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A novel Transposable element-derived microRNA participates in plant immunity to rice blast disease

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Summary

MicroRNAs (miRNAs) are small non-coding RNAs that direct post-transcriptional gene silencing in plant development and stress responses through cleavage or translational repression of target mRNAs. Here, we report the identification and functional characterization of a new member of the miR812 family in rice (named as miR812w) involved in disease resistance. miR812w is present in cultivated *Oryza* species, both *japonica* and *indica* subspecies, and wild rice species within the *Oryza* genus, but not in dicotyledonous species. miR812w is a 24nt-long that requires DCL3 for its biogenesis and is loaded into AGO4 proteins. Whereas overexpression of miR812w increased resistance to infection by the rice blast fungus *Magnaporthe oryzae*, CRISPR/Cas9-mediated *MIR812w* editing enhances disease susceptibility, supporting that miR812w plays a role in blast resistance. We show that miR812w derives from the *Stowaway* type of rice MITEs (Miniature Inverted-Repeat Transposable Elements). Moreover, miR812w directs DNA methylation *in trans* at target genes that have integrated a *Stowaway* MITE copy into their 3′ or 5′ untranslated region (*ACO3*, *CIPK10, LRR* genes), as well as *in cis* at the *MIR812w* locus. The target genes of miR812 were found to be hypo-methylated around the miR812 recognition site, their expression being up-regulated in transgene-free CRISPR/Cas9-edited miR812 plants. These findings further support that, in addition to post-transcriptional regulation of gene expression, miRNAs can exert their regulatory function at the transcriptional level. This relationship between miR812w and Stowaway MITEs integrated into multiple coding genes might eventually create a network for miR812w-mediated regulation of gene expression with implications in rice immunity.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that direct gene silencing at the post-transcriptional level in eukaryotes. In plants, miRNAs are transcribed from *MIR* genes as long single-stranded primary transcripts containing a unique stem-loop structure which is processed in two steps by a RNAse III DICER-LIKE (DCL) protein to produce double-stranded miRNA duplexes (miRNA-5p/miRNA-3p, previously named miRNA/miRNA* duplexes) (Arikit *et al.,* 2013; Axtell, 2013; Jones-Rhoades *et al.,* 2006; Rogers and Chen, 2013). The two strands of the miRNA-5p/miRNA-3p duplex are methylated at the 3′ end and the functional strand of the duplex is loaded into an ARGONAUTE (AGO)-containing RNA-induced silencing complex (RISC) (Vaucheret, 2008). miRNAs guide the RISC to the target mRNA via base pairing and mediate gene silencing through target cleavage or translational inhibition (Brodersen *et al.,* 2008; Llave *et al.,* 2002).

Besides miRNAs, many other classes of small RNAs are produced in plants, collectively termed small-interfering RNAs (siRNAs) that are distinguished from miRNAs by their distinct biogenesis pathways. Whereas miRNAs derive from single-stranded RNA precursors with stem-loop structures, siRNAs arise from double-stranded RNA precursors produced by RNA-dependent RNA polymerases (RDRs) or from sense-antisense transcript pairs.

Plant *MIR* genes are thought to have originated by duplication events that created perfect inverted repeat loci that evolved by random mutation into short, imperfectly paired, stem-loop structures characteristic of *MIR* genes (Allen *et al.,* 2004). The spontaneous evolution from hairpin structures in the genome is also believed to be the origin of plant miRNAs (De Felippes *et al.,* 2008; Nozawa *et al.,* 2012). Additionally, transposable elements (TEs) are regarded as an important source of inverted repeats and several miRNAs have been shown to derive from TEs (Cho, 2018; Li *et al.,* 2011; Piriyapongsa and Jordan, 2008). Ancient *MIR* genes give rise predominantly to canonical 21-nt miRNAs that are generated by DCL1 (Kurihara and Watanabe, 2004). By contrast, recently evolved miRNAs are processed by DCL3 or DCL4 (rather than by DCL1) to produce miRNAs of variable length, usually 23- to 25-nt long-miRNAs (referred to as long miRNAs or lmiRNA) (Cuperus *et al.,* 2011; Nozawa *et al.,* 2012; Rajagopalan *et al.,* 2006; Vazquez *et al.,* 2008). Changes in the fold-back structure of a miRNA precursor have been proposed to determine DCL usage in precursor processing (from DCL3/DCL4 to DCL1) (Cuperus *et al.,* 2011; Nozawa *et al.,* 2012). In rice and *Arabidopsis*, the production of functional 24 nt miRNAs by DCL3 is well documented (Chellappan *et al.,* 2010; Cuperus

et al., 2011; Vazquez *et al.,* 2008; Wu *et al.,* 2010; Zhu *et al.,* 2008).

MiRNAs function as central regulators of gene expression in many aspects of plant growth and development (Chen, 2009; Song *et al.,* 2019), as well as in adaptive responses to abiotic and biotic stresses, including pathogen infection (Baldrich and San Segundo, 2016; Li *et al.,* 2010; Navarro *et al.,* 2006; Staiger *et al.,* 2013; Sunkar *et al.,* 2012; Weiberg *et al.,* 2014). Even though, most miRNAs so far identified in plants guide cleavage or translational repression of target genes, certain miRNAs have been shown to direct DNA methylation (Bao *et al.,* 2004; Wu *et al.,* 2010).

The plant immune system consists of many interconnected processes that are induced upon pathogen recognition which are defined principally according to the molecules recognized by the host plant. Plants recognize pathogen epitopes, known as Pathogen-Associated Molecular Patterns (PAMPs), also known as elicitors, which then activates a general defence response referred to as PAMP-triggered immunity (PTI) (Boller and Felix, 2009; Jones and Dangl, 2006; Li *et al.,* 2020a). PTI components include protein phosphorylation/dephosphorylation, reinforcement of cell wall, production of reactive oxygen species (ROS) and induction of defence-associated genes (i.e. *Pathogenesis-Related* or *PR* genes). During co-evolution, pathogens have evolved effectors that are delivered to plant cells and suppress PTI leading to disease susceptibility. In turn, some plants have evolved another immune response in which microbial effectors, or host proteins modified by these effectors, are recognized by proteins encoded by Resistance (R) genes. This recognition triggers a rapid and effective host defence called Effector-Triggered Immunity (ETI). Most studies to date on plant immunity focused on transcriptional reprogramming induced by pathogen infection, and less is known about post-transcriptional processes controlling disease resistance.

Contrary to antiviral innate immunity which relies on RNA silencing mechanisms, antibacterial and antifungal immune responses have been traditionally considered as protein-based defence mechanisms, largely independent of RNA silencing mechanisms. Nowadays, the scenario has changed and RNA-based silencing mechanisms are known to play an important role both in PTI and ETI to bacterial and fungal pathogens in plants (Staiger *et al.,* 2013). Most of our knowledge about miRNAs involved in plant immunity comes from studies in the dicotyledonous model plant *A. thaliana* during interaction with the bacterial pathogen *Pseudomonas syringae*. Here, treatment with the flagellin-derived elicitor peptide flg22 from *P. syringae* causes an increase in miR393, and subsequent down-regulation of auxin receptor genes, leading to antibacterial resistance (Navarro *et al.,* 2006). Other miRNAs with a known function in disease resistance in *Arabidopsis* are: miR156, miR160, miR396, miR398, miR400, miR472, miR773, miR844, miR858 and miR863 (Boccara *et al.,* 2014; Camargo-Ramírez *et al.,* 2018; Lee *et al.,* 2015; Li *et al.,* 2010; Niu *et al.,* 2016; Park *et al.,* 2014; Salvador-Guirao *et al.,* 2018a; Soto-Suárez *et al.,* 2017; Yin *et al.,* 2019). However, even though an important number of miRNAs have been shown to be pathogen-responsive in different plant species, the biological function and mode of action of most of these pathogen-regulated miRNAs in immunity remains elusive. Key challenges remain with respect to the role of miRNAs controlling disease resistance in crop species.

Rice (*Oryza sativa* L) is one of the most important cereal crops and the primary source of food for more than half of the world's population. Rice is also considered the model organism for functional genomics in monocotyledonous plants and reference genomes are available for both *indica* and *japonica* subspecies (Goff *et al.,* 2002; Yu *et al.,* 2002). At present, rice is the third plant species with the highest number of miRNAs annotated in miRBase with 738 mature miRNAs (after *Glycine max* and *Medicago truncatula*, with 756 miRNAs each (miRBase release 22) (Kozomara *et al.,* 2019). The biological function of only a small fraction of rice miRNAs has been deciphered.

One of the major factors limiting rice production is the rice blast disease caused by the fungal pathogen *Magnaporthe oryzae* (Wilson and Talbot, 2009). Previous studies revealed that a substantial fraction of the miRNAome is regulated during *M. oryzae* infection, and/or treatment with *M. oryzae* elicitors in rice plants (Baldrich *et al.,* 2015; Campo *et al.,* 2013; Li *et al.,* 2016). However, only few miRNAs with a function in blast resistance have been reported, which include positive (miR7695, miR160, miR162, miR398 and miR166k-166h) and negative (miR156, miR164, miR167, miR169, miR319, miR396, miR442 and miR1873) regulators of immune responses (Campo *et al.,* 2013; Chandran *et al.,* 2019; Li *et al.,* 2014, 2017, 2020b; Salvador-Guirao *et al.,* 2018b; Sánchez-Sanuy *et al.,* 2019; Wang *et al.,* 2018; Zhang *et al.,* 2018b, 2020; Zhao *et al.,* 2020; Zhou *et al.,* 2019). Clearly, the involvement of miRNAs in rice immunity has only begun to emerge.

