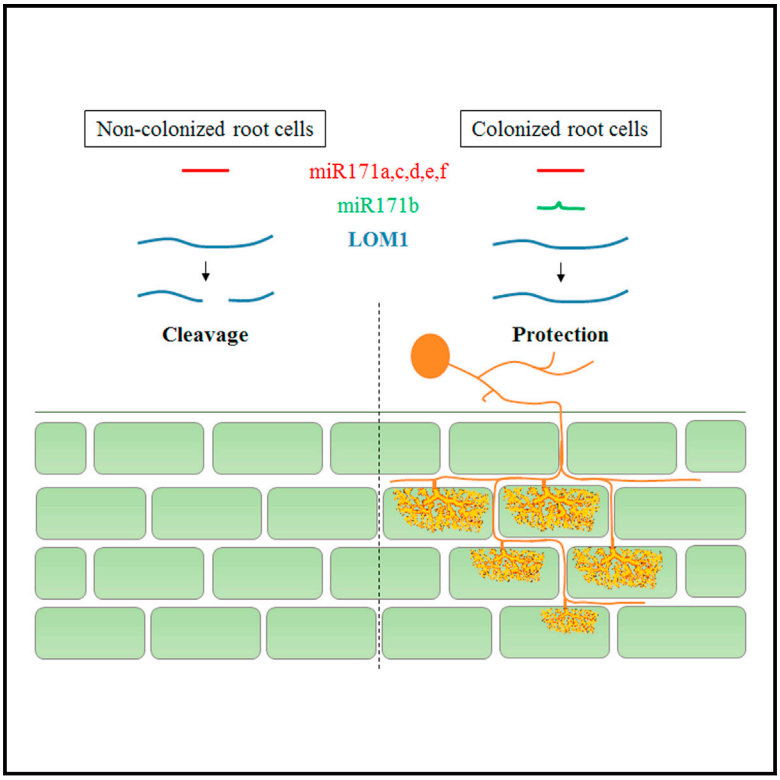
Short Article

Cell Host & Microbe

Positive Gene Regulation by a Natural Protective miRNA Enables Arbuscular Mycorrhizal Symbiosis

Graphical Abstract



Highlights

- miR171b is specific to mycotrophic species and has a target gene cleavage site mismatch
- miR171b accumulation activated target gene *LOM1* expression and enhanced mycorrhization
- miR171b is preferentially expressed in arbuscule-containing cells
- miR171b expression protects LOM1 from silencing by other miR171 family members

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In Brief

Arbuscular mycorrhizal symbiosis, a plant-fungal mutualistic relationship, is regulated by the miR171 family. Couzigou et al. discover that miR171b has evolved a target site mismatch such that instead of silencing, miR171b protects its target gene LOM1 from cleavage by other miR171 family members and thereby enables AM symbiosis.

Couzigou et al., 2017, Cell Host & Microbe *21*, 1–7
January 11, 2017
http://dx.doi.org/10.1016/j.chom.2016.12.001

CellPress

Please cite this article in press as: Couzigou et al., Positive Gene Regulation by a Natural Protective miRNA Enables Arbuscular Mycorrhizal Symbiosis, Cell Host & Microbe (2016), http://dx.doi.org/10.1016/j.chom.2016.12.001

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Short Article

Positive Gene Regulation by a Natural Protective miRNA Enables Arbuscular Mycorrhizal Symbiosis

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http://dx.doi.org/10.1016/j.chom.2016.12.001

SUMMARY

Arbuscular mycorrhizal (AM) symbiosis associates most plants with fungi of the phylum Glomeromycota. The fungus penetrates into roots and forms within cortical cell branched structures called arbuscules for nutrient exchange. We discovered that miR171b has a mismatched cleavage site and is unable to downregulate the miR171 family target gene, *LOM1* (*LOST MERISTEMS 1*). This mismatched cleavage site is conserved among plants that establish AM symbiosis, but not in non-mycotrophic plants. Unlike other members of the miR171 family, miR171b stimulates AM symbiosis and is expressed specifically in root cells that contain arbuscules. MiR171b protects LOM1 from negative regulation by other miR171 family members. These findings uncover a unique mechanism of positive post-transcriptional regulation of gene expression by miRNAs and demonstrate its relevance for the establishment of AM symbiosis.

INTRODUCTION

MicroRNAs (miRNAs) are short RNAs, generally around 21 nt, that negatively regulate gene expression. They anneal by homology with mRNAs of target genes to direct their degradation or the inhibition of their translation. In plants, most miRNAs belong to miRNA families. All the members of one family generally target the same set of genes, but they differ in their expression patterns, thus allowing fine-tuning of target gene expression (Llave et al., 2002; Mallory et al., 2004; Combier et al., 2006; Rogers and Chen, 2013; Couzigou and Combier, 2016). Plant miRNAs usually have near-perfect pairing with their mRNA targets; the mRNA-miRNA duplex is then targeted for degradation by cleavage at a specific cleavage site (Liu et al., 2014). Whereas miRNAs were generally considered as non-coding RNAs, we have recently shown that the primary transcripts of miRNAs encode small peptides, the miPEPs, which specifically activate the transcription of their miRNA in a positive loop. We also showed that this activation of miRNA synthesis, as well as the subsequent downregulation of target genes, can be obtained by treatments with exogenous synthetic miPEPs. Given that the miPEPs encoded by the primary transcripts of an miRNA family are all different, it is thus possible to activate the synthesis of a specific member of an miRNA family by treatment with the appropriate synthetic miPEP. This approach allows the study of the role of each member of an miRNA family (Lauressergues et al., 2015; Couzigou et al., 2015).

Arbuscular mycorrhizal (AM) symbiosis is one of the most ancient symbioses between plants and microorganisms. It associates the roots of most land plants to fungi of the Glomeromycota. In exchange for sugars coming from photosynthesis, the fungus provides minerals, mainly phosphorus, to its plant host. Moreover, the AM symbiosis improves plant resistance to biotic and abiotic stresses. After a molecular dialog between the two partners, the fungus penetrates the roots and forms in cortical cells highly branched structures called arbuscules in which the exchange of nutrients occurs (Gutjahr and Parniske, 2013). This process is tightly controlled by the plant to avoid over-colonization of the roots by the fungus. Indeed, the *Medicago truncatula* miR171h, which is only retrieved in species able to form AM symbiosis, controls the fungal colonization by downregulating NSP2, a GRAS transcription factor (Lauressergues et al., 2012).

Whereas miR171h targets NSP2, it contains strong sequence dissimilarities and targets different genes compared to other miR171s. The miR171 family is known to target LOM genes (also known as *Hairy Meristem* genes), which are transcription factors belonging to the GRAS family (Schulze et al., 2010; Xue et al., 2014). In *Arabidopsis thaliana*, *LOM* genes control shoot and root indeterminacy by regulating meristem cell differentiation (Stuurman et al., 2002; Schulze et al., 2010; Engstrom et al., 2011). Moreover, their regulation by miR171 is involved in trichome distribution (Xue et al., 2014).

RESULTS AND DISCUSSION

The miR171 family of *M. truncatula* is involved in root development and AM symbiosis (Lauