The Polycistronic miR166k-166h Positively Regulates Rice Immunity via Post-transcriptional Control of EIN2

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MicroRNAs (miRNAs) are small RNAs acting as regulators of gene expression at the post-transcriptional level. In plants, most miRNAs are generated from independent transcriptional units, and only a few polycistronic miRNAs have been described. miR166 is a conserved miRNA in plants targeting the HD-ZIP III transcription factor genes. Here, we show that a polycistronic miRNA comprising two miR166 family members, miR166k and miR166h, functions as a positive regulator of rice immunity. Rice plants with activated MIR166k-166h expression showed enhanced resistance to infection by the fungal pathogens Magnaporthe oryzae and Fusarium fujikuroi, the causal agents of the rice blast and bakanae disease, respectively. Disease resistance in rice plants with activated MIR166k-166h expression was associated with a stronger expression of defense responses during pathogen infection. Stronger induction of MIR166k-166h expression occurred in resistant but not susceptible rice cultivars. Notably, the ethylene-insensitive 2 (EIN2) gene was identified as a novel target gene for miR166k. The regulatory role of the miR166h-166k polycistron on the newly identified target gene results from the activity of the miR166k-5p specie generated from the miR166k-166h precursor. Collectively, our findings support a role for miR166k-5p in rice immunity by controlling EIN2 expression. Because rice blast is one of the most destructive diseases of cultivated rice worldwide, unraveling miR166k-166h-mediated mechanisms underlying blast resistance could ultimately help in designing appropriate strategies for rice protection.

Keywords: blast, ethylene-insensitive 2 (EIN2), miR166, Oryza sativa, Magnaporthe oryzae, rice

INTRODUCTION

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs that mediate post-transcriptional gene silencing in eukaryotes (Jones-Rhoades et al., 2006). They are transcribed as long primary transcripts (pri-miRNAs), forming an imperfect fold-back structure, and are sequentially processed by a DICER-like ribonuclease (typically DCL1) to produce a pre-miRNA and finally a double-stranded miRNA duplex, the miRNA-5p/miRNA-3p duplex (previously named miRNA/miRNA* duplex) (Kurihara and Watanabe, 2004). The miRNA-5p/miRNA-3p duplexes are then transported...
to the cytoplasm, where the functional miRNA strand is incorporated into an ARGONAUTE1 (AGO1)-containing RNA-induced silencing complex (RISC) (Baumberger and Baulcombe, 2005; Jones-Rhoades et al., 2006; Rogers and Chen, 2013). miRNAs guide post-transcriptional gene silencing via sequence-specific cleavage or translational repression of target transcripts (Llave et al., 2002; Brodersen et al., 2008).

The crucial role of miRNAs in controlling plant developmental processes and response to abiotic stress is well documented (De Lima et al., 2012). Alterations in the accumulation of a substantial fraction of the miRNAome during pathogen infection is also described in different pathosystems, and for some miRNAs a role in plant immunity has been described (Shivaprasad et al., 2012; Campo et al., 2013; Boccara et al., 2014; Li et al., 2014; Baldrich and San Segundo, 2016; Soto-Suárez et al., 2017). However, our current knowledge of the biological roles of pathogen-regulated miRNAs in plant immunity is still limited, and most comes from studies in the interaction of Arabidopsis thaliana with the bacterial pathogen Pseudomonas syringae (Staiger et al., 2013; Weiberg et al., 2014; Fei et al., 2016; Kuan et al., 2016).

miRNAs are thought to have originated by duplication of pre-existing protein-coding genes with subsequent mutations (Allen et al., 2004; Rajagopalan et al., 2006). The spontaneous evolution from hairpin structures in the genome, or derivation from transposable elements, has also been proposed to explain the origin of plant miRNAs (Felippes et al., 2008; Nozawa et al., 2012). Whole-genome duplication events, and tandem or segmental duplications of MIR genes, are believed to be responsible for the expansion and diversification of miRNA gene families in plants (Maher et al., 2006; Nozawa et al., 2012). In animals, the occurrence of miRNA clusters is common, but only a few miRNA clusters have been described in plants, mainly in Arabidopsis (Boualem et al., 2008; Merchan et al., 2009; Barik et al., 2014; Baldrich et al., 2016). These clustered miRNAs can be transcribed independently or simultaneously as polycistronic transcripts. Furthermore, transcripts of polycistronic miRNAs might contain copies of members belonging to the same miRNA family (homologous polycistron), or unrelated miRNAs (non-homologous polycistron).

The miR166 family comprises multiple members in monocotyledonous and dicotyledonous plants that are transcribed independently (monocistrons). This is a highly conserved family of miRNAs with conserved target genes, the Class III homeodomain-leucine zipper (HD-ZIP III) transcription factors. These transcription factors, such as the Arabidopsis PHABULOSA (PHB) and PHABOLUTA (PHV), are involved in diverse developmental processes (Emery et al., 2003; Itoh et al., 2008). Altered accumulation of miR166 during abiotic stress also led to the notion that miR166 might play a role in the plant response to diverse abiotic stresses. Very recently, it has been described that miR166 knockdown triggers drought resistance in rice (Zhang et al., 2018). Evidence for miR166 in adapting to pathogen infection in plants has not been reported.

Recently, we described the occurrence of a rice polycistronic miRNA, miR166k-166h, comprising two miR166 family members (miR166k and miR166h). Expression profiling revealed that mature miRNAs generated from the miR166k-166h precursor are co-expressed in rice leaves (Baldrich et al., 2016). In other studies, various miR166 species were found to differentially respond to infection by the rice blast fungus M. oryzae or to differentially accumulate in blast-resistant and blast-susceptible rice varieties (Li et al., 2014, 2016).