In the present study, we describe a previously uncharacterized miRNA, a member of the miR812 family of rice miRNAs. The newly identified miRNA is provisionally designated as miR812w. We show that CRISPR/Cas9-induced mutations in *MIR812w* results in enhanced susceptibility to *M. oryzae* infection. Conversely, *MIR812w* overexpression confers resistance to infection by the rice blast fungus *M. oryzae*. miR812w is a DCL3-dependent 24 nt long miRNA that specifically associates with AGO4. We also show that miR812w directs cytosine DNA methylation in *trans* at miR812w target genes as well as at its own locus. Collectively, results here presented support a role of miR812w in controlling disease resistance in rice, most probably, through methylation of target genes.

Results

miR812w, a novel miRNA from rice

In previous studies, we reported a set of small RNAs representing novel miRNA candidates from rice (Baldrich *et al.,* 2015; Campo *et al.,* 2013). In this study, one of those miRNA candidates (previously named as miR-75; Campo *et al.,* 2013) was selected for further investigation.

Initially, we investigated whether this small RNA fulfils the criteria already established for the assignment of small RNA sequences as miRNAs (Axtell and Meyers, 2018). They included: the structural features of the miRNA precursor and small RNAs mapping to this precursor, evidence of excision from the stem-loop precursor structure and expression (RNA gel blotting and data from small RNA-seq libraries), and existence of small RNA sequences forming a miRNA-5p/miRNA-3p duplex with 2 nucleotide (nt) 3′ overhands (a typical feature of DCL cleavage). We also examined the accumulation of this small RNA in DCL and RDR mutants, and its association to AGO proteins.

The predicted secondary structure for the precursor of the miRNA candidate under study is presented in Figure 1a. By mapping all small RNAs identified in small RNA libraries from rice tissues (leaves, roots) (Baldrich *et al.,* 2015), a centred distribution

of sequences mapping to the opposite arms of this structure (or isomiRs) was observed (Figure S1). miRNA isomers are known to be produced during miRNA biogenesis and have been proposed to be caused by DCL slippage (Gozmanova *et al.,* 2017). Most of the small RNAs mapping to the stem-loop region of this precursor were 24 nt in length (Figure S1).

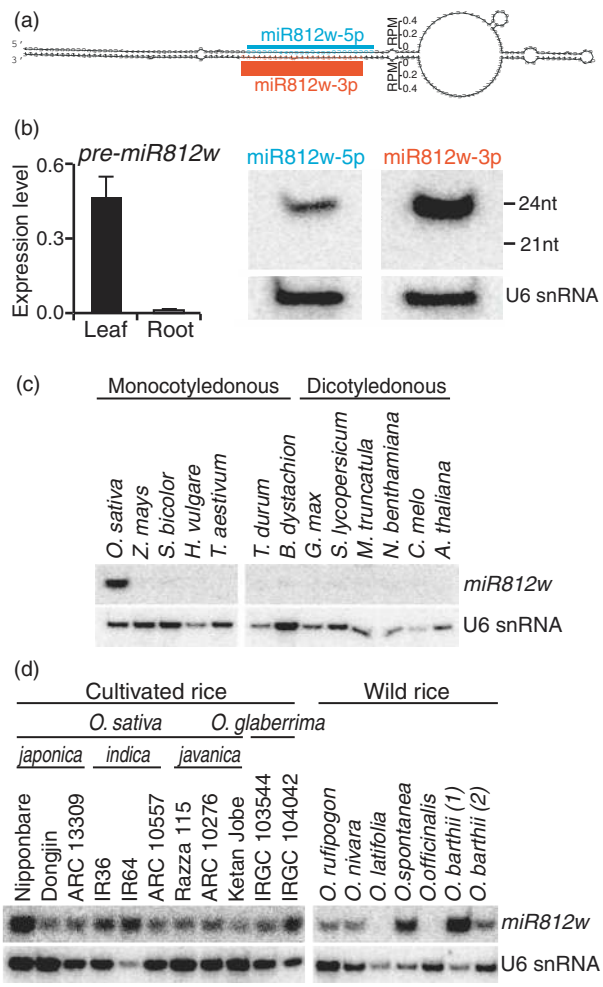


Figure 1 Validation of the novel miRNA candidate under study as a novel miRNA from rice. The newly identified miRNA is a member of the rice miR812 family, which has been named as miR812w. (a) Structure of the miRNA precursor. The small RNAs identified in small RNA-seq datasets (Baldrich *et al.,*2015) mapping to the 5′ and 3′ arm of this precursor (named as miR812w-5p and miR812w-3p) are 24-nt in length and have 2-nt overhangs at both 3′ ends. RPM, Reads per million. (b) Accumulation of miR812w precursor transcripts in leaves and roots of 21 days-old rice plants (left panel) as determined by RT-qPCR. Northern blot detection of miR812w-5p and miR812w-3p (right panel) in leaves of 21 days-old rice plants. A representative blot out of three independent biological replicates is shown. (c) Northern blot analysis of osa-miR812w in monocotyledonous and dicotyledonous species. (d) Northern blot analysis of osa-miR812w in cultivated varieties of the genus *Oryza* (*O. sativa*, Asian rice; *O. glaberrima*, African rice) and wild species of the genus *Oryza*. The small RNA fraction obtained from 100 μg of total RNA was probed with a P^{32}-labelled synthetic oligonucleotide complementary to the miR812w-3p sequence (indicated in Table S4). RNA blots were also probed with the U6 probe for loading control (lower panel). Oligonucleotide sequences used as primers are listed Table S4. The complete list and geographical distribution of *Oryza* species analysed in this study is indicated at Table S1

A search in the miRBase registry revealed homology of the nucleotide sequence of this precursor with that of miR812 precursors from rice, the miR812 family consisting of 22 members (miR812a to miR812v) (Figure S2). The small RNA sequence mapping to the 3p arm of the precursor structure also showed partial homology with mature miR812 sequences registered in miRBase (Figure S3). Here, it should be mentioned that the annotated mature miR812 family members have substantial differences among them (Figure S3). Likely previously performed annotations of miR812 species need to be revised. Following naming in miRBase, we provisionally named this miRNA as miR812w.

MIR812w was expressed in leaves of rice plants, but not in roots (Figure 1b, left panel). Most notably, DNA sequencing of the RT-PCR products using specific primers designed to amplify the precursor structure revealed that both 5p and 3p small RNAs are comprised in the miR812w precursor transcription unit, confirming the identity of the miR812w precursor and excluding the possible amplification of other miR812 precursors. Small RNA northern blot analysis confirmed the accumulation of both miR812w-5p and miR812w-3p small RNA species in rice leaves, of which miR812w-3p sequences accumulated at the highest level (Figure 1b, right panel). The higher accumulation of miR812w-3p sequences was also observed by sRNAseq analysis in rice leaves (Figure 1a; Figure S1). Together, these findings support that miR812w represents a previously uncharacterized member of the miR812 family.

miR812w is detected in rice, but not in other plant species

According to miRBase, miR812 family members are identified only in rice. To investigate whether miR812w is present in plant species other than rice, we examined miR812w accumulation in several monocotyledonous and dicotyledonous species. As shown in Figure 1c, miR812w was detected only in rice but not in any of the other monocotyledonous or dicotyledonous species assayed in this work. This finding suggests miR812w might be a rice-specific miRNA that could have evolved after monocot-dicot divergence.

Next, we examined miR812w accumulation in cultivated and wild rice species. The genus *Oryza* comprises two cultivated species, *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice). *Oryza sativa* is the most extended cultivated rice and comprises *japonica* and *indica* subspecies. Further, *japonica* rice is classified into temperate *japonica* (*japonica*) and tropical *japonica* (*javanica*). In this work, rice varieties representative of *O. sativa* (*japonica*, *javanica* and *indica*) and *O. glaberrima* were examined for miR812w accumulation (rice accessions and genome type are indicated in Table S1). Although at a different level, miR812w accumulated in all the cultivated rice varieties, both *O. sativa* and *O. glaberrima* cultivars (Figure 1d). When examining miR812w accumulation in wild rice, miR812w accumulation was observed in *O. rufipogon, O. nivara* and *O. spontanea*, but not in *O. latifolia* or *O. officinalis* (Figure 1d). miR812w also accumulated in *O. barthii*, the wild relative of *O. glaberrima* (African rice) (Figure 1d). All the wild species in which miR812w was present have an AA-genome type, while *Oryza* species in which miR812 was absent have a CC (*O. latifolia*) or CCDD (*O. officinalis*) genome (Table S1). The observation that miR812w is present in the ancestors of modern *Oryza* cultivars, namely *O. nivara* and *O. rufipogon* (the two closest relatives of Asian rice *O. sativa*) (Choi *et al.,* 2017; Wang *et al.,* 2014) and *O. barthii* (the wild

ancestor of African rice *O. glaberrima*) (Choi *et al.,* 2017; Wang *et al.,* 2014) might be indicative of conservation during domestication of *Oryza* rice (Asian and African rice).

