoculation of a virulent M. oryzae isolate GZ8. GZ8 was a GFP-tagged isolate that derived from a paddy yard in the north of China and virulent to Nipponbare (Li et al., 2019a). LTH showed increased miR1871 abundance following the inoculation of GZ8 in comparison with the mock samples at 12 and 48 hours post-inoculation (hpi), whereas displayed decreased abundance at 24 hpi. In contrast, IRBLkm-Ts exhibited indistinctive changes in miR1871 accumulation at all examined time points (Figure S1). These results indicated that miR1871 was involved in rice–M. oryzae interaction.

We then investigated the role of miR1871 in rice immunity. We constructed the transgenic lines overexpressing miR1871 (OX1871), which showed significantly higher amounts of miR1871 than the Nipponbare (NPB) control (Figure 1a). We examined the sensitivity of OX1871 to three M. oryzae isolates GZ8, CRB-01 and TJ-1. CRB-01 is an isolate derived from the northeast of China without the known avirulence genes identified (Wang et al., 2018b). TJ-1 is an isolate derived from the paddy yard in Tong-Jiang County, Sichuan basin, the southwest of China. OX1871 lines were more sensitive to the three M. oryzae isolates with larger disease lesions and supported more fungal growth than the Nipponbare control following punch or spray inoculation (Figure 1b-c, Figure S2a-b). These results indicated that miR1871 facilitated the colonization of blast fungus in rice plants.

Blocking miR1871 enhances rice resistance against M. oryzae

As overexpressing miR1871 compromised rice blast disease resistance, we tried to explore whether blocking miR1871 could improve resistance. We then constructed the transgenic lines blocking miR1871 from binding its target genes by expressing a target mimic of miR1871 (MIM1871), which could bind miR1871 to form a double-stranded complex (Figure S3a). MIM1871 lines displayed significantly decreased accumulation of miR1871 in comparison with the Nipponbare control (Figure 1a). As expected, MIM1871 lines showed enhanced resistance with smaller disease lesions and supported less fungal growth than the Nipponbare control after punch or spray inoculation (Figure 1b-c, Figure S2a-b). These results indicated that blocking miR1871 enhanced rice blast disease resistance.

As blocking miR1871 enhanced rice blast disease resistance, we examined whether miR1871 affected the defence
responses triggered by blast fungus, including the induction of defence-related genes and \( \text{H}_2\text{O}_2 \) accumulation. NAC domain-containing protein 4 (NAC4), pathogenesis-related genes 10b (PR10b) and Os04g10010 are defence-response marker genes induced by \( M. \text{oryzae} \) (Li et al., 2020). MIM1871 displayed significantly enhanced expression of all these genes at 36 hpi of \( M. \text{oryzae} \) in comparison with the Nipponbare control, whereas OX1871 exhibited decreased or unchanged expression of these genes (Figure 1d). Similarly, MIM1871 showed more accumulation of \( \text{H}_2\text{O}_2 \) in the invasive cells than that of the Nipponbare control at 48 hpi of GZ8, whereas OX1871 displayed less accumulation (Figure 1e). These results indicated that blocking

Figure 1 miR1871 regulates rice resistance against \( M. \text{oryzae} \). (a) The amounts of miR1871 in transgenic lines overexpressing the \( MIR1871 \) gene (OX1871), blocking miR1871 (MIM1871) and the Nipponbare (NPB) control. The levels of miR1871 were examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (b) Disease symptom on represented leaves of OX1871, MIM1871 and the Nipponbare control following inoculation of \( M. \text{oryzae} \) isolates GFP-tagged Zhong8-10-14 (GZ8), CRB-01 and TJ-1, respectively. The photograph of OX1871 lines with their Nipponbare control was captured five days post-inoculation (dpi). The photograph of MIM1871 lines with their Nipponbare control was captured seven days post-inoculation (dpi). Size bar, 5 mm. (c) The relative fungal biomass of indicated isolates on OX1871, MIM1871 and the Nipponbare control. The fungal biomass was shown as the ratio of DNA levels of \( M. \text{oryzae} \) MoPot2 against DNA levels of rice ubiquitin. (d) The mRNA levels of defence-related genes (NAC4, PR10b and Os04g10010) in OX1871, MIM1871 and the Nipponbare control at 12 hours post-inoculation (hpi) of GZ8. The mRNA levels were normalized to that in Nipponbare. (e) The accumulation of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) in OX1871, MIM1871 and the Nipponbare control at 48 hpi of GZ8. \( \text{H}_2\text{O}_2 \) was stained by 3,3’-diaminobenzidine (DAB) and indicated by the intensity of brown. The photographs at the upper portion were taken with a stereomicroscope. Size bars, 1 mm. The photographs at the down portion were taken with a microscope (Zeiss imager A2). The red arrows indicate appressoria. Size bars, 40 \( \mu \)m. For a, c and d, data are shown as mean ± SD (n = 3 independent repeats), and different letters above the column indicate significant differences (\( P < 0.01 \)) as determined by the one-way Tukey–Kramer test. These experiments were repeated at least two times with similar results.