In this work, we present evidence supporting that MIR166k-166h plays a role in rice immunity. We show that rice plants with activated MIR166k-166h expression exhibit resistance to infection by the fungal pathogens M. oryzae and Fusarium fujikuroi, the causal agents of the rice blast and bakanae disease, respectively. Rice blast is one of the most devastating diseases of cultivated rice due to its widespread distribution and destructiveness (Wilson and Talbot, 2009). The phenotype of disease resistance is associated with a stronger induction of defense responses during pathogen infection. MIR166h-166k expression was strongly induced by M. oryzae infection in blast-resistant but not in blast-susceptible rice varieties. Moreover, we identified a novel target gene for miR166k, the ethylene-insensitive 2 (EIN2) gene (targeted by miR166k-5p in the miR166k-166h polycistron). Overall, our results support that the polycistronic miR166k-166h positively regulates rice immunity through modulation of EIN2 expression.

MATERIALS AND METHODS

Plant Material

Rice (Oryza sativa) plants were grown at 28°C/22°C under 16-h light/8-h dark conditions. The T-DNA insertion line for MIR166k-166h (M0110144) and wild-type genotype (O. sativa japonica cv Taining 67) were obtained from the Taiwan Rice Insertional Mutant collection (TRIM1). Genotyping of the TRIM mutant was carried out by PCR on genomic DNA using a T-DNA-specific primer located at the left border of the T-DNA and a primer located in the vicinity of the insertion site. PCR products were confirmed by DNA sequencing. Quantitative PCR (qPCR) was used to determine the T-DNA copy number in the rice mutant with the monocopy sucrose phosphate synthase gene used as the endogenous reference (Ding et al., 2004) (primers are listed in Supplementary Table S1).

The rice cultivars Saber, TeQing, Kanto 51, Maratelli and Vialone Nano were obtained from the germplasm seed bank of the Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA-Rice Research Unit, Vercelli, Italy).

Infection Assays and Elicitor Treatment

The fungus M. oryzae (strain Guy-11) was grown on complete media as described (Campos-Soriano et al., 2012). For infection assays with M. oryzae, 3-week-old plants were spray-inoculated with a spore suspension (5 × 10⁵ spores/ml), or mock-inoculated. Development of disease symptoms was followed over time. Lesion area was determined by using Assess 2.0 software (American Phytopathological Society). For infection assays with Fusarium fujikuroi, the fungus was grown on PDA (Difco, Franklin Lakes, NJ, United States). Rice seeds were

1http://trim.sinica.edu.tw/
pregerminated for 24 h on Murashige and Skoog (MS) medium and then inoculated with a suspension of *F. fujikuroi* spores (1 \( \times 10^6 \) spores/ml), or sterile water. Seedlings were allowed to continue germination for 1 week. Three independent infection experiments were performed, with at least 24 plants per genotype in each experiment. Statistically significant differences were determined by one-way ANOVA. qPCR was used to quantify fungal DNA in infected leaves with specific primers for the 28S DNA gene of the corresponding fungus (Qi and Yang, 2002; Jeon et al., 2013). For this, standard curves were prepared by using *M. oryzae* or *F. fujikuroi* DNA.

For elicitor treatment, 3-week-old plants were sprayed with an elicitor suspension of *M. oryzae* (3 \( \times 10^2 \) µg/ml) or mock-inoculated as described (Casacuberta et al., 1992).

### 1-Aminocyclopropane-1- Carboxylic Acid (ACC) Treatment

Three-week old rice plants were treated with ACC (Merck, Darmstadt, Germany) at a concentration of 50 µM for 15 min, 1, 4, and 24 h. Control plants were mock-inoculated.

#### RT-qPCR, Stem-Loop RT-PCR and 5’ RACE-PCR

Total RNA was extracted by using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from DNAse-treated total RNA (1 µg) with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, United States) and oligo-dT. RT-qPCR was performed with Light Cycler 480 and SYBR Green (Invitrogen, Carlsbad, CA, United States). For this, standard curves were prepared by using *M. oryzae* or *F. fujikuroi* DNA.

For elicitor treatment, 3-week-old plants were sprayed with an elicitor suspension of *M. oryzae* (3 \( \times 10^2 \) µg/ml) or mock-inoculated as described (Casacuberta et al., 1992).

### RESULTS

**MIR166k-166h Activation Enhances Resistance to Infection by the Rice Blast Fungus *M. oryzae***

The rice genome contains several loci encoding monocistronic miR166s distributed on 7 chromosomes: miR166a, miR166b, miR166c, miR166d, miR166e, miR166f, miR166g, miR166i, miR166j, miR166l and miR166m (miRBase release 21) (Supplementary Figure S1). Furthermore, a polycistronic miR166 encoding two miR166 family members, the miR166k-166h precursor, was identified on chromosome 2 (Baldrich et al., 2016). The mature miR166k and miR166h species locate in one or another hairpin of the miR166k-166h precursor structure (Figure 1A, left panel). Of note, loci encoding monocistronic transcripts for miR166k or miR166h have not been identified in the rice genome.