miR812w is processed by DCL3 and specifically associates with rice AGO4

As previously mentioned, ancient miRNAs are generated by DCL1 which gives rise to 21-nt canonical miRNAs, whereas recently evolved miRNAs are preferentially processed by DCL3 and/or DCL4 to produce different sizes of miRNAs (Cuperus *et al.,* 2011; Rajagopalan *et al.,* 2006; Vazquez *et al.,* 2008). In this study, we investigated the accumulation of miR812w sequences in knockdown *Osdcl1*, *Osdcl3* and *Osdcl4* rice mutants (Liu *et al.,* 2005, 2007; Song *et al.,* 2012). Northern blot analysis of small RNAs revealed that, compared with the wild-type plants, the accumulation of miR812w was compromised in the *dcl3*, but not in the *dcl1* or *dcl4* mutants (Figure 2a). Definitive probe of DCL3 dependence for miR812w production was obtained by surveying the pre-miR812w-derived small RNA sequences in available data sets of rice *dcl* mutants (Wei *et al.,* 2014), observing a strong decrease in miR812w species in *dcl3a* mutants (Figure 2b), but not in the *dcl1* mutant (Figure S4). More importantly, miR812w accumulation was not abolished in the *rdr2-2* mutant, indicating that the biogenesis of miR812w does not require RDR2 (Figure S4). Instead, miR812w accumulated in the *rdr2-2* mutant plants, a phenomenon that has been previously observed for other rice and *Arabidopsis* miRNAs in *rdr2* mutants (Kasschau *et al.,* 2007; Wu *et al.,* 2010). An overrepresentation of miRNAs appears to occur when the production of RDR2-dependent 24-nt siRNAs is lost in the *rdr2* mutant plants (Wu *et al.,* 2010). Altogether, these results support the involvement of DCL3 in the production of mature 24-nt *MIR812w* species.

Apart from their biogenesis, the mode of action of small RNAs is tightly related with the Argonaute protein, the effector protein of the RISC, to which they can bind (Vaucheret, 2008). Whereas canonical 21-nt miRNAs having a 5′-terminal uridine (5′ U) preferentially and stably associate with AGO1, AGO4 predominantly recruits 24-nt small RNAs with a 5′-terminal adenine (5′A). Examining AGO-bound sRNA is then a starting point in distinguishing functional from non-functional miR812w species.

To determine miR812w AGO loading preference, we examined available datasets of AGO-associated small RNAs from rice, namely AGO1 (AGO1a and AGO1b) and AGO4 (AGO4a and AGO4b) (Wu *et al.,*2009, 2010). Sequences from the miR812w-3p strand were found to be specifically associated with AGO4b and to a lesser extent also to AGO4a (Figure 2c). Conversely, no miR812w enrichment was observed in any of the AGO1 proteins (Figure 2d). This fact agrees with preferential loading of AGO4, as miR812w-3p is a 24-nt small RNAs with a 5′-terminal adenine (5′A). These findings suggest that miR812w-3p exerts its function through the AGO4-containing RISC and that miR812w-3p is the functional strand of the miR812w duplex.

CRISPR/Cas9-mediated mutagenesis of *MIR812w* enhances susceptibility to infection by *M. oryzae*

The functional analysis of *MIR* genes has been challenging, in part, due to the lack of effective approaches to obtain loss-of-function mutations of *MIR* genes. Traditional approaches to study the function of *MIR* genes (e.g. mutagenesis) are largely ineffective due to the small size of *MIR* genes, and most of our current understanding of miRNA function comes from transgenic lines overexpressing either a miRNA or a miRNA-resistant target

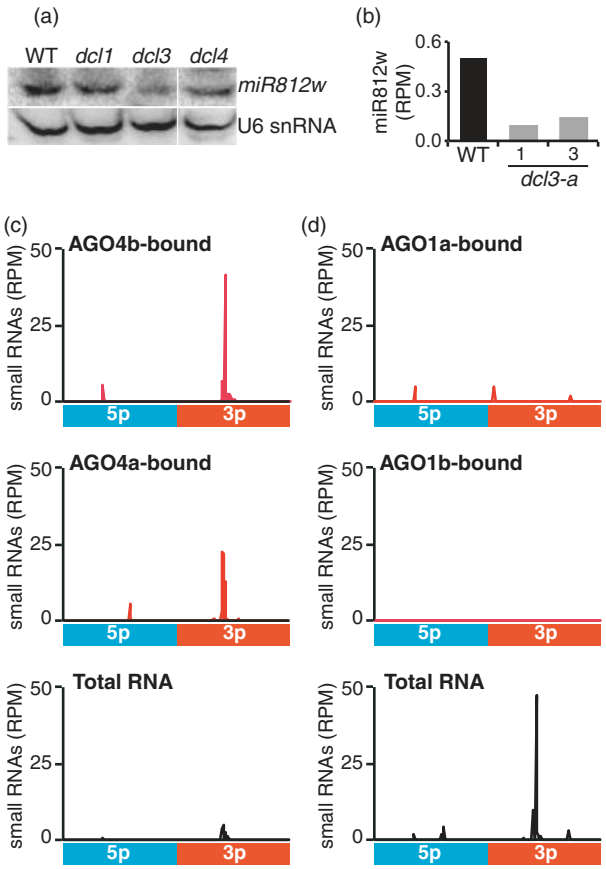


Figure 2 Biogenesis of miR812w. (a) Northern blot analysis of miR812w in rice *dcl1, dcl3* and *dcl4* mutants. RNA blots were also probed with the U6 probe for loading control (lower panel). (b) Abundance of miR812w-3p in *dcl3a* rice mutant lines. (c-d) Total sRNA and AGO-bound reads plotted over the miR812w precursor. The 5p and 3p arms are indicated. (b-d) Small RNA data were obtained from published libraries from seedlings of *O. sativa* cv Nipponbare of *dcl3a* mutant lines (Wei *et al.,*2014) and immunoprecipitation with AGO proteins (Wu *et al.,*2009, 2010). Scale bar indicate small RNA read number normalized per one million reads (RPM)

gene or expressing an artificial target mimics. The target mimicry technology, however, does not discriminate among miRNA family members sharing mature miRNA sequences.

miR812w was originally identified in a search for miRNAs whose accumulation is regulated by treatment with elicitors obtained from the rice blast fungus *M. oryzae* (Campo *et al.,* 2013; previously named as miR-75). To investigate whether *MIR812w* plays a role in blast resistance, we used the CRISPR/Cas9 technology. A sgRNA was designed for targeting the *MIR812w* precursor sequence which contains a Protospacer Adjacent Motif (PAM) sequence (UGG) close to the 5′ end of the mature miR812w sequence (Figure 3a). PCR analysis of genomic DNA followed by DNA sequencing using gene-specific primers located upstream and downstream of the target site was used to identify CRISPR/Cas9-induced mutations. In this way, three independent mutant alleles were identified in hygromycin-resistant T0 plants, which contained a deletion of 22 nucleotides (Δ22nt), a deletion of 3 nucleotides (Δ3nt), or an insertion of 1 nucleotide (+1nt) (Figure 3a). Mutations detected in T0 followed Mendelian inheritance without the occurrence of mutations

different from those mentioned above. Plants bearing one of these alleles (Δ22nt, Δ3nt or +1nt,) were selected for subsequent analyses.

An examination of the predicted secondary structure of the CRISPR/Cas9-edited mutant alleles revealed that the Δ22nt deletion removes the lower stem region of the *miR812w* precursor, whereas the Δ3nt or +1nt mutations does not substantially affect its secondary structure (Figure 3b). We reasoned that the 22 nt deletion may have a significant impact on miR812w production. Along with this, small RNA northern blot analysis revealed a drastic reduction on the accumulation of mature miR812w sequences in the Δ22nt mutant (hereafter named as miR812w-Δ22 plants) (Figure 3c). In contrast, miR812w accumulation was not affected in the +1nt mutant plants (Figure 3c). The Δ22nt deletion, however, did not totally abolish mature miR812w accumulation, which can be attributed to either inefficient processing of the miR812w precursor or cross-hybridization with other miR812 family members.

Homozygous Cas9-free CRISPR/Cas9-edited miR812 plants harbouring the −22 deletion at the miR812w locus were identified (Figure S5a). The *miR812w-Δ22* plants grew and developed normally, although at the adult stage these plants were slightly smaller in height than wild-type plants (Figure S5b).