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miR1871 enhanced rice defence responses triggered by M. oryzae.

Blocking miR1871 enhances PTI-related defence responses

To explore how blocking miR1871 enhanced rice blast resistance, we performed RNA-seq experiments and compared the transcriptome profiles of MIM1871 and the Nipponbare control with three repeats to analyse the miR1871-involved signalling pathways. The filtered data, including the numbers of total reads, mapped reads and the percentage of mapped reads, were listed in Table S1. 35,772 genes were identified (Table S2). Based on one-way ANOVA analysis, 2,698 genes were significantly affected by miR1871 (P value < 0.05 and Log2 >1 or Log2<−1; Table S3, Figure S4). Gene Ontology (GO) analysis revealed that the up-regulated genes in the MIM1871 line were enriched in pathways related to defence-related hormone-activated signalling, and responses to cold or salt stress, as well as responses to chitin treatment (Figure 2a, Table S4). To validate the RNA-seq data, we selected ten genes that were clustered in more than two GO groups for expression analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Consistently, all the ten genes were constitutively up-regulated in MIM1871 (Figure 2b). Among these genes, five genes were clustered in the GO-enriched genes that respond to chitin, including WRKY45, WRKY53, WRKY71, ERF020 and MAPKKK17. These results indicate that our RNA-seq data are reliable, and blocking miR1871 constitutively up-regulates rice defence-related genes that may prime immune responses.

To explore the priming effect of blocking miR1871, we examined the PTI responses, such as the burst of reactive oxygen species (ROS) and callose deposition, in the transgenic lines and the Nipponbare control. The MIM1871 lines displayed higher induction of ROS in comparison with the Nipponbare control following the treatment of flg22, whereas the OX1871 lines showed the reversed phenotype (Figure 2c), suggesting a negative role of miR1871 in the regulation of flg22-triggered ROS burst. As failed in detection of the chitin-induced ROS in rice, we transiently expressed miR1871 or MIM1871 in Nicotiana benthamiana (N. benthamiana), respectively, to further examine the effects of miR1871 on ROS. With the treatment of flg22 and chitin, the leaves expressing miR1871 displayed suppressed ROS responses compared with the control leaves expressing GFP, whereas the leaves expressing MIM1871 showed enhanced ROS (Figure S5), indicating that blocking miR1871 enhanced PAMP-triggered ROS. Moreover, both flg22 and chitin induced more callose deposits in the MIM1871 lines than that in the Nipponbare control, whereas induced fewer callose deposits in OX1871 lines (Figure 2d-e). These results were consistent with the enrichment of genes that were responsive to chitin in the MIM1871 transgenic rice lines (Figure 2a), suggesting that blocking miR1871 could improve the PTI responses in rice.

miR1871 suppresses the expression of LOC_Os06g22850

As miRNAs regulate plant development and resistance by suppressing the expression of target genes, we then tried to explore the target genes of miR1871 to learn the signalling pathways controlled by miR1871. Three genes were predicted as the targets of miR1871, namely LOC_Os01g28300, LOC_Os06g22850 and LOC_Os11g19610 (http://plantgrn.noble.org/rsRNATarget/; Wu et al., 2009; Figure S3a). However, the miRNA levels of LOC_Os11g19610 were under the detecting threshold, and LOC_Os01g28300 was specifically expressed in seeds. LOC_Os06g22850 encodes an MFAP (hereafter named as MFAP1) and expressed in all rice organs (http://rice.plantbiology.msu.edu). We next explored the expression of MFAP1 in leaves of OX1871 and MIM1871. The mRNA levels of MFAP1 were significantly enhanced in all three examined MIM1871 lines, whereas only obviously suppressed in one of the three detected OX1871 lines (Figure S3b). miR1871 is a 24-nt miRNA and is predicted to mediate DNA methylation via a DCL3-dependent pathway (Wu et al., 2010). We then analysed the cytosine methylation pattern at the miR1871-targeted site of MFAP1 in the Nipponbare control and a dcl3a mutant. We found that the methylation occurred in the Nipponbare control but not in the dcl3a mutant (Figure S3c), indicating that miR1871 controlled the cytosine methylation near the targeted sites. We then examined whether miR1871 suppressed the protein levels of MFAP1. The target site of MFAP1 was located at the intron of the 3'-UTR region (Figure S3a). We made constructs expressing YFP-fused 3'-UTR of MFAP1, in which the target site of MFAP1 was located (3SS: YFP-UTRMFAP1) (Figure 3a). We co-expressed the fused protein with miR1871 alone or with miR1871 and MIM1871 together. The YFP intensity and protein levels were decreased when the construct 3SS: YFP-UTRMFAP1 was co-expressed with miR1871 in N. benthamiana, whereas were recovered when MIM1871 was added (Figure 3b-c), indicating that miR1871 suppressed the expression of MFAP1, and MIM1871 could release the suppression by miR1871.