In this work, a T-DNA tagged line (M0110144) carrying the T-DNA insertion upstream of the *MIR166k-166h* locus was identified in the TRIM collection produced in the Tainung 67 (TN67) background (Hsing et al., 2007). Of note, mutant alleles for miRNAs are not easily found in insertional mutant collections because of the small size of *MIR* genes. The T-DNA contains 8 copies of the *CaMV35* enhancer near the left border, and genes within 15 kb of the T-DNA left border and 5 kb of the right border might be activated by these enhancers. Knowing this, we hypothesized that this mutant might be an activation mutant for *MIR166k-166h*. The T-DNA insertion site was confirmed by PCR genotyping followed by DNA sequencing of the PCR products (Supplementary Figure S2A). No homozygous *MIR166k-166h* plants were identified in PCR genotype screens. Most importantly, heterozygous mutant plants accumulated higher levels of miR166k-166h precursor transcripts, which correlated well with an increase in the accumulation of mature...
FIGURE 1 | Characterization of polycistronic miR166k-166h mutant plants. (A) Structure of the miR166k-166h precursor and location of mature miR166 sequences (left panel). The accumulation of miR166k-166h precursor transcripts and mature miR166 sequences in wild-type (TN67) and miR166k-166h-Ac mutant plants was determined by RT-qPCR and stem-loop RT-qPCR, respectively (right panels). Note that the stem-loop RT-qPCR does not discriminate among miR166k-3p and miR166h-3p sequences (indicated as miR166kh-3p). (B) Phenotype of wild-type and miR166k-166h-Ac mutant plants at 7 days post-inoculation with M. oryzae spores (5 $\times$ 105 spores/ml). The percentage of leaf area affected by blast lesions was determined by image analysis (APS Assess 2.0) (right upper panel). Quantification of M. oryzae DNA was determined by qPCR with specific primers of the M. oryzae 28S gene (right lower panel). (C) Resistance to infection by F. fujikuroi in miR166k-166h-Ac mutant plants. Pictures were taken at 7 days after inoculation with fungal spores. Quantification of fungal DNA was carried out by qPCR using specific primers for F. fujikuroi (right panel). (D) Accumulation of transcripts for the defense marker genes OsPBZ1 and OsPR1a in wild-type and miR166k-166h-Ac plants in response to M. oryzae infection determined by RT-qPCR. Plants were inoculated with M. oryzae spores (5 $\times$ 105 spores/ml) or mock-inoculated (+ and –, respectively). Ct values obtained in the PCR reactions were normalized to the average Ct values for the cyclophilin 2 gene (for graphical representation, the values are multiplied by 100). Data are mean ± SD (**P ≤ 0.01; ***P ≤ 0.001; ANOVA test, M. oryzae-inoculated versus mock-inoculated).
miR166k and miR166h sequences (Figure 1A, right panel). Since the rice genome does not contain monocistronic miR166k and miR166h loci, the miR166k and miR166h mature sequences accumulating in rice leaves are expected to be generated from the polycistronic miR166k-166h precursor. These observations confirmed that the TRIM mutant is an activation mutant for MIR166k-166h (hereafter referred to as miR166k-166h-Ac). However, miR166h has been shown to repress the seed maturation program in Arabidopsis, and difficulties in generating transgenic lines overexpressing miR166 were previously reported (Tang et al., 2012). Presumably, high levels of miR166 expression and concomitant silencing of HD-ZIP III might compromise normal plant development. Therefore, it is not surprising that homozygous miR166k-166h-Ac mutant plants could not be identified in this study. The miR166k-166h-Ac mutant harbors a single copy of the T-DNA inserted in its genome (Supplementary Table S2).

We considered the possibility that the expression of genes other than MIR166k-166h might be activated in the miR166k-166h-Ac mutant. Two genes, OsSAUR12 (Os02g52990) and Erwinia-induced protein (Os02g53000), were identified upstream and downstream, respectively, of the T-DNA insertional site (Supplementary Figure S2A). However, we found no altered accumulation of OsSAUR12 or Erwinia-induced protein transcripts in the miR166k-166h-Ac mutant (Supplementary Figure S2B). There were no obvious phenotypic differences between miR166k-166h mutant and wild-type plants under controlled greenhouse conditions (Supplementary Figure S2C).

To investigate whether miR166k-166h miRNA plays a role in rice immunity, we performed blast disease resistance assays. Wild-type (cv TN67) and miR166k-166h-Ac plants were spray-inoculated with spores of the rice blast fungus M. oryzae, and disease symptoms were followed over time. The miR166k-166h-Ac plants consistently showed reduced disease symptoms as compared with wild-type plants (Figure 1B, left panel). Blast resistance was confirmed by quantification of fungal biomass and determination of lesion area in the infected leaves (Figure 1B, right panels).

The miR166k-166h-Ac mutant plants also showed enhanced resistance to infection by the fungus F. fujikuroi, the causal agent of bakanae in rice (Ou, 1985). The fungus infects the plant through the roots (or crowns) and grows systemically within the plant. At 7 days after inoculation, the miR166k-166h-Ac seedlings exhibited more vigorous growth of the root system compared to wild-type seedlings which also had extensive necrosis in their roots (Figure 1C, left panel). Quantification of fungal biomass confirmed limited fungal growth in roots of miR166k-166h-Ac seedlings (Figure 1C, right panel).

To obtain further insights into the mechanisms underlying disease resistance in the miR166k-166h-Ac mutant, we determined the expression pattern of the defense genes OsPBZ1 (Probenazole-inducible 1) and OsPR1a (Pathogenesis-Related 1a) in mutant and wild-type plants at different times after infection with M. oryzae (24, 48, and 72 h post-inoculation [hpi]). OsPBZ1 (a member of the PR10 family of PR genes) and OsPR1a genes are markers for the activation of the rice defense response to M. oryzae infection (Midoh and Iwata, 1996; Agrawal et al., 2001). As expected, fungal infection induced OsPR1a and OsPBZ1 expression in wild-type plants. Importantly, transcript levels of these defense genes were higher in M. oryzae-inoculated miR166k-166h-Ac than M. oryzae-inoculated wild-type plants at all times of infection (Figure 1D). These findings support that the miR166k-166h-Ac mutant responds to pathogen challenge with a super-induction of defense genes, which is consistent with the phenotype of disease resistance observed in these plants.