Next, we examined whether the Δ22 mutation generated in the *MIR812w* precursor by the CRISPR/Cas9 system has a consequence in blast disease resistance of rice. For this, three-week-old *mir812w-Δ22* mutant and control plants were spray-inoculated with *M. oryzae* spores, and disease symptoms were followed over time. Compared with wild-type plants, the homozygous *mir812w-Δ22* plants consistently exhibited higher

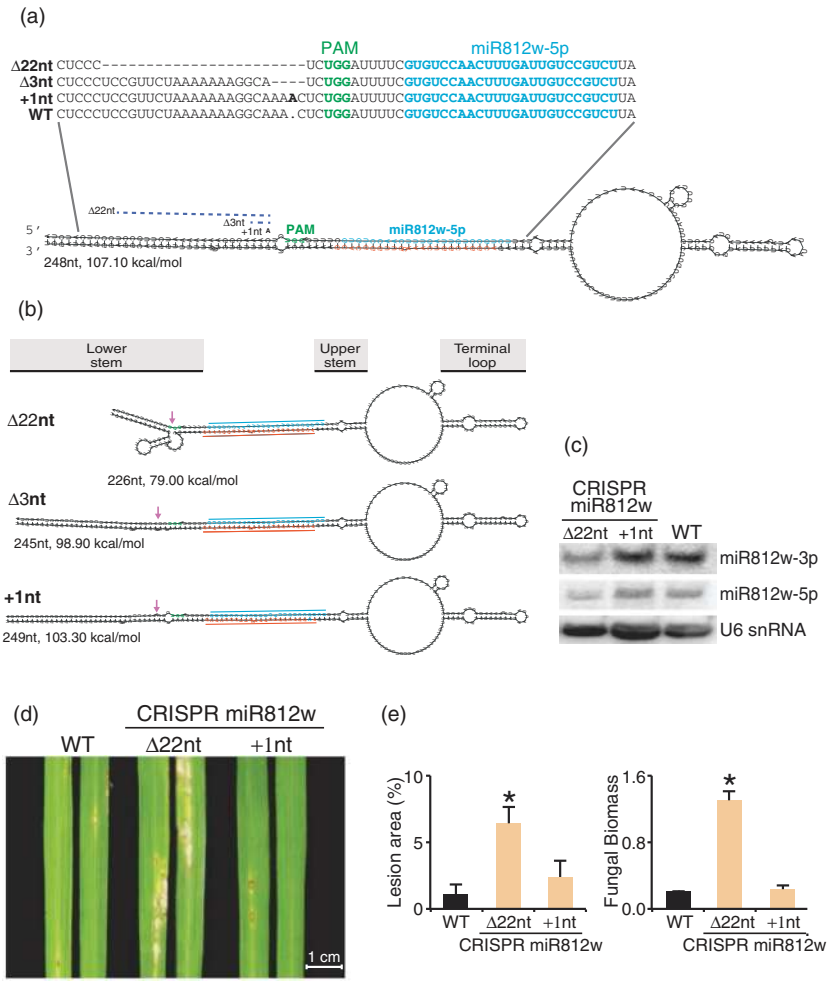


Figure 3 CRISPR/Cas9-induced mutations in the *MIR812w* gene and infection phenotype to *M. oryzae*. (a) Mutations generated by CRISPR/Cas9 editing are shown in the upper part of the miR812w precursor structure. Three miR812w alleles were obtained: Δ22nt, Δ3nt and +1nt. Dashes indicate deletions. In bold, single nucleotide insertion. The PAM motif (UGG) is next to miR812w-5p. WT, Wild-type. (b) Predicted secondary structure of the mutated miR812w precursor (Δ22nt, Δ3nt, +1nt) using RNAfold. The length of the precursor (nt) and the minimum free energy of folding (MFE, kcal/mol) are indicated. The position of the CRISPR/Cas9 mutation is indicated by an arrow. (c) Accumulation of miR812w as determined by northern blot using a ^{32}P-labelled oligonucleotide for detection of miR812w-3p and miR812w-5p (Table S4). The small RNA fraction was isolated from total RNA of leaves (WT, Δ22nt, +1nt plants). RNA blots were also probed with the U6 probe for loading control (lower panel). (d–e) Disease phenotype of *mir812w-Δ22* and *mir812w+1* rice plants. (d) Representative images of *M. oryzae*-infected CRISPR/Cas9-edited miR812w plants at 7dpi. (e) Quantification of blast lesions was carried out by image analysis (left panel). Relative quantification of fungal biomass was determined by qPCR using specific primers for *M. oryzae* 28S DNA (values are fungal DNA levels normalized against the rice *Ubiquitin 1* gene (right panel). Primers are listed in Table S4. Data from one representative experiment of three independent experiments are presented as the mean ± SE (n = 15) (Student t-test, $*P < 0.05$), which gave similar results

susceptibility to *M. oryzae* infection (Figure 3d). The enhanced susceptibility of *mir812w*-Δ*22* plants was confirmed by quantification of the area of blast lesions and the amount of fungal biomass in the infected leaves (Figure 3e). No clear differences were observed in disease susceptibility between CRISPR/Cas9-edited *MIR812w* plants carrying the +1nt insertion (*mir812w+1*) compared to wild-type plants (Figure 3d, e), which is consistent with the observation that the 1 nt insertion does not greatly affect miR812w accumulation (Figure 3c). It is tempting to hypothesize that removal of the lower stem region of miR812w precursor structure in the *mir812w*-Δ*22* mutant might affect miR812w precursor processing, hence, miR812w function.

Taken together, these results demonstrate that a deletion of 22nt in the *MIR812w* precursor is accompanied by an increase on susceptibility to infection by the blast fungus *M. oryzae*. These findings also illustrate the usefulness of the CRISPR/Cas9 technology for the analysis of miRNA function.

MIR812w overexpression increases resistance to *M. oryzae* infection

Knowing that a mutation generated by CRISPR/Cas9 editing results in enhanced susceptibility to *M. oryzae* infection, we sought to investigate the effect of miR812w overexpression in pathogen resistance. For this, we generated transgenic rice (*O. sativa* cv Nipponbare) constitutively expressing *MIR812w* (hereafter named as miR812w-OE plants) (Figure S6a). Before transforming rice, we confirmed efficient processing of the cloned pre-osa-miR812w precursor into mature miR812w by transient expression in *Nicotiana benthamiana* (Figure S6b).

Compared with control plants (wild-type, empty vector transgenic plants and azygous lines segregated from T0 plants), the miR812w-OE lines accumulated higher levels of precursor and mature miR812w sequences (Figure 4a). No obvious phenotypical differences between homozygous miR812w-OE plants and wild-type plants were observed (Figure S6c). The transgenic miR812w-OE lines contained a single copy of the transgene (T3 generation) as revealed by qPCR using the singly copy *SPS* (*sucrose phosphate synthase*) gene for normalization (Table S2).

Blast resistance assays were performed with three independent homozygous miR812w-OE lines. By visual inspection, the miR812w-OE plants consistently showed reduced disease symptoms as compared with control plants (Figure 4b). Quantification of symptoms in leaves confirmed that miR812w-OE plants exhibited smaller areas of blast lesions and less fungal biomass than those on control plants (Figure 4c). Same results were observed at the T2 and T3 generation. Consistent with the phenotype of blast resistance, miR812w-OE plants exhibited a higher induction of *PR1* expression (a marker of pathogen-induced defence responses) compared to that in wild-type plants (Figure S7). In contrast, *M. oryzae* inducibility was compromised in *mir812w*-Δ*22* (Figure S7). Collectively, results here presented indicated that *MIR812w* overexpression enhances resistance to infection by the blast fungus *M. oryzae,* while CRISPR/Cas9-edited *mir812w*-Δ*22* rice plants exhibited blast susceptibility. These findings further support for the involvement of miR812w in blast resistance in rice.

H_2O_2 accumulation in *M. oryzae*-infected leaves of miR812w overexpressor and CRISPR/Cas9-edited plants

Knowing that an alteration in *MIR812w* expression by either overexpression or CRISPR/Cas9-induced mutation has an effect on the host response to pathogen infection, we sought to determine whether *MIR812w* expression was itself pathogen-responsive in wild-type plants. Compared with non-infected plants, *MIR812w* expression was transiently activated during *M. oryzae* infection (at 48 h post-inoculation) (Figure 5a, left panel). Treatment with a crude preparation of *M. oryzae* elicitors also increased *MIR812w* expression (Figure 5a, right panel).

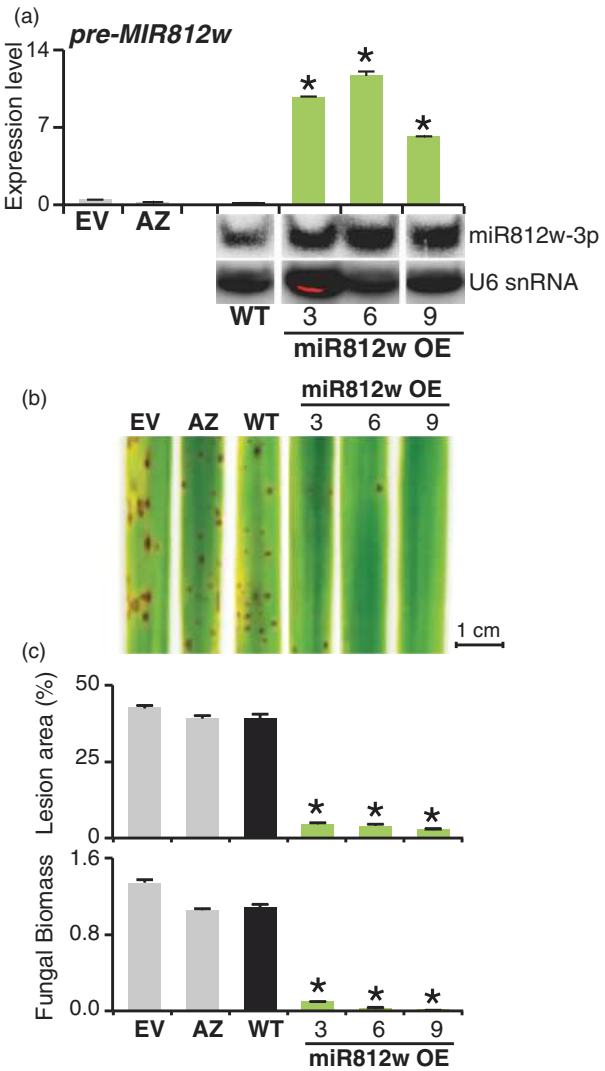


Figure 4 Resistance of rice plants overexpressing *MIR812w* (miR812w-OE) to *M. oryzae* infection. Three independent miR812w-OE lines and control plants (EV, AZ and WT) were assayed. WT, wild-type; EV, transgenic empty vector; AZ, segregated azygous plants. (a) Accumulation of miR812w precursor transcripts (upper panel) and mature miR812w (lower panel) sequences in transgenic rice lines determined by RT-qPCR and small RNA northern blot analysis, respectively. Lower panels show U6 small RNA (snRNA) as loading controls. (b–c) Disease phenotype of miR812w-OE plants (d) Representative images of *M. oryzae*-infected miR812w-OE plants at 7dpi. Scale bar (1 cm). (e) Quantification of blast lesions and fungal biomass was carried out by image analysis and qPCR using specific primers for *M. oryzae* 28S DNA, respectively (as in Figure 3). Primers are listed in Table S4. Data from one representative experiment of three independent experiments are presented as the mean ± SE (n = 15) (Student t-test, $*P < 0.05$), which gave similar results