Mutations of MFAP1 compromise rice blast disease resistance and PTI responses

We next examined the role of MFAP1 in rice immunity. We constructed mutant lines of MFAP1 using the CRISPR/Cas9 technology in the Nipponbare background. We designed two gRNA sites for this gene to make sure that at least one gRNA is working (Figure S6a) and identified two homozygous mutants (mfap1 and mfap2). mfap1-1 carried a 21-bp deletion nearby gRNA1 site and a 1-bp deletion nearby gRNA2 sites resulting in deletion of 7 amino acids (aa), and changes of several amino acids, as well as a premature stop (Figure S6b and d). mfap1-2 carried an insertion of C nearby gRNA1 site and a 1-bp deletion nearby gRNA2 sites resulting in changes from aa 57 and led to a premature stop after aa 70 (Figure S6c and e).

We then assessed the sensitivity of mfap1 mutants to M. oryzae. Consistent with the phenotype of OX1871, mfap1 mutants displayed compromised resistance against M. oryzae accompanied by bigger disease lesions and more fungal biomass than the Nipponbare control (Figure 4a-b). Moreover, like OX1871, mfap1 mutants showed lower induction of ROS and fewer callose deposits than the Nipponbare control (Figure 4c-f). These results indicated that miR1871 possibly repressed rice blast disease resistance by suppressing the expression of MFAP1, the mutations of which compromised rice immunity and PTI responses.

MFAP1 contributes to MIM1871-confferred blast disease resistance

To further study the roles of MFAP1 in miR1871-regulated resistance against M. oryzae, we constructed the transgenic lines
overexpressing MFAP1 in Nipponbare background (OXMFAP1) and in OX1871 background (OXMFAP1/OX1871), respectively. All the transgenic lines exhibited significantly increased expression of MFAP1 in comparison with the controls and the null segregants (Figure S7a-c). Like MIM1871, the OXMFAP1 lines were more resistant than the Nipponbare control and the null segregants, associated with smaller lesions and lower fungal biomass (by ~50%) (Figure 5a-b). Moreover, the OXMFAP1/OX1871 lines also showed enhanced resistance in comparison with the Nipponbare control, the OX1871 lines, and the null segregants (Figure 5a-b). These results suggest that MFAP1 contributes to MIM1871-conferred resistance to M. oryzae.

We tried to explain how MFAP1 regulated rice immunity. We first tested the location of MFAP1 in plant cells by transient expression of a YFP-fused MFAP1 (35S: MFAP1-YFP) in N. benthamiana. The YFP signals were enriched nearby the propidium iodide (PI)-dyed cell wall (Figure S8a). Plasmolysis assay further revealed that MFAP1 was in apoplast nearby the cell wall (Figure S8b). As MFAP1 was located nearby the cell wall, and mfap1 mutants displayed compromised PTI responses, we anticipated that MFAP1 could enhance PTI responses. We transiently expressed GFP-fused MFAP1 (MFAP1-GFP) in N. benthamiana to examine the effect of MFAP1 on PTI responses. As anticipated,
examined the expression of several genes characterized as the increase in yield per plant (Figure 6b-f, Table S5). We then grains per panicle and heavier grains leading to a remarkable contrast, MIM1871 showed lower SSR but more panicles, more decrease in grain yield per plant (Figure 6b-f, Table S5). In lower SSR but heavier grain weight resulting in a significant increase in MIM1871. Moreover, when miR1871 and were consistent with the defence responses triggered by PAMPs that transient expression of YFP-UTRMFAP1 deposits triggered by flg22 and chitin, compared with the control (Figure 3a, Table S5). OX1871 displayed similar plant architecture but shorter plants in comparision with the Nipponbare control (Figure 6a, Table S5). MIM1871 and these parameters and some other yield traits of OX1871, and immunity, we performed a time-course experiment and examined the expression of miR1871 and MFAP1 in rice leaves, panicles and basal internode. The amounts of miR1871 were significantly changed in all the examined lines in comparison with the Nipponbare control (Figure 6g), indicating the involvement of the two genes in the miR1871-MFAP1 module-regulated tillering. OsSPL14 is a well-known negative regulator of tiller number in rice (Jiao et al., 2010a; Miura et al., 2010; Wang et al., 2018a; Miura et al., 2010b; Kang et al., 2017; Wang et al., 2019). Consistently, MIM1871 showed enhanced expression of OsSPL2 but suppressed expression of OsAAP5, whereas OsX1871 and mfap1 mutant displayed a reverse expression pattern of the two genes (Figure 6g), indicating the involvement of the two genes in the miR1871-MFAP1 module-mediated regulation of tillering. OsSPL14 was not significantly changed in all the examined lines in comparison with the Nipponbare control (Figure 6g), indicating OsSPL14 was not involved in miR1871-mediated regulation of tiller number. Altogether, these results suggested that miR1871 possibly regulates these yield traits via MFAP1, and blocking miR1871 is helpful for the improvement of grain yield by enhancing panicle number and size.