**MIR166k-166h Expression During Fungal Infection and Treatment With Elitcitors**

Given that activation of MIR166k-166h affects disease resistance, we sought to investigate whether MIR166k-166h expression is itself regulated during the normal host response to infection. Upon pathogen challenge, miR166k-166h precursor transcript level was increased in leaves of M. oryzae-inoculated compared with non-inoculated wild-type TN67 plants, with a parallel increase in mature miR166k and miR166h sequences (both miRNA-5p and miRNA-3p species) (Figure 2A). Interestingly, accumulation of precursor and mature miR166 sequences also increased in response to treatment with a crude preparation of elicitors (Figure 2B). Elicitor treatment resulted in faster induction of miR166k-5p and miR166h-5p species versus miR166kh species. Induction of marker genes of defense activation, OsPBZ1 and OsPR1a, confirmed that the host plant detects and responds to elicitor treatment (Supplementary Figure S3A). Finally, we examined the elicitor-responsiveness of the monocistronic miR166s, miR166a and miR166c. The accumulation of precursor transcripts for these miR166 family members (pre-miR166a and pre-miR166c) was found to be transiently, but not significantly, regulated during elicitor treatment (Supplementary Figure S3B).

From these results, we concluded that pathogen infection and also treatment with fungal elicitors upregulates MIR166k-166h expression, which suggests a role of this polycistronic miRNA in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI).

The promoter region of protein-coding genes often includes cis-acting regulatory elements responsible for pathogen inducibility. Knowing that fungal infection and elicitor treatment induced MIR166k-166h expression, we scanned the MIR166k-166h promoter region for the presence of cis-regulatory elements related to biotic stress. The sequence upstream of the precursor structure for the miR166k-166h precursor was extracted from the NCBI database and the transcription start site (TSS) was identified by using the TSSP Softberry program for identifying TSS in plants\(^1\). cis-acting elements present in the 1.6 Kb DNA region upstream of the TSS were searched in the PLACE database\(^2\). The MIR166k-166h promoter was found to contain an important number of cis-elements required for response to pathogen infection or elicitor treatment (Supplementary Figure S4 and Supplementary Table S3). We identified several W-boxes (TGAC core sequences), such as WBOXATNPR1 (TTGAC), elicitor responsive element (ERE, TTCAGG), WRKY710S\(^3\).

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\(^1\)http://softberry.com/

\(^2\)http://www.dna.affrc.go.jp/PLACE
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FIGURE 2 | MIR166k-166h expression in response to M. oryzae infection or treatment with M. oryzae elicitors in wild-type (cv. TN67) plants. The accumulation of miR166k-166h precursor and mature miR166 sequences was determined by RT-qPCR and stem-loop RT-qPCR, respectively, at different times after inoculation with M. oryzae spores (5 × 10^8 spores/ml) (A) or treatment with M. oryzae elicitors (3 × 10^2 µg/ml) (B). Values represent the fold-induction of gene expression (Ct) in M. oryzae-inoculated versus mock-inoculated plants. Three independent experiments were carried out (P ≤ 0.05, ANOVA test).

(TGAC), WBOXNTERF3 (TGACY), and ASF1 (TGACG) cis-elements (Supplementary Figure S4). These regulatory cis-elements are the binding sites for salicylic acid-induced WRKY transcription factors and are also found in many pathogen- and elicitor-responsive genes. The SEBF regulatory element (SEBFCONSSTPR10A, YTGTCWC), initially characterized in the promoter of the pathogen and elicitor inducible potato PR-10a gene and later in the promoter of several other PR genes, was also identified in the MIR166k-166h promoter. Other functional pathogen/elicitor-responsive elements identified were the GT1-SCAM4 (GAAAAA) and PAL-responsive (CCGTCC) elements. Finally, regulatory elements associated with defense-related hormone signaling also present in the MIR166k-166h promoter included the ethylene (ERELEEE4, ethylene-responsive element; AWTTCAAA) and methyl jasmonic acid (T/G BOXPIN2, AACGTG) regulatory elements.

MIR166k-166h Expression in Resistant and Susceptible Rice Varieties

We examined MIR166k-166h expression in rice varieties showing a phenotype of disease resistance against the rice blast fungus: Kanto 51, Saber and TeQing (resistant varieties), and Vialone Nano and Maratelli (susceptible varieties). The resistant genotypes are characterized by the presence of the resistance (R) genes: Pik in Kanto51, and Pib in Saber and TeQing (Tacconi et al., 2010). The basal level of expression varied among the different rice varieties (Figure 3). At 72 hpi with M. oryzae. MIR166k-166h expression was strongly induced in the three resistant rice genotypes here assayed, whereas its expression was barely affected or was even decreased by M. oryzae infection in the susceptible cultivars Vialone Nano and Maratelli (Figure 3). Thus, induction of MIR166k-166h expression appears to occur in resistant but not susceptible rice cultivars.

Prediction and Experimental Validation of a Novel Target for miR166

As previously mentioned, HD-ZIP III genes are conserved target genes for miR166 in plants. In monocistronic miR166s, the mature miR166 sequences that direct cleavage of HD-ZIP III transcripts are located at the 3’ arm of the precursor structure, namely miR166h-3p and miR166k-3p. In rice, five HD-ZIP III genes have been described: Oshox9 (Os10g33960), Oshox10 (Os03g01890), Oshox29 (Os01g10320), Oshox32 (Os03g43930) and Oshox33 (Os12g41860) (Agalou et al., 2008). Degradation tags indicative of miR166-mediated cleavage of Oshox9, Oshox10, Oshox32, and Oshox33 were identified by degradome analysis, which supports that they are real targets of rice miR166s (Li et al., 2010; Baldrich et al., 2015). In addition, RT-qPCR analysis revealed reduced levels of Oshox9, Oshox10, and Oshox32 in miR166k-166h-Ac mutant versus wild-type plants (Figure 4A),
FIGURE 3 | Accumulation of miR166k-166h precursor transcripts in susceptible and resistant rice varieties M. oryzae spores or mock-inoculated (+ and −, respectively). RT-qPCR analysis of accumulation of miR166k-166h precursor transcripts at 72 hpi. Data are mean ± SD (∗∗P ≤ 0.01; ∗P ≤ 0.05, ANOVA test).

which confirms the functionality of mature miRNAs encoded by the polycistron. As for Oshox29 and Oshox33, these genes were found expressed at very low levels in wild-type plants, and their expression was not significantly affected in miR166k-166h-Ac mutant plants as compared with wild-type plants (Figure 4A).