The accumulation of reactive oxygen species (ROS) is a common feature of basal resistance to pathogen infection in plants (Torres *et al.,* 2006). ROS act as antimicrobial agents and signalling molecules during the plant response to pathogen infection. In this work, 3,3′-diaminobenzidine (DAB) staining was used to visualize H_2O_2 accumulation in leaves of miR812w-OE and CRISPR/Cas9-edited (*mir812w-Δ22*) plants. Histochemical analyses showed that, compared to wild-type plants, miR812w-OE plants accumulated more hydrogen peroxide (H_2O_2) after infection with *M. oryzae* spores or treatment with *M. oryzae* elicitors (Figure 5b, c). To note, miR812w-OE plants does not accumulate H_2O_2 in a constitutive manner, but only after inoculation with fungal spores (or elicitor application). As for the CRISPR/Cas9-edited rice plants, H_2O_2 was barely detected in *M. oryzae*-infected or elicitor-treated leaves of these plants (Figure 5b, c). The enhanced accumulation of H_2O_2 in leaves of mi812w-OE plants might well help to prevent disease progression, which is consistent with the phenotype of disease resistance observed in these plants.

miR812w is a MITE-derived miRNA

Earlier studies in rice and *Arabidopsis* reported that lmiRNAs (e.g. 24-nt long miRNAs) might arise from Transposable elements (TE) (Vazquez *et al.,* 2008; Wu *et al.,* 2010). In this work, we carried out a search for homologous sequences in transposable elements using the RepeatMasker web server (www.repeatmasker.org/). This analysis revealed strong sequence similarity between *MIR812w* and the miniature inverted-repeat element (MITE) *stowaway* family of non-autonomous DNA transposons from rice (Figure S8). MITEs are non-autonomous Transposable elements (TEs) that are often found in untranslated regions (UTRs) of coding genes (Casacuberta *et al.,* 1998; Santiago *et al.,* 2002), and *Stowaway* is one of the most abundant type of MITEs in the rice genome (Feschotte *et al.,* 2003). It is also known that the presence of the *Stowaway* family is highly associated with speciation in the AA-type genome lineage of *Oryza* species (Kanazawa *et al.,* 2000), which is consistent with our finding that miR812w is detected in wild species that have an AA-type genome, but not in wild species with a CC (*O. officinalis*) or CCDD (*O. latifolia*) genome (shown in Figure 1d). Whether miR812w is specific of AA-genome wild rice species deserves further investigation.

Rice genes carrying an insertion of a Stowaway MITE are predicted as mi812w target genes

Unlike animal miRNAs, plant miRNAs require extensive complementarity for target recognition (Axtell and Meyers, 2018). In this work, the psRNATarget server was used for prediction of miR812w target genes (Dai *et al.,* 2018). By using a score threshold of 3, up to 39 genes were predicted as target genes for miR812w, these genes functioning in diverse biological processes, including signalling processes and defence responses (Table S3). Furthermore, we noticed that 36 out of the 39 predicted target genes of miR812w contained a *Stowaway* MITE insertion embedded within their untranslated region (UTR), most of them at the 3′ UTR (29 genes at the 3′ UTR; 6 genes at the 5′ UTR) (Table S3). On this basis, we reasoned that *Stowaway*-containing target genes could be target genes of miR812w.

We investigated whether miR812w could direct cleavage of predicted target transcripts. For this, we initially searched for cleavage products of the predicted target genes in previously reported Parallel Analysis of RNA Ends (PARE) libraries obtained from rice tissues (Baldrich *et al.,* 2015). PARE analyses did not yield high-confidence miR812w targets. Equally, no target cleavage was detected by RLM-RACE for any of the predicted miR812w targets here examined (e.g. *bHLH064, RIP1, CIPK10, LRR, ACO3, Glucan endo-1,3-β-glucosidase 7, UDP-glucoronosyl and UDP-glucosyl transferase*). This observation suggests that miR812w is unlikely to function by directing cleavage of *Stowaway*-containing target genes.

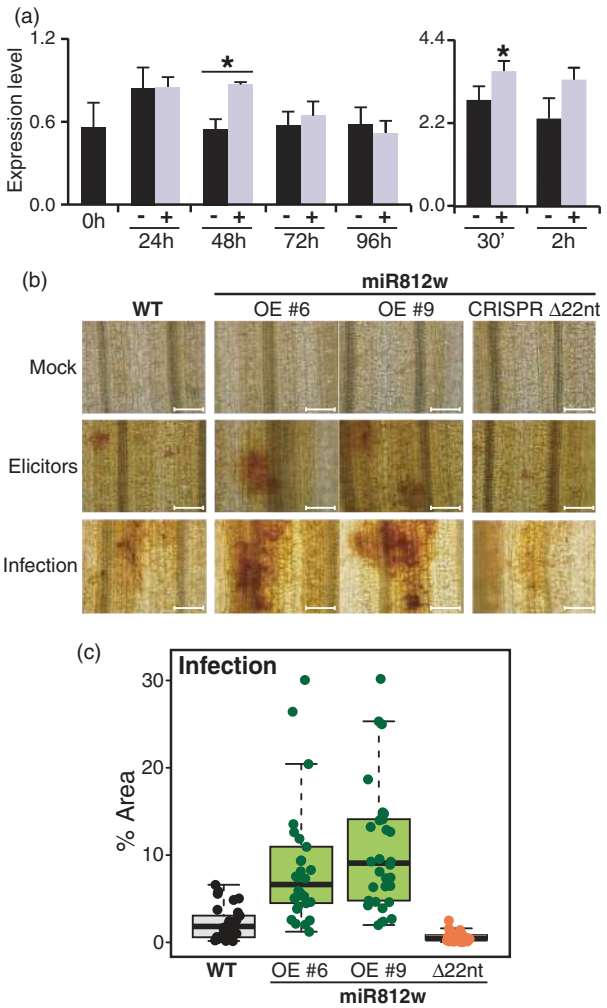


Figure 5 *MIR812w* expression in wild-type rice plants during *M. oryzae* infection, and H_2O_2 accumulation in miR812-OE and CRISPR/Cas9-edited plants. (a) The accumulation of miR812w precursor transcripts was determined by RT-qPCR at the indicated times after inoculation with a suspension of *M. oryzae* spores (left panel) or treatment with *M. oryzae* elicitors (right panel). Data from one representative experiment of three independent experiments are presented as the mean ± SE ($n = 3$; each sample consisted of a pool of 10 individual leaves; $^*P < 0.05$, ANOVA test). (b) DAB staining to visualize H_2O_2 accumulation in wild-type, miR399-OE and CRISPR/Cas9-edited plants carrying the *Δ22* deletion. miR812w-OE plants inoculated with *M. oryzae* accumulated more H_2O_2 that did wild-type plants, whereas little DAB staining could be observed in *M. oryzae*-inoculated leaves of *mir812w-Δ22* plants. (c) H_2O_2 accumulation in *M. oryzae*-infected leaves was quantified on digital photographs using imageJ (https://imagej.nih.gov/ij/)

miR812w mediates DNA methylation of target genes and at its own locus

In rice, DCL3-dependent 24nt-long miRNAs are known to direct DNA methylation at their target genes (in *trans*), as well as at loci of their origin (in *cis*) in rice (Hu *et al.,* 2014; Wu *et al.,* 2010). This piece of evidence together with the observation that miR812w was found to associate with AGO4, which is involved in DNA methylation, prompted us to investigate whether miR812w can direct DNA methylation either in *trans* (at its predicted target transcripts), or in *cis* (at its own locus).