**Figure 3** miR1871 suppresses the expression of MFAP1. (a) Confocal images of YFP-UTRMFAP1 fluorescence signals suppressed by miR1871. The 35S: YFP-UTRMFAP1 reporter construct was transiently expressed alone or co-expressed with 35S: MIR1871 in N. benthamiana leaves by Agrobacterium-mediated infiltration at indicated optical density (O.D.) concentrations. Size bars, 100 μm. (b) Confocal images of YFP-UTRMFAP1 fluorescence signals co-expressed with miR1871 and MIM1871. The 35S: YFP-UTRMFAP1 reporter construct was transiently expressed alone or co-expressed with 35S: MIR1871 and 35S: MIM1871 in N. benthamiana leaves by Agrobacterium-mediated infiltration at indicated optical density (O.D.) concentrations. Size bars, 100 μm. GV3101 was used to complement the expression of total bacteria. (c) Western blotting analysis showed the protein amounts of YFP in N. benthamiana leaves in (a). Protein extracted from the infiltrated leaves was subjected to Western blot analysis with anti-GFP sera. The Ponceau-stained Rubisco was used as the control indicating the equal sample loading. These experiments were repeated two times with similar results.

overexpression of MFAP1-YFP led to higher ROS and more callose deposits triggered by flg22 and chitin, compared with the control that transient expression of YFP only (Figure S9a-d). These results were consistent with the defence responses triggered by PAMPs in MIM1871. Moreover, when miR1871 and MFAP1-YFP were co-expressed, MFAP1-YFP partially recovered miR1871-suppressed ROS burst (Figure 5c), indicating that MFAP1 played a positive role in MIM1871-conferred blast disease resistance by improving the PTI responses.

The miR1871-MFAP1 module regulates rice agronomic traits and yield

Intriguingly, we found that the miR1871-MFAP1 module also regulates rice yield. The yield of rice is determined by several components, including panicle number, grain number per panicle, seed setting rate (SSR) and grain weight. We then examined these parameters and some other yield traits of OX1871, MIM1871 and mfap1 planted in a paddy field in the rice-growing season of 2019 and 2020. All the examined lines displayed similar plant architecture but shorter plants in comparison with the Nipponbare control (Figure 6a, Table S5). OX1871 and mfap1 generated fewer panicles, fewer grains per panicle, lower SSR but heavier grain weight resulting in a significant decrease in grain yield per plant (Figure 6b-f, Table S5). In contrast, MIM1871 showed lower SSR but more panicles, more grains per panicle and heavier grains leading to a remarkable increase in yield per plant (Figure 6b-f, Table S5). We then examined the expression of several genes characterized as the regulators of rice tiller numbers in OX1871 and MIM1871, namely OsLRK2, OsAAP5 and OsSPL14. While OsLRK2 acted as a positive regulator, OsAAP5 and OsSPL14 acted as negative regulators in tillering (Jiao et al., 2010b; Kang et al., 2017; Wang et al., 2019). Consistently, MIM1871 showed enhanced expression of OsLRK2 but suppressed expression of OsAAP5, whereas OX1871 and mfap1 mutant displayed a reverse expression pattern of the two genes (Figure 6g), indicating the involvement of the two genes in the miR1871-MFAP1 module-mediated regulation of tillering. OsSPL14 is a well-known negative regulator of tiller number in rice (Jiao et al., 2010a; Miura et al., 2010; Wang et al., 2018a; Zhang et al., 2020). The expression of OsSPL14 was not significantly changed in all the examined lines in comparison with the Nipponbare control (Figure 6g), indicating OsSPL14 was not involved in miR1871-mediated regulation of tiller number. Altogether, these results suggested that miR1871 possibly regulates these yield traits via MFAP1, and blocking miR1871 is helpful for the improvement of grain yield by enhancing panicle number and size.