Knowing that MIR166k-166h activation has an impact on blast resistance, we considered the possibility that this phenotype might be caused by the activity of miR166 species encoded in the miR166k-166h precursor on novel, non-conserved target genes. We performed a target prediction analysis by using the psRNATarget tool. Similar to other species, the target search predicted HD-ZIP III as target genes of miR166s encoded in the miR166k-166h polycistron (miR166k-3p and miR166h-3p). This computational prediction identified a putative target gene for the miR166k-5p sequence, the EIN2 gene. As for miR166h-5p, a possible binding site for this miRNA in a ferredoxin-nitrite-reductase gene was predicted.

A function for EIN2 as mediator of ethylene-dependent defense responses in plants is well established (Iwai et al., 2006; Helliwell et al., 2013, 2016; Yang et al., 2017). Accordingly, in this work we investigated whether EIN2 is a target gene for miR166k-5p. In contrast to Arabidopsis, in which EIN2 is encoded by a single gene, the rice genome possesses four EIN2 genes: OsEIN2.1 (also named MHZ7; Os07g06130), OsEIN2.2 (Os03g49400), OsEIN2.3 (Os07g06300), and OsEIN2.4 (Os07g06190) (Ma et al., 2013; Yang et al., 2015). Based on sequence homology, OsEIN2 genes can be classified into two groups, the first comprising OsEIN2.1 and OsEIN2.2 and the second OsEIN2.3 and OsEIN2.4. The four OsEIN2 genes have the binding site for miR166k-5p (Supplementary Figure S5A). RT-qPCR analysis was used to quantify OsEIN2.1, OsEIN2.2 and OsEIN2.3/4 expression in wild-type (TN67) and mutant plants (with the high sequence homology between OsEIN2.3 and OsEIN2.4, we could not design PCR-specific primers for these genes). Location of the primers used for detection of EIN2.1, EIN2.2 and EIN2.3/2.4 is shown in Supplementary Figure S5B. This analysis revealed downregulation of OsEIN2.1 and OsEIN2.2 in miR166k-166h-Ac plants (Figure 4B, left panel). The observed inverse correlation between mature miR166k-5p levels and EIN2.1 and EIN2.2 transcripts in miR166k-166h-Ac plants already indicated a possible miR166k-5p-mediated downregulation of this particular OsEIN2 family members. Intriguingly, OsEIN2.3/4 transcripts accumulated to a higher level in miR166k-166h-Ac mutant than wild-type plants. The amount of uncleaved OsEIN2 transcripts was determined by using PCR primers flanking the miR166k-5p cleavage site. Although the accumulation of uncleaved EIN2 transcripts was notably reduced in the activation mutant, uncleaved transcripts still accumulated to an important level in these plants, likely due to the contribution of EIN2.3/EIN2.4 transcripts (Figure 4B, right panel). This observation suggests the existence of complex regulatory mechanisms governing the expression of OsEIN2 in rice in which downregulation of OsEIN2.1 and OsEIN2.2 expression is accompanied by an increase in EIN2.3/EIN2.4 transcripts.

These observations prompted us to further investigate whether EIN2 gene is a real target gene for miR166k-166h by performing RNA ligase-mediated 5′ RACE (5′-RACE). Sequencing of the 5′-RACE PCR clones revealed cleavage
FIGURE 4 | Expression of miR166 targets and identification of a novel target gene for miR166. (A) RT-qPCR analysis of accumulation of Oshox transcripts encoding HD-ZIP III transcription factors in wild-type and miR166k-166h-Ac plants. (B) Expression of OsEIN2 genes. Left panel: Gene-specific PCR primers were used for quantification of OsEIN2.1, OsEIN2.2 or OsEIN2.3/2.4 transcripts (EIN2.3 and EIN2.4 sequences are closely related each other, and specific primers for these genes could not be designed). Right panel: RT-PCR using primers spanning the miR166 target site in EIN2 transcripts. Data (A, B) are mean ± SD (***P ≤ 0.001; **P ≤ 0.01, ANOVA test). (C,D) Experimental validation of miR166-mediated cleavage of OsEIN2.1 (C) and Oshox32 (D) transcripts by 5′ RACE. Gene-specific primers were used for 5′ RACE and the resulting PCR products (right panels) were sequenced. The identified cleavage sites are indicated by an arrow, and the numbers below indicate the detected cleavage site of independent clones. WT, TN67. Data are mean ± SD (***P ≤ 0.001; **P ≤ 0.01, ANOVA test).
fragments of EIN2.1 transcripts (Figure 4C, left panels). Transcripts were found cleaved at the canonical position of miRNA/target mRNA pairing (between nucleotides 10 and 11 from the 5' end of the miRNA), which supports that EIN2 is indeed a target gene for miR166 in rice. As well, cleavage products of EIN2.1 accumulated to a lower level in wild-type than miR166k-166h-Ac plants (Figure 4C, right panel). As a control, miR166-guided cleavage products of hox32 were also identified by 5'-RACE (Figure 4D). Altogether, these results demonstrated that miR166 cleaves EIN2.1 transcripts and that the miR166k-5p strand in the miR166k-166h precursor is functional.

miR166k-166h Mediates Cleavage of EIN2 Transcripts That Reduce Levels of EIN2 Protein

Among the newly identified miR166 targets, EIN2 is worth describing specifically. This gene is a central signal transducer in the ethylene signaling pathway in plants, and ethylene signaling is known to modulate plant immune responses (Solano and Ecker, 1998; Jun et al., 2004; Denancé et al., 2013; Ma et al., 2013).