Bisulfite sequencing was used to analyse the status of DNA methylation of 3 genes that were predicted as miR812w target genes. They were: *1-aminocyclopropane-1-carboxylate oxidase3* (*ACO3*, Os*02g53180*), *Calcineurin B Like* (*CBL)-interacting serine–threonine protein kinase* (*CIPK10*, Os03g22050) and *Leucine Repeat-containing protein* (*LRR*, Os06g04830) genes (Figure 6a). The *Stowaway* MITE was present at the 5′ UTR (*ACO3*) or the 3′ UTR (*CIPK10, LRR*), and contained the miR812w-3p target sequences (the alignment of the *MIR812w* precursor sequence with that of the MITE sequence embedded in *ACO3, CIPK10 and LRR* genes is shown in Figure S9). Regarding the function of these genes, *ACO* genes are known to be involved in the final step of ethylene biosynthesis, this hormone being a modulator of disease resistance in plants (van Loon *et al.,* 2006). A function of disease resistance (*R*) gene is predicted for the *LRR* gene examined in this work (Plant Resistance Genes database, from Pythozome, http://prgdb.crg.eu). CIPK serine–threonine protein kinases are known to interact with CBL proteins (Ca^{2+} sensor proteins) for the regulation of biotic stress responses. Distinct CIPKs from rice were reported to play a role in PTI and ROS production in rice (Kurusu *et al.,* 2010).

DNA methylation of *ACO3*, *CIPK10* and *LRR* was examined in wild-type and CRISPR/Cas9-edited (*miR812w-Δ22*) plants. In wild-type plants, cytosine methylation was identified in the CG, CHG and CHH sequence contexts in the 3 target genes (Figure 6a), with maximum methylation levels in the CG context (Figure S10a). Cytosine methylation was limited to the 75nt region around the miR812w-3p recognition site (Figure 6a). Of interest, the methylation levels in *miR812w-Δ22* plants were reduced compared to wild-type plants, at a different extent depending on the methylation site and the target gene (Figure 6a). These findings support that miR812w function is required for DNA methylation of the *ACO3*, *CIPK10* and *LRR* loci.

We then investigated whether miR812w could mediate methylation at its own locus. Bisulfite sequencing showed cytosine methylation in all the sequence contexts of the *MIR812w* locus, which were concentrated at the two arms of the stem region of the miR812 precursor structure, with less methylated sites at the loop region (Figure S10b). Similar results were previously reported for another miRNA from rice, miR1873, that directs cytosine DNA methylation at its own locus (Wu *et al.,* 2010; Zhang *et al.,* 2018a). Compared to wild-type, *miR812w-Δ22* plants showed reduced levels of DNA methylation at the miR812w sequence (Figure 6b). Thus, miR812w appears to mediate methylation not only at target genes, but also at its own locus.

Methylation of cytosine residues is generally associated with transcriptional silencing. Accordingly, we examined *ACO3*, *CIPK10* and *LRR* expression in wild-type and *miR812w-Δ22* plants. Compared to wild-type plants, a significant increase in *ACO3*, *CIPK10* and *LRR* expression occurred in *miR812w-Δ22* plants (Figure 7). The observed reduction of DNA methylation caused by a mutation in the miR812w locus (*miR812w-Δ22* plants) is associated to the activation of *ACO3, CIPK10* and *LRR* expression. This observation is indicative of a miR812w-mediated regulation of *ACO3*, *CIPK10* and *LRR* expression, most probably, through DNA methylation, with potential influence on disease susceptibility. However, the possibility that other targets of miR812w may also function in resistance to *M. oryzae* infection in rice plants should not be ruled out.

Discussion

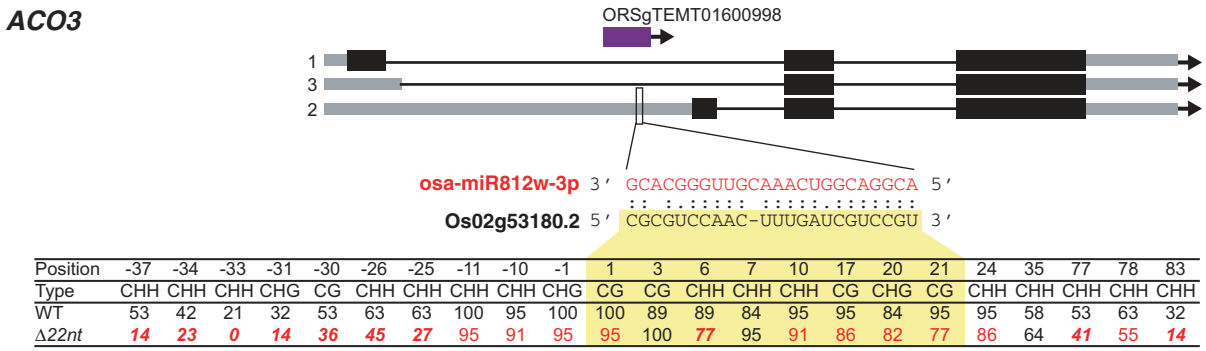
In this study, we provide evidence that miR812w represents a new member of the miR812 family of rice miRNAs that mediates pathogen resistance. We demonstrated that constitutive expression of *MIR812w* confers resistance to *M. oryzae* infection. Conversely, CRISPR/Cas9-edited *MIR812w* rice plants are more susceptible to infection. Not only pathogen infection, but also treatment with fungal elicitors is accompanied by a transient increase in *MIR812w* expression.

Several pieces of data support that miR812w is a novel, evolutionarily recent *MIR* gene from rice. First, the *MIR812w* precursor is processed by DCL3 and gives rise to 24-nt long mature miR812w species (as revealed by northern blotting and analysis of small RNA populations in *dcl* mutants). The miR812w precursor structure also shows a high degree of complementarity in the stem region, as it is generally observed in precursor structures of evolutionary young *MIR* genes. Second, the 24-nt-derived miR812w-3p species were found to associate with AGO4, supporting that miR812w is functional. In this respect, it is well known that DCL3-processed 24-nt long miRNAs are specifically sorted into AGO4 (Wu *et al.,* 2010), whereas ancient 21-nt miRNAs are recruited in AGO1 complexes. Finally, miR812w is detected in rice, but not in any of the other monocotyledonous or dicotyledonous species examined in this study.

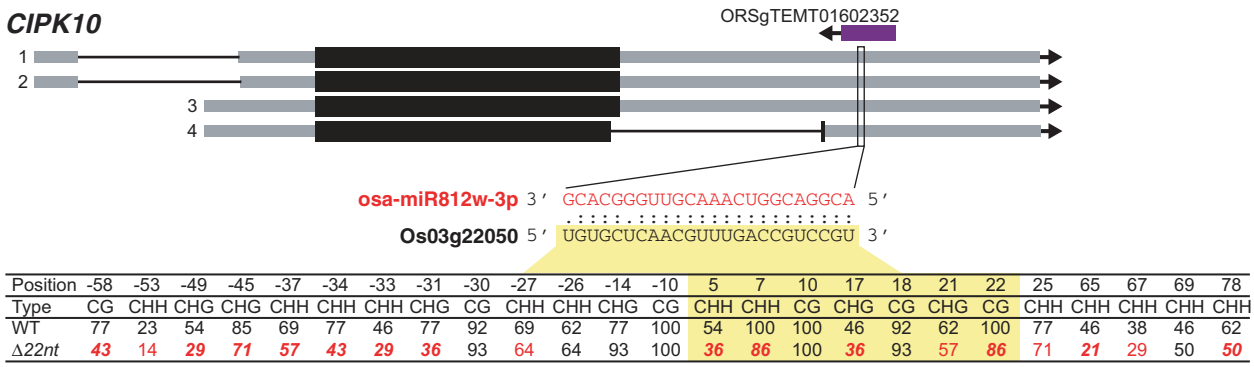
Because 24nt siRNAs can be produced from long, double-stranded (dsRNAs) that are processed by DCL3 and are loaded into AGO4, the two classes of 24-nt small RNAs (e.g. those derived from *MIRNA* precursors or from long dsRNAs) can be confounded. Nevertheless, an important distinction is made between 24-nt miRNAs and 24-nt siRNAs with respect to their origin. Thus, the 24-nt miRNAs are produced from single-stranded precursors that adopt a fold-back structure, while siRNAs are produced from long double-stranded RNAs with perfect complementarity. Importantly, in this study we show that mature miR812w sequences, miR812w-5p and miR812w-3p, derive from a single-stranded precursor transcript, as revealed by DNA sequencing of RT-PCR products of *MIR812w* precursor transcripts and agroinfiltration experiments in *N. benthamiana* leaves. Hence, it can be concluded that miR812w represents a *bona fide* 24-nt miRNA.