**Figure 3** miR1871 suppresses the expression of MFAP1. (a) Confocal images of YFP-UTRMFAP1 fluorescence signals suppressed by miR1871. The 35S: YFP-UTRMFAP1 reporter construct was transiently expressed alone or co-expressed with 35S: MIR1871 in N. benthamiana leaves by Agrobacterium-mediated infiltration at indicated optical density (O.D.) concentrations. Size bars, 100 μm. (b) Confocal images of YFP-UTRMFAP1 fluorescence signals co-expressed with miR1871 and MIM1871. The 35S: YFP-UTRMFAP1 reporter construct was transiently expressed alone or co-expressed with 35S: MIR1871 and 35S: MIM1871 in N. benthamiana leaves by Agrobacterium-mediated infiltration at indicated optical density (O.D.) concentrations. Size bars, 100 μm. GV3101 was used to complement the expression of total bacteria. (c) Western blotting analysis showed the protein amounts of YFP in N. benthamiana leaves in (a). Protein extracted from the infiltrated leaves was subjected to Western blot analysis with anti-GFP sera. The Ponceau-stained Rubisco was used as the control indicating the equal sample loading. These experiments were repeated two times with similar results.

miR1871-MFAP1 module is dynamically expressed during the growing period

To find out how the “miR1871-MFAP1” module controlling yield and immunity, we performed a time-course experiment and examined the expression of miR1871 and MFAP1 in rice leaves, panicles and basal internode. The amounts of miR1871 were decreased gradually in leaves during the vegetative stage and in panicles during the productive stage (Figure 7a-b). Moreover,
miR1871 was decreased significantly in basal internode before tillering but increased remarkably after tillering (Figure 7c). Conversely, the mRNA levels of MFAP1 were increased accordingly during the vegetative and reproductive stages (Figure 7a-b).

This expression pattern of miR1871 and MFAP1 was consistent with the enhanced blast disease resistance and development of panicles in mature rice plants. When the rice plants grew up, the amounts of miR1871 were decreased gradually in leaves and panicles leading to enhanced expression of MFAP1, which improved blast disease resistance and panicle formation (Figure 7d). Moreover, during the tillering stage, the decreased miR1871 amounts in basal internode led to the increase in tiller number, whereas after tillering stage, the increased miR1871 suppressed the tillering to avoid the invalid tiller (Figure 7c-d). Thus, the dynamical and spatial expression of miR1871 fine-tunes rice immunity and yield via MFAP1. However, the mRNA levels of MFAP1 in the basal internode were not reversely consistent with the amounts of miR1871 before and after tillering (Figure 7c).

Such an inconsistency indicated that MFAP1 is also regulated by other regulators, or there existed other target genes involved in the regulation of panicle number.

Discussion

miRNAs fine-tune the trade-off between plant immunity and yield. In this work, we reported that blocking miR1871 improved both immunity and yield. While OX1871 exhibited compromised blast disease resistance and reduced grain yield (Figures 1 and 6), MIM1871 showed enhanced resistance and increased grain yield compared with the Nipponbare control (Figures 1 and 6). Moreover, consistent with the resistance phenotype of OX1871, mfap1 displayed the compromised resistance and grain yield (Figure 4). Conversely, OXMFAP1 and OXMFAP1/OX1871 lines all displayed enhanced resistance like MIM1871 (Figure 5). These results indicated that the miR1871-MFAP1 module was an important regulator for rice resistance and yield traits and could
be used in rice breeding to improve resistance and yield simultaneously.

Intriguingly, the miR1871-MFAP1 module regulated PTI responses. Overexpression of miR1871 or mutations of MFAP1 resulted in suppressed induction of ROS and callose deposition. Conversely, blocking miR1871 or overexpression of MFAP1 enhanced these defence responses (Figures 2, 4 and 5). Moreover, blocking miR1871 constitutively enhanced the expression of genes responsive to chitin (Figure 2), which in turn primes PAMP-triggered immune responses. These results indicated that the miR1871-MFAP1 module controls rice immunity by regulating PTI responses. However, current data did not address whether the miR1871-MFAP1 module participated in the regulation of ETI against *M. oryzae*, which could be an interesting future research focus.