To further investigate the functional relationship between miR166h-166k activity and EIN2 expression, we performed agroinfiltration experiments in N. benthamiana leaves in which the miR166k-166h precursor and a GFP-tagged EIN2.1 gene were co-expressed. As controls, constructs bearing the empty vector, the miR166k-166h precursor alone, or the EIN2.1-GFP chimeric gene alone were also assayed in agroinfiltration experiments. RT-PCR analysis revealed the accumulation of precursor miR166k-166h transcripts in agroinfiltrated leaves (Figure 5A, left panel). Accumulation of mature miR166 sequences derived from this precursor was confirmed by ST-RT-qPCR and Northern blot analyses (Figure 5A and Supplementary Figure S6). These analyses indicated that the miR166k-166h precursor is properly expressed and processed in N. benthamiana leaves when expressed alone or with EIN2.1-GFP. However, levels of miR166k-166h transcripts were higher in miR166k-166h-only agroinfiltrated leaves versus leaves in which the miR166k-166h precursor was co-expressed with EIN2.1 (Figure 5A, left panel, pre-miR166 and pre-miR166+EIIN2), an aspect that deserves further investigation.

When examining the transcript accumulation of EIN2.1, co-expression of the miR166k-166h precursor with EIN2.1-GFP reduced the EIN2.1-GFP transcript level as compared with expression of EIN2.1-GFP alone (Figure 5B, left panel, EIN2 and pre-miR166+EIN2). The observed reduction in EIN2.1-GFP transcripts was accompanied by a reduced EIN2-GFP protein level, as revealed by immunoblotting of protein extracts with an anti-GFP antibody (Figure 5B, right panel). From these results, we conclude that miR166k-166h targets and cleaves OsEIN2.1 and that cleavage of OsEIN2.1 transcripts reduces EIN2 protein accumulation.

Finally, knowing that MIR166k-166h expression is upregulated during M. oryzae infection in wild-type (cv TN67) plants (Figure 2A), and that OsEIN2.1 is a target gene for miR166, we investigated the expression of OsEIN2 family members during pathogen infection. OsEIN2.1 and OsEIN2.2 were downregulated during M. oryzae infection (Figure 5C), which is consistent with the observed increase in miR166k-5p level in the same tissues. In contrast, OsEIN2.3/4 expression was upregulated during pathogen infection. Presumably, the increased level of miR166k-5p in M. oryzae-infected leaves would be responsible for downregulation of OsEIN2.1 during pathogen infection.

Expression of Ethylene Signaling Components in miR166k-166h-Ac Plants

In the absence of ethylene, phosphorylation of EIN2 prevents transduction of ethylene signaling. However, in the presence of ethylene, EIN2 phosphorylation is reduced and the C-terminal fragment of EIN2 is cleaved and translocated to the nucleus where the downstream EIN3 and EIL1 transcriptional cascade is activated. In addition, EIN2 and EIN3/EIL1 are regulated by proteasomal degradation via EIN3-binding F-box protein 1 and 2 (EBF1/2). Then, EIN3 and EIL1 regulate the expression of ethylene-responsive genes including Ethylene Response Factor 1 (ERF1) which, in turn, modulates the expression of various ethylene-responsive genes such as PDF1.2 and chitinase genes (Lorenzo et al., 2003; Abiri et al., 2017). It is generally assumed that EIN2 functions as a positive regulator of ethylene signaling, as revealed by repression of ethylene-inducible defense genes in ein2 antisense rice plants (Jun et al., 2004). The construct used to obtain ein2 antisense rice plants covered a 638-bp DNA fragment of the EIN2.1 cDNA encompassing the C-terminal region of EIN2, a region with high sequence conservation among OsEIN2 family members. Thus, silencing of all four OsEIN2 genes is expected to occur in the ein2 antisense plants previously described (Jun et al., 2004).

Accumulating evidence also indicates that ethylene signaling is required in rice for basal resistance against the blast fungus M. oryzae (Singh et al., 2004; Iwai et al., 2006; Helliwell et al., 2013, 2016; Yang et al., 2017). Thus, the observed increase in miR166k-166h accumulation and concomitant downregulation of OsEIN2.1 and OsEIN2.2 expression in miR166k-166h-Ac plants (Figures 1, 4, respectively) apparently contradicts OsEIN2 positively regulating ethylene signaling in the rice response to M. oryzae infection.

To address the apparent contradiction of downregulation of OsEIN2 expression in miR166h-166k-Ac plants, showing blast resistance, we investigated the expression of genes acting downstream of EIN2 in the ethylene signaling pathway in mutant plants. OsEIN3 and OsEIL1, as well as OsERF1, were upregulated in miR166k-166h-Ac plants as compared with wild-type plants, whereas OsEBF2 expression was downregulated (Figure 6A). Consistent with upregulation of OsERF1, the expression of ethylene-responsive defense genes, such as PDF1.2 and chitinase genes (e.g., CHIT8 and CHIT14, members of the PR3 family of PR genes; and WIP5, a PR4 family member) was also upregulated in miR166k-166h-Ac plants (Figure 6B). These data indicate that although miR166k-166h activation downregulates OsEIN2.1 and OsEIN2.2, components in the pathway for ethylene signal transduction downstream of OsEIN2 are induced in miR166k-166h-Ac plants, which would agree with the resistance phenotype.
that is observed in miR166k-166h-Ac mutant plants. Knowing that OsEIN2.3/2.4 is activated in the miR166k-166h-Ac mutant (see Figure 4B), OsEIN2.3/2.4 activation is likely responsible for the observed induction of downstream components of ethylene signaling in these plants, including ethylene-regulated defense genes.
FIGURE 6 | Expression of ethylene signaling components in miR166k-166h-Ac mutant plants, and MIR166k-166h expression in response to treatment with the ethylene precursor ACC. RT-qPCR analysis of the accumulation of transcripts for the indicated genes with use of gene-specific PCR primers. Ct values obtained in the PCR reactions were normalized to the average Ct values for the cyclophilin 2 gene. (A) Expression of genes acting downstream of EIN2 (OsEIN3, OsEIL1, OsERF1 and OsEBF2) in wild-type and miR166k-166h-Ac plants. (B) Expression of OsPDF1.2, OsWIP5 (PR4 family), OsCHIT8 and OsCHIT14 (PR3 family). (C) Accumulation of miR166k-166h precursor transcripts in control and ACC-treated (50 µM) wild-type plants. (D) Accumulation of EIN2.1, EIN2.2 and EIN2.3/2.4 in control and ACC-treated wild-type plants. Data are mean ± SD (**P ≤ 0.01; *P ≤ 0.05; ANOVA test).
To provide additional clues for the function of miR166k-166h in rice immunity, we investigated whether MIR166k-166h expression itself is regulated by ethylene in wild-type plants. For this, wild-type plants were treated with the ethylene precursor ACC, and the accumulation of miR166k-166h precursor transcripts was determined at different times after ACC treatment (15 min, 1, 4, and 24 h). ACC treatment resulted in a clear and gradual increase in the accumulation of miR166k-166h precursor transcripts in wild-type plants (Figure 6C).