An interesting finding of this work was the observation that miR812w originated from a *Stowaway* MITE. Although in animals it is generally accepted that TEs are responsible for the formation of *MIR* loci, our understanding on *MIR* genes originating from TEs in plants is still limited (Cho, 2018; Poretti *et al.,* 2020; Roberts *et al.,* 2014; Wei *et al.,* 2014; Zhang *et al.,* 2011). Intriguingly, an examination of the genomic context of plant miRNAs registered in miRBase revealed that a considerable number of plant miRNAs are identical or homologous to TEs (Li *et al.,* 2011; Piriyapongsa and Jordan, 2008). Furthermore, in that study the authors identified four members of the rice miR812 family (miR812f,

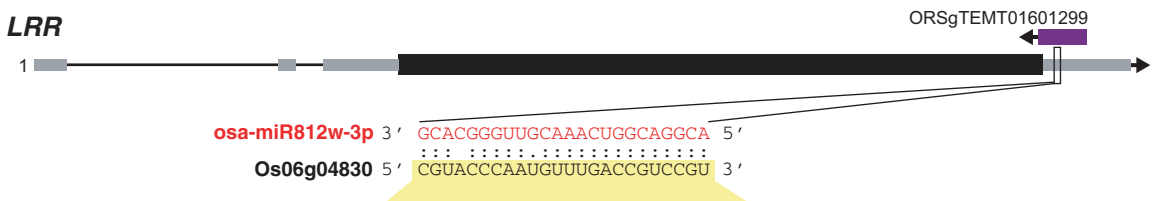
(a)



Position	-37	-34	-33	-31	-30	-26	-25	-11	-10	-1	1	3	6	7	10	17	20	21	24	35	77	78	83
Type	CHH	CHH	CHH	CHG	CG	CHH	CHH	CHH	CHH	CHG	CG	CG	CHH	CHH	CHH	CG	CHG	CG	CHH	CHH	CHH	CHH	CHH
WT	53	42	21	32	53	63	63	100	95	100	100	89	89	84	95	95	84	95	95	58	53	63	32
Δ22nt	***14***	***23***	***0***	***14***	***36***	***45***	***27***	95	91	95	95	100	***77***	95	91	86	82	77	86	64	***41***	55	***14***



Position	-58	-53	-49	-45	-37	-34	-33	-31	-30	-27	-26	-14	-10	5	7	10	17	18	21	22	25	65	67	69	78
Type	CG	CHH	CHG	CHG	CHH	CHH	CHH	CHG	CG	CHH	CHH	CHG	CG	CHH	CHH	CG	CHG	CG	CHG	CG	CHH	CHH	CHH	CHH	CHH
WT	77	23	54	85	69	77	46	77	92	69	62	77	100	54	100	100	46	92	62	100	77	46	38	46	62
Δ22nt	***43***	14	***29***	***71***	***57***	***43***	***29***	***36***	93	64	64	93	100	***36***	***86***	100	***36***	93	57	***86***	71	***21***	29	50	***50***



Position	-9	-8	-1	1	5	6	7	17	18	21	22	25	35	60	67	69	71	83	93	96	100
Type	CHH	CHG	CHG	CG	CHH	CHH	CHH	CHG	CG	CHG	CG	CHH	CHH	CHH	CG	CHH	CHH	CHH	CHH	CHH	CHH
WT	76	100	88	100	76	76	88	18	100	76	100	53	18	41	100	41	41	53	71	65	71
Δ22nt	***65***	***88***	82	***82***	82	***59***	***76***	59	100	71	94	***41***	18	35	100	***24***	35	***41***	***47***	59	***47***

(b)

																miR812w-5p							
Position	-52	-50	-45	-41	-38	-36	-35	-34	-32	-31	-27	-16	-12	-10	-1	5	6	9	19	20	23	47	55
Type	CG	CHG	CHH	CHH	CHH	CHH	CHH	CHH	CHG	CG	CHH	CHH	CHH	CHG	CG	CHH	CHH	CHH	CHG	CG	CHH	CG	CG
WT	50	22	33	39	28	44	72	33	56	67	78	89	94	100	100	78	83	83	78	94	83	100	100
22nt	***28***	28	***7***	***21***	36	***21***	71	71	92	***0***	***0***	***0***	***0***	***0***	100	93	100	86	93	93	86	***86***	100

Δ22nt

miR812w-3p

Position	-34	-19	2	6	10	14	21	23	29	30	40	54
Type	CHH	CHH	CG	CG	CHH	CG	CHH	CG	CHH	CHH	CHH	CG
WT	33	67	100	100	100	94	89	100	89	89	72	100
22nt	29	71	93	100	***86***	93	93	100	***79***	86	71	93

Figure 6 miR812w directs DNA methylation at DNA sequences (in *trans*) as well as at its own locus (in *cis*). (a) Analysis of DNA methylation at the miR812w target transcripts in wild-type (WT) and *miR812w-Δ22* (*-Δ22nt)* by bisulfite sequencing. The target genes examined were: *ACO3* (Os02g53180, upper panel), *CIPK10* (Os03g22050, medium panel) and *LRR* (Os06g04830, lower panel). Data was analysed using the Kismeth software (Gruntman *et al.,*2008). Numbers represent the percentage of methylation at each cytosine methylation context (CG, CHG and CHH). The first nucleotide of the target site is set as position +1. A 75nt surrounding the mir812w target site (shaded in yellow) is shown. Red colour indicates those cytosine contexts with lower methylation in *miR812w-Δ22* (*-Δ22nt)* compared to wild-type (WT) plants (in bold italics are indicated those with a decrease higher than 10%). Schematics representation for each target gene is shown (wide black boxes, exons; grey thin boxes, Untranslated regions (UTR); thin lines, introns). Purple boxes indicate the position and code ID for the *Stowaway* MITE inserted at the UTR of each target gene. (b) miR812w directs DNA methylation in *cis* at its own locus. DNA methylation status was analysed and represented as in (a). The 5′ nucleotide of mature miR812w-5p (shaded in blue) and miR812w-3p (shaded in pink) is set as position +1. The 22nt deletion next to miR812w-5p is indicated in *miR812w-Δ22* rice plants with an orange square. Details on DNA methylation at the *MIR812w* precursor sequence can be found in Figure S10b. These experiments were carried out two times with similar results

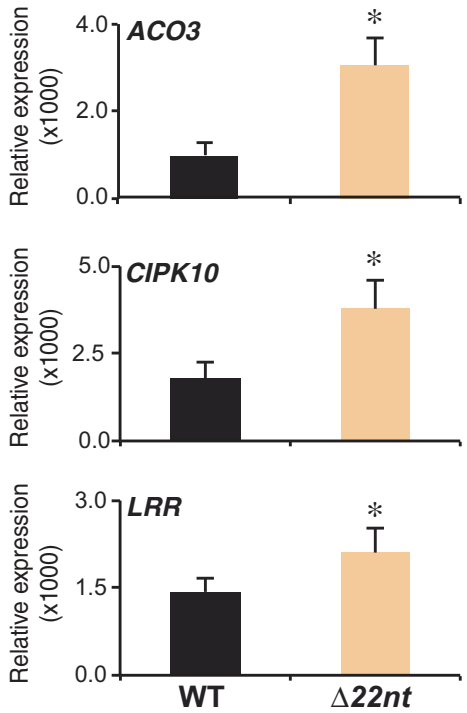


Figure 7 Expression analysis of *ACO3* (Os02g53180), *CIPK10* (Os03g22050) and *LRR* (Os06g04830) in wild-type and *miR812w-Δ22* rice plants by qRT-PCR. The rice *ubiquitin1* gene was used for normalization. Data from one representative experiment of three independent experiments are presented as the mean ± SE (n = 3, each biological replicate is a pool of 3 individual leaves) (Student t-test, *$P < 0.05$). Primers are indicated in Table S4

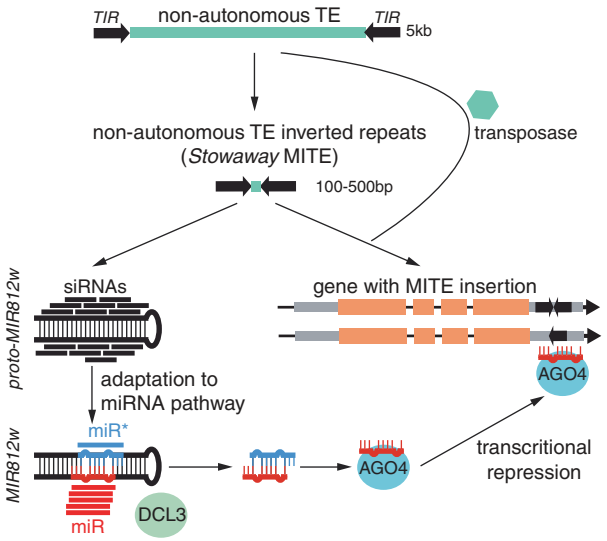


Figure 8 Proposed model for the origin and mode of action of miR812w in rice. MITEs are non-autonomous transposable elements (TEs) that derive from autonomous TEs and depend on their transposases for mobilization and integration elsewhere in the genome (i.e protein-coding genes). Terminal inverted repeats (TIR, 10–15 bp) are indicated by black arrows. A *proto-MIR812w* would be formed from a *Stowaway* MITE that further evolved to become a *MIR812w* precursor that is processed DCL3. During miR812w evolution, small RNAs might have been generated from the *proto-MIR812w*. The functional strand of the miR812w-5p/miR812w-3p duplex (miR812w-3p) would be loaded into the AGO4 clade protein to mediate DNA methylation at their target transcripts (in *trans*), as well as at its own locus (in *cis*). During evolution, MITEs from the Stowaway family would have integrated into the 3′ UTR region of protein-coding genes, such as the *ACO3*, *CIPK10* and *LRR* genes. The miR812w could then be derived from a *Stowaway* MITE, while the *Stowaway* MITE would serve as a source for the origin of potential target genes for miR812w. Adapted from Li *et al.,* (2011)

miR812h, miR812i and miR812j) having homology with TEs, but the significance of this homology was not investigated (Li *et al.,* 2011). In wheat, TamiR113 was reported to originate from a member of the MITE VRN family (Yu *et al.,* 2014).

A model is presented in Figure 8 to explain the possible origin and mechanism of action of miR812w in rice. According to this model, miR812w evolved from a *Stowaway* MITE, and the incorporation of cognate MITEs into protein-coding genes would have created target sites for miR812w. In favour of this possibility, a search for target genes of miR812w revealed multiple genes containing the recognition site for miR812w, mostly at their 3′-UTR. In this way, the presence of cognate target sequences in protein-coding genes might have conferred miR812w regulation to these genes while allowing the creation of a network of miR812w-mediated gene regulation. Such regulatory system would allow the plant to activate an appropriate spectrum of responses that would enhance the plant's ability to stop the spread of the pathogen. The existence of regulatory systems contributing to the evolution of miRNA regulatory systems has long been proposed (Piriyapongsa and Jordan, 2008; Roberts *et al.,* 2014).