MFAP1 protein was first identified as the extracellular matrix glycoproteins in animals (Horrigan et al., 1992) and later found to be involved in spliceosome preparations (Neubauer et al., 1998). MFAP proteins play important roles in microfibril assembly, tissue homeostasis, cell viability and tumour progression. MFAPs contain five subfamily members, namely from MFAP1 to MFAP5. MFAP1 was first cloned in the human genome and characterized as a regulator of inherited diseases affecting microfibrils (Yeh et al., 1994). In the fungus *Cenangium ferruginosum*, MFAP1 is identified as a growth-related marker gene (Ryu et al., 2018). In Drosophila, a homolog of MFAP1 was identified as a nucleolar protein involving in pre-messenger RNA processing, spliceosome assembly and cell survival (Rode et al., 2017; Ulrich et al., 2016). In *C. elegans*, a *mfap1* gene encodes a nuclear-located splicing factor that can affect alternative splicing of miRNAs (Ma et al., 2012). In this study, we demonstrated that the rice MFAP1 protein was located near the cell wall. This location resembles the location of some animal MFAP family members in animals, which are in the extracellular matrix (Horrigan et al., 1992). Moreover, the location of the MFAP1 protein was consistent with its roles in physical defence responses such as callose deposition (Figure S9), the component of which was β-1,3 glucan and located on the cell wall. However, it is still unclear how MFAP1 participates in the regulation of ROS burst.

miR1871-MFAP1 module also regulated yield traits (Figure 6). Both OX1871 and mfap1 mutants exhibited decreased grain yield due to decreased panicle number, grain number per panicle and SSR (Figure 6, Table S5). Conversely, MIM1871 displayed the reverse phenotype. These results indicated that miR1871 regulated rice yield traits via MFAP1. Further study about the agronomic traits of OXMFAP1 and MFAP1/OX1871 would greatly contribute to our understanding of the roles of the miR1871-MFAP1 module. Moreover, how the cell wall-located MFAP1 contributes to rice yield traits is unclear. Screening the interacting proteins of MFAP1 and learning about their biochemical function would be helpful to reveal how MFAP1 functioned in rice immunity and growth.
MFAP1 was possibly not the only target gene of miR1871. For example, Os01g28300 and Os06g19610 were also predicted as the targets of miR1871 (Wu et al., 2009). LOC_Os01g28300 encodes an F-box domain-containing protein, and LOC_Os11g19610 encodes a retrotransposon. However, both genes are not expressed in leaves or under the detecting threshold, indicating an impossibility of involvement of rice leave resistance. Further study is required to test whether these genes were targets of miR1871 and involved in the regulation of yield traits. For example, LOC_Os01g28300 is specifically expressed in seeds (http://rice.plantbiology.msu.edu). Whether LOC_Os01g28300 participates in miR1871-regulated seed development needs further study.

Methods

Plant materials and growth conditions

We use LTH and IRBLkm-Ts to examine the expression pattern of miR1871. We use rice (Oryza sativa) japonica accession Nipponbare (NPB) to construct the miR1871 overexpressing lines (OX1871), miR1871 blocking lines by expressing a target mimic of miR1871 (MIM1871), mfap1 mutants, MFAP1 overexpressing lines (OXMFAP1) and the double overexpressing lines (OXMFAP1/OX1871). For resistance and defence response assay, the transgenic lines and the Nipponbare control were planted in a glasshouse at 28°C/2 with 70% relative humidity and 12-h/12-h light/dark cycles.

Trait measurements

For the analysis of agronomic traits, the transgenic lines and the controls were grown in a paddy yard in Wenjiang District, Sichuan Province, China, in the rice-growing season from mid-April to mid-September in 2019 and 2020. The morphology of the rice lines was captured at the maturity stage. For each line, 30 rice plants were planted with a 15-cm × 20-cm of plant-row spacing, and five or more individual plants were selected for yield trait analysis, including panicle number per plant, grain number per panicle, seed setting rate (the ratio of filled grains to the total grains per plant), 1,000-grain weight and grain yield per plant. The 1,000-grain weight and grain yield per plant were obtained after the filled grains were dried in a 42°C oven for one week using an SC-A grain analysis system (Wanshen Ltd., Hangzhou, China). All the data were analysed by one-way ANOVA followed by post hoc Tukey HSD analysis at P value < 0.05.
Plasmid construction and transformation