Finally, expression analysis was performed to determine the accumulation of EIN2 transcripts in wild-type in response to ACC treatment. Consistent with up-regulation of MIR166k-166h in response to ACC treatment, EIN2.1 and EIN2.2 were found to be down-regulated during the same period of treatment (Figure 6D). However, EIN2.3/2.4 transcripts accumulated at a higher level in ACC-treated plants compared to control plants (Figure 6D). Thus, a different trend in the regulation of EIN2 family members occurs in response to ACC treatment which correlates with differences previously observed between miR166k-166h-Ac mutant plants and wild-type plants (see Figure 4B).

**DISCUSSION**

In this work, we provide evidence that the polycistronic miR166k-166h plays a role in rice immunity. Thus, activation of MIR166k-166h in miR166k-166h-Ac plants, and concomitant increase in mature miR166s derived from the miR116h-166k polycistron in the rice genome of both M. truncatula and African rice. These observations support conservation of the miR166 clusters in the rice genome. An intriguing aspect of this study was the finding of a different trend in the regulation of OsEIN2 expression in miR166k-166h-Ac plants depending on the family member. Whereas EIN2.1 and EIN2.2 are downregulated in the rice mutant of these miR166 clusters has not been demonstrated (Boualem et al., 2008; Zhang et al., 2009; Barik et al., 2014; Li et al., 2017).

Our evidence supports that EIN2 is a novel target gene for miR166, this gene being targeted by miR166k-5p in the MIR166k-166h polycistron. Supporting this conclusion, we found opposite expression patterns of miR166k-5p and OsEIN2.1 in miR166k-166h-Ac mutant plants. Also, miR166k-5p and its target gene showed opposite expression patterns in response to fungal infection (upregulation and downregulation, respectively). Definitive proof of a miR166k-5p-mediated cleavage of EIN2.1 transcripts came from 5′-RACE analyses and agroinfiltration experiments in N. benthamiana leaves. The observed miR166-guided cleavage of EIN2.1 transcripts was accompanied by reduced EIN2 protein level. From these results, we conclude that EIN2.1 represents a novel target gene for miR166k-5p species encoded by the polycistronic miR166k-166h precursor.

Clearly, the existence of multiple miR166 family members might contribute to diversification and functional specialization of miR166 in plants. In line with this, miR166b has been reported to target rice RDD1 (rice Dof daily fluctuations 1), a non-HD-ZIP III transcription factor involved in nutrient uptake and accumulation (Iwamoto and Tagiri, 2016). Very recently, miR166-guided cleavage of ATHB14-LIKE transcripts encoding a homeobox-leucine zipper protein has been described in soybean (Li et al., 2017). In M. truncatula, a miR166 polycistron containing two copies of miR166a targeting HD-ZIP III transcripts was found to control root architecture and nodule development after infection by Sinorhizobium meliloti (Boualem et al., 2008). Presumably, mature miRNAs encoded by the miR166k-166h polycistron might have evolved to mediate rice defense responses to pathogen infection.

When considering the mature miR166s encoded by the miR166k-166h precursor, we noticed that miR166 species targeting OsEIN2.1 correspond to miR166-5p in monocistronic miR166s, while miR166-3p sequences target hox genes. Hence, the two strands of the miR166 duplex in the miR166k-166h precursor appear to be functional. There are other examples in which the two strands of a miRNA are functional, as for miR939 in Arabidopsis. Here, the miR939 strand guides cleavage of transcripts encoding auxin receptor genes (TIR1, AFB2, AFB3), and the miR939-3p strand cleaves MEMB12 transcripts encoding a SNARE protein involved in exocytosis of the PR1 protein (Zhang et al., 2011). Degradome analysis revealed miR166e-3p and miR166h-5p-mediated events for genes involved in the arbuscular mycorrhizal symbiosis in Medicago truncatula (e.g., Sucrose synthase, Tyr protein kinase and protein phosphatase 2C) (Devers et al., 2011). In addition to being represented by multiple copies in the rice genome, the ability of miR166 precursors to produce two mature functional strands in the same miRNA-5p/miRNA-3p duplex also represents an effective strategy to diversify miR166 function.

Our results indicate that MIR166k-166h activation enhances defense gene expression, most probably by modulating OsEIN2 expression. An intriguing aspect of this study was the finding of a different trend in the regulation of OsEIN2 expression in miR166k-166h-Ac plants depending on the family member.
either (the two genes being more closely related to one another than and the existence of regulatory mechanisms in which miR166k-5p and OsEIN2.3/2.4 OsEIN2 interconnecting networks controlling the expression of ethylene-regulated defense genes is in turn would regulate components of the ethylene signaling pathway leading to induction of ethylene-regulated defense genes (PDF1.2, chitinases). We propose an interlocking regulation mechanism governing the expression of OsEIN2 family members and downstream signaling components leading to activation of defense gene expression. Further studies are required to determine the interlocking mechanisms among OsEIN2 family members and among miR166k-miR166h and EIN2.