Our results show that miR812w can direct cytosine DNA methylation in *trans* at target genes (e.g. *ACO3*, *CIPK10* and *LRR*), as well as *in cis* at the locus from which miR812w is produced. Consistent with results previously reported by other authors, miR812w-mediated methylation occurs approximately within the 80nt region around the miR812w recognition sites (Wu *et al.,* 2010; Zhang *et al.,* 2018a). A reduction in cytosine methylation of *ACO3*, *CIPK10* and *LRR* in *miR812w-Δ22* plants was accompanied by a higher expression of these genes in *miR812w-Δ22* plants. In *Arabidopsis*, a miR156/166-dependent methylation of the *PHABULOSA (PHB)* and *PHABOLUTA (PHV)* genes controlling leaf development was previously described (Bao *et al.,* 2004). It is worth mentioning that for another member of the miR812 family, miR812q, a miR812q-guided cleavage of *CIPK10* was reported (Jeong *et al.,* 2011). It is tempting to assume that during evolution, the various miR812w family members might have evolved a dual function for regulation of target gene expression at the transcriptional (e.g. miR812w-mediated DNA methylation) and post-transcriptional (e.g. miR812q-mediated cleavage of *CIPK10*) levels. Regarding methylation at the *MIR812w* locus, the possibility that changes in DNA methylation in the miR812 precursor DNA sequence has an effect on *MIR812w* transcription, hence, accumulation of mature miR812w-3p sequences, should be considered. A recent study in soybean/Cyst nematode interactions demonstrated that differentially methylated *MIR* genes modulate susceptibility to infection by soybean Cyst nematodes, indicating that DNA methylation of *MIR* genes might be

important in the adaptive response of plants to biotic stress (Rambani *et al.,* 2020).

Although we provide evidence for a function of miR812w in resistance to infection by the blast fungus, the challenge is now to understand how a miR812w-directed methylation at target sites, as well as at its own locus, might regulate immune responses in rice. In this work, only 3 of the *Stowaway*-containing target genes predicted for miR812w were investigated, whose expression was found to be up-regulated in *miR812w-Δ22* plants compared with wild-type plants. Given that miR812w appears to be involved in the methylation of *ACO3*, *CIPK10* and *LRR*, one would expect that *Stowaway*-containing genes, other than *ACO3*, *CIPK10* and *LRR*, might also be regulated by miR812w, which incidentally, might modulate blast disease resistance. Along with this, multiple candidate target genes have been identified *in silico* as potential targets of miR812w, and for many of them a regulation during *M. oryzae* infection has been described (Table S3). Here, it should be taken into account that, even though DNA methylation is most commonly associated with transcriptional repression, positive associations of DNA methylation to gene expression have also been reported (Erdmann and Picard, 2020; Harris *et al.,* 2018). Therefore, both repressive and activating transcriptional effects should be considered when investigating the impact of miR812w-mediated methylation on target genes. Based on the results here presented on the accumulation of H_2O_2 in miR812w-OE and CRISPR/Cas9-edited miR812w rice plants, it will be also of interest to investigate how miR812w might exert its regulatory role in oxidative stress responses, either directly or indirectly. In other studies, a MITE-derived miRNA from wheat, Tae-miR1436, has been shown to have a MITE copy integrated into mildew resistance genes which led the authors to propose that MITE-derived miRNAs might contribute to the wheat immune response (Poretti *et al.,* 2020). Further investigation is needed to identify MITE-derived miRNAs in plant species and to elucidate the mechanisms by which these miRNAs contribute to plant immunity.

Finally, results here presented reinforce the notion that, in addition to post-transcriptional regulation of gene expression, miRNAs can also exert their regulatory function at the transcriptional level by directing DNA methylation. A better understanding of gene regulatory networks in which miR812w participates will provide useful tools for developing novel strategies for crop protection.

Experimental procedures

Blast resistance assays

The fungus *M. oryzae* (strain Guy-11) was grown in Complete Media Agar (CMA, containing 30 mg/L chloramphenicol) for 15 days under controlled conditions (28°C, 16 h/8 h). Soil-grown plants at the 3–4 leaf stage were infected by spraying a *M. oryzae* spore suspension (10^5 spores/ml; 0.2 ml/plant; prepared as described, (Campo *et al.,* 2013) with an aerograph at 2 atmospheres of pressure (Sesma and Osbourn, 2004). Plants were maintained overnight in the dark under high humidity. The percentage of leaf area affected by blast lesions was determined at 7 days post-inoculation by image analysis. Quantification of fungal DNA biomass was carried out by real-time PCR using specific primers for the *Magnaporthe oryzae* 28S and normalized to the rice *Ubiquitin1* an internal control (Table S4) (Qi and Yang, 2002). At least, 4 independent experiments were carried out with similar results.

Target prediction

Target prediction was done using the Plant Small RNA Target Analysis Server (psRNATarget) program by aligning mature miR812w sequences with MSU Rice Genome Annotation (v7) (http://bioinfo3.noble.org/psRNATarget; release 17). Default settings were used (Dai *et al.,* 2018).

Bioinformatics analysis of sequencing data

Raw reads of sRNAs and PARE data were downloaded from the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra). Rice sRNA datasets used in this study included GSE66611 (for leaves and roots treated with *M. oryzae* elicitors) (Baldrich *et al.,* 2015), GSE18251 (for OsAGO1a and OsAGO1b IPs) (Wu *et al.,* 2009), GSE20748 (for *OsDCL1IR-2* and *Osrdr2* mutants, and OsAGO4a and OsAGO4b IPs) (Wu *et al.,* 2010) and GSE50778 (for the *Osdcl3a* mutant) (Wei *et al.,* 2014). PARE datasets used in this study included GSE66611 (for leaves and roots treated with *M. oryzae* elicitors) (Baldrich *et al.,* 2015). All datasets were trimmed for adaptors using Trimmomatic v0.32 (Bolger *et al.,* 2014). sRNA datasets were mapped to miR812 precursors using Bowtie2 (Langmead and Salzberg, 2012). PARE datasets were analysed using CleaveLand v4.5 (Addo-Quaye *et al.,* 2009) and MSU Rice Genome Annotation Project Release 7 (Kawahara *et al.,* 2013).

Bisulfite sequencing

Genomic DNAs from wild-type (WT) and CRISPR-edited (Δ22nt) rice plants were extracted as described (Sánchez-Sanuy *et al.,* 2019). Bisulfite conversion of gDNAs (500 ng) was done with the EZ DNA Methylation-Gold kit following the manufacturer's instructions (ZYMO Research). Then, 3 μl of bisulfite-treated DNAs were used for PCR amplification of different loci using the GoTaq® G2 Flexi DNA Polymerase (Promega) with the following conditions: 95°C, 2 min; 45 cycles of (95°C, 30 s; 52°C, 30 s; 72°C, 30 s); 72°C, 5 min. PCR products were gel purified and cloned into pGEM-T easy vector (Promega). For each region, 20 individual top-strand clones were sequenced. Primers were designed at flanking regions of the miRNA-binding sites and avoiding repetitive regions with the Kismeth software (Gruntman *et al.,* 2008) (Table S4). Sequencing data analysis was done with the Kismeth software selecting 75 bp up- and downstream of the miRNA target sites.

Other methods

Details on Growth of Plant Material, Elicitor treatment, Vector construction, Rice transformation, Transient expression in *N. benthamiana*, Histological detection of ROS accumulation, northern blot and quantitative RT-PCR analysis are available in Methods S1.

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Author contributions

SC and BSS planned and designed the research. SC, FSS, RCR, JGA, LCS and MSS performed the experiments. SC and PB carried out the bioinformatics analyses. SC, BSS and FSS analysed data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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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 Nucleotide sequence of the novel miRNA candidate under study.
Figure S2 Alignment of the precursor sequences of the rice miR812 family.
Figure S3 Alignment of mature sequences of the rice miR812 family with miR812w-3p.
Figure S4 Abundance of miR812w-3p in *dcl1* and *rdr2* rice mutants.
Figure S5 Characterization of wild-type (WT) and CRISPR-edited rice plants harbouring the *Δ22nt* mutant allele (*mir812w-*Δ*22*).
Figure S6 Constitutive expression of *MIR812w* in rice.
Figure S7 Accumulation of *PR1* transcripts in miR812w overexpressor and CRISPR-edited rice plants.
Figure S8 *MIR812w* has homology to the MITE from the *Stowaway* family.
Figure S9 Alignment of the *MIR812w* precursor sequence and the MITE sequence embedded in the miR812w target transcript.
Figure S10 miR812w directs DNA methylation at its target genes (in trans) and at its own locus (in *cis*).
Table S1 *Oryza* species analysed in this study, their genome type, accession number and geographical region of cultivation.
Table S2 T-DNA copy number in mir812w overexpressor and empty vector rice plants.
Table S3 Putative target genes for osa-miR812w.
Table S4 Oligonucleotides used in this study.
Methods S1 Supplemental Experimental procedures.