We amplified the genomic sequence of MIR1871 from the genomic DNA of Nipponbare with the specific primers miR1871-F-KpnI and miR1871-R-SalI containing 492 bp upstream and 457 bp downstream sequences of MIR1871 (Table S6). The plasmid 35S-pCAMBIA1300 was used as the vector to express the MIR1871 gene promoted by constitutive promoter 35S (OX1871). We constructed a target mimic of miR1871 (MIM1871) by cloning a double-stranded fragment reversely complementary to miR1871 with three inserted bases (AAACCAACATGATAACAT-CAGAGCCAT) into the KpnI-SalI sites of the binary vector 35S-pCAMBIA1300. The fragment substituted the target site of miR399 in the vector as described previously (Franco-Zorrilla et al., 2007; Li et al., 2017) and acted as a target mimic to absorb miR1871 thus blocked the binding of miR1871 and its authentic target genes. The target mimic fragments were obtained by annealing with primers MIM1871-F-KpnI and MIM1871-R-SalI (Table S6). To construct the transgenic lines overexpressing MFAP1, we amplified the CDS of MFAP1 from cDNA of Nipponbare with the specific primers MFAP1-F and MFAP1-R. The fragment was inserted into the binary vector 35S-pCAMBIA1300 to express the protein MFAP1. To make the transgenic lines overexpressing MFAP1 in OX1871, the CDS of the MFAP1 gene were cloned into the binary vector 35S-pCAMBIA2300 (Table S6).

The CRISPR/Cas9 plasmid construct and mutant screen

We constructed the mutants of MFAP1 following previous reports (Li, W et al., 2017) with CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas9 technique. The guide RNA sequences (Table S6) of MFAP1 were screened by a Cas-
Off-target sites with screen parameters including less than three base-pair mismatches and one DNA/RNA bulge. The maize ubiquitin promoter (UBI) upstream of a codon-optimized hSpCas9 (Cong et al., 2013) was inserted into the binary vector pCAMBIA1300 with hygromycin selection (via hygromycin B phosphotransferase). The original BsaI site present in the pCAMBIA1300 backbone was removed using a point mutation kit (TransGen, China). A fragment containing an OsU6 promoter (Feng et al., 2013) and a negative selection marker gene ccdB flanked by two BsaI sites and an snRNA derived from pX260 (Cong et al., 2013) was inserted into this vector using an In-fusion cloning kit (Takara, Japan) to produce the CRISPR/Cas9 binary vector pBGK32. E. coli strain DB3.1 was used for maintaining this binary vector. The 23 bp targeting sequences (including the WT genome sequence to identify the mutation sites. The PCR products (500-600 bp) were sequenced and blasted to using the primers flanking the designed target sites (Table S6). For resistance assay, we incubated the bacteria at 800 g for 5 min and resuspended them in an MMA buffer (10mM MES, 10mM MgCl2, 100 mM AS). We performed Western blotting analyses to determine the amounts of miR1871 by qRT-PCR with the specific primers (Table S6) and selected snRNA U6 as the internal reference to normalize the amounts of miR1871.

Inoculation and microscopy assay
We used the three- to five-leaf-stage seedlings for resistance and defence response assay. We inoculated the transgenic lines and the controls with or without the spores (1 x 10^5 spores/mL) of isolated isolates by punch or spray inoculation. For resistance assays, we observed the symptom on the inoculated leaves at 5-7 days post-inoculation (dpi) and examined the fungal biomass as previously reported (Park et al., 2012). In brief, we extracted the DNA from the inoculated leaves and determined the relative fungal biomass by qRT-PCR using the DNA levels of the M. oryzae Mop02 gene against that of the rice Ubiquitin gene. For detection of H2O2 amounts, we collected the spray-inoculated leaves at 36 hpi and placed the leaves in 3, 3'-diaminobenzidine (DAB) (1 mg/ ml, Sigma, Alorich, USA) for staining at room temperature without light treatment for 12 hours. Subsequently, we decolourised the stained leaves in 90% ethyl alcohol at 65°C several times (Xiao et al., 2003). The H2O2 accumulation was captured with a stereomicroscope (Zeiss imager A2).