Basal resistance to M. oryzae has been reported to require activation of ethylene biosynthesis and signaling networks during the biotrophic phase of the infection process (Singh et al., 2004; Iwai et al., 2006; Helliwell et al., 2013, 2016; Yang et al., 2017). However, the mechanisms by which the pathogen induces ethylene biosynthesis remain unknown. Because MIR166k-166h expression is itself regulated by treatment with the ethylene precursor ACC (Figure 6), the M. oryzae-induced production of ethylene might induce MIR166k-166h expression. Furthermore, the M. oryzae-mediated ethylene accumulation has been found to affect JA signaling (Yang et al., 2017). Whether defense hormone networking is altered in miR166k-166h-Ac plants deserves further investigation.

It is also known that ethylene has antagonistic effects in controlling the rice defense response depending on the pathogen lifestyle and also on the type of pathogen. Whereas the accumulation of ethylene appears to be required for resistance against M. oryzae (Iwai et al., 2006), repression of ethylene signaling has been shown to enhance resistance against the necrotrophic rice brown spot fungus Cochliobolus miyabeanus (Vleeshouwer et al., 2010). A major future challenge is to determine the molecular processes by which MIR166k-166h function is integrated in the complex regulatory mechanisms involved in ethylene-regulated immune responses to M. oryzae infection and whether activation of MIR166h-166k expression confers resistance to pathogens other than M. oryzae.

Besides playing a role in plant responses to pathogen infection, ethylene is considered a phytohormone involved in regulation of plant growth and development. Because excessive ethylene production under pathogen infection might negatively affect plant development, the host plant must then maintain a tight control of ethylene homeostasis to cope with pathogenic infections with no growth penalty. In this respect, negative feedback mechanisms have been proposed to coordinate plant growth and ethylene/salinity responses (Tao et al., 2015).

Given the well-established roles of miR166 and its HD-ZIP III target genes in controlling developmental processes in a broad range of plant species, an intriguing question is why MIR166k-166h activation does not affect normal growth in the miR166k-166h mutant. A possible threshold of miR166k-166h level (and subsequent miR166-regulated Oshox transcripts) might explain this observation. Under heterozygosity, the miR166k-166h-Ac mutant plants would not accumulate sufficient levels of M. oryzae elictors

![Diagram](https://via.placeholder.com/150)

**FIGURE 7** Proposed model for the function of miR166k-166h in ethylene signaling during infection of rice plants by the blast fungus M. oryzae. In the absence of ethylene, active ethylene receptors negatively regulate OsEIN2 via phosphorylation, thus repressing the downstream signaling transduction. Pathogen recognition would trigger ethylene biosynthesis, which is perceived by its receptors. Upon ethylene perception, the EIN2 phosphorylation is reduced and the carboxy-terminal fragment of EIN2 is cleaved and translocated to the nucleus for activation of EIN3/EL1 and Ethylene Response Factor 1 (ERF1), thereby activating defense gene expression. Pathogen-induced ethylene production would also induce MIR166k-166h expression, which would then regulate the expression of OsEIN2 family members (downregulation of OsEIN1.2 and OsEIN2.2; upregulation of OsEIN2.3/2.4). MIR166k-166h activation in miR166k-166h mutant plants would mimic the activation of ethylene signaling pathways induced by M. oryzae infection in the host plant. Arrows and blunt ends indicate positive and negative regulation, respectively. Arrows with broken lines indicate still unknown interlocked regulatory mechanisms among EIN2 family members.
of miR166kh species to alter normal developmental programs due to excessive downregulation of miR166 HD-ZIP III target genes. Moderate levels of mature miR166s produced by the miR166k-166h polycistron would provide a means to mount a more successful defense response without no penalty on normal development.

The functional significance of the organization of miRNAs as polycistrons is still debated. Polycistronic transcription can fine-tune gene expression in related or unrelated biological processes (e.g., defense responses and developmental processes). A single promoter drives the expression of polycistronic miRNAs, which allows for the expression of multiple miRNAs in a coordinated spatial and/or temporal manner.

CONCLUSION

Our results support that miR166k-166h is a positive regulator of rice immunity via regulation of OsEIN2. A better knowledge of miR166k-166h functioning in blast resistance will help in deciphering the functional consequences of polycistronic expression of miRNAs in plants. Because blast is one of the primary causes of rice losses worldwide, unraveling miR166k-166h-mediated mechanisms underlying blast resistance could ultimately help in designing novel strategies for crop protection.

AUTHOR CONTRIBUTIONS

RS-G performed the experiments and analyzed the data. Y-iH and BSS designed and conceived the work. All the authors contributed to the manuscript writing.

FUNDING

This work was supported by grants from the Spanish Ministry of Economy and Competitiveness [BIO2012-32838, BIO2015-67212-R] and the CSIC/NSC (Spanish Research Council/National Science Council of Taiwan)-Cooperative Research Project-Formosa Program (2009TW0041). We also acknowledge financial support from the CERCA Program from the Generalitat de Catalunya, and MINECO through the “Severo Ochoa Program for Centers of Excellence in R&D” 2016-2019 [SEV-2015-0533]”. RS-G is a recipient of a Ph.D. grant from the Spanish Ministry of Economy and Competitiveness (BES-2013-065521).

ACKNOWLEDGMENTS

We thank Dr. J-S Zhang (CAS, Beijing) for the EIN2 cDNA, Dr. D. Baulcombe (University of Cambridge, United Kingdom) for the N. benthamiana RDR6-IR line, and Drs. E. Lupotto and G. Valé for resistant and susceptible rice cultivars. We also thank N. Fernández for assistance with parts of this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00337/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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