Transient expression assay in Nicotiana benthamiana
We fused YFP with the full-length 3'-UTR of MFAPI at its C-terminus (35S: YFP- UTRMFAP1). The sequence of UTRMFAP1 was amplified from the Nipponbare genomic DNA (Table S6). We inserted the isolated fragment into KpnI-SpeI sites of the binary vector 35S-pCAMNIA1300-YFP and got the binary vector 35S-pCAMNIA1300-YFP-UTRMFAP1. We transformed the vector into Agrobacterium strain GV3101 and conducted the agroinfection assay in N. benthamiana. In brief, we incubated the Agrobacterium strains harbouring the respective expression constructs (35: YFP-UTRMFAP1, 35S: miR1871, 35S: MIM1871) at 28°C overnight in LB media containing kanamycin (50 mg/ mL) and carbenicillin (50 mg/ mL) on a shaking at 250 rpm. We collected the bacteria at 800 g for 5 min and resuspended them in an MMA buffer (10mM MES, 10mM MgCl2, 100 mM AS). We infiltrated the Agrobacteria into leaves of N. benthamiana for transient expression assay and examined the GFP expression. To examine the PTI-triggered callose deposition in N. benthamiana,
the leaves expressing miR1871, or MIM1871, or MFAP1, or MFAP1 and miR1871 together were syringe-infiltrated with 1 µM flg22 or 20 µg/ml chitin for 16 hours. Then, the treated leaves were collected and stained with 0.01% aniline blue for half an hour following a previous report (Zhang et al., 2007). We examined callose deposition in rice following a previous report (Liu et al., 2012). The first leaves of the five-day-old seedling were treated with flg22 or chitin for 12 hours and then fixed in ethanol: acetic acid (3:1 [v/v]) solution for 5 h. The fixed leaves were then rehydrated successively in 70% and 50% ethanol for 2 h, respectively, and in water overnight. Then, the leaves were treated with 10% NaOH for 1 hour to make the tissues transparent. After washed three times with water, the leaves were incubated in the staining buffer containing 150 mM K2HPO4, pH 9.5, 0.01% aniline blue (Sigma-Aldrich) for 4 hours. The images of callose deposition were captured with a fluorescence microscope under a UV channel (340 to 380 nm) (Zeiss imager A2.0), and the callose deposits were calculated using ImageJ software.

RNA-seq and data analysis

We collected the leaf samples of MIM1871 and the Nipponbare control at the three-leaf stage. We extracted the total RNAs of these samples and determined the RNA quantity and integrity by using NanoDrop 2000 (Thermo Scientific, Waltham, MA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA-seq libraries were constructed using the Illumina TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA) following standard protocols and sequenced on an Illumina HiSeq X Ten platform, and 150 bp paired-end reads were generated. Average 50 M read (Pair-end 125bp model) for each library was output. The raw reads of fastq format were firstly processed using Trimmomatic (Bolger et al., 2014). All clean reads for each sample were aligned to a manufacture reference containing Oryza sativa. After reads mapping, a new GTF file was constructed by Cufflinks (Trapnell et al., 2012) and gene quantity was calculated by Htseq (Anders et al., 2015). Then, an R package DESeq2 (Love et al., 2014) was applied to different gene expression analyses. Other data statistics and visualizing were performed by self-R scripts. P value < 0.05 and fold change > 2 or fold change < 0.5 was set as the threshold for significantly differential expression. Hierarchical clustering analysis of DEGs was run with hclust in R version 3.2.0. GO enrichment and KEGG (Kanehisa et al., 2008) pathway enrichment analysis of DEGs were performed, respectively, using R based on the hypergeometric distribution.

Author contributions

Y. L. and W-M. Wang conceived the experiment, and together with T-T. L., X-R. H., Y. Z., Q. F., X-M. Y. and X-H. Z. carried it out; G-B. L., J-H. Zhao, J-W. Zhang, Z-X. Z., J. F. and Y. Huang analysed the data; S-X. Z., Y-P. J. and M. Pu carried out the field trial; Y. L. and W. Wang wrote the paper.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 miR1871 is responsive to rice blast fungus Magnaporthe oryzae.

Figure S2 Blocking miR1871 enhances rice resistance against Magnaporthe oryzae following spray-inoculation.

Figure S3 miR1871 is predicted to regulate the methylation of MFAP1 in rice.

Figure S4 The hot map of the genes differentially expressed in MIM1871 and the Nipponbare control detected by RNA-seq.

Figure S5 miR1871 regulates PAMPs-induced Burst of reactive oxidative species (ROS).

Figure S6 Mutation sites of MFAP1 in mfap1 mutants.

Figure S7 Overexpression of MFAP1 in the Nipponbare background and OX1871 background, respectively.

Figure S8 MFAP1 is located nearby the cell wall.

Figure S9 MFAP1 positively regulates PTI responses.

Table S1 The report of filtered data.

Table S2 The list of genes identified in MIM1871-1 and the control NPB.

Table S3 The list of genes affected by miR1871.

Table S4 The GO profile of the up-regulated genes in MIM1871 line.

Table S5 The agronomic straits of OX1871, MIM1871, mfap1, and the NPB control in 2019 and 2020.

Table S6 The primers used in this study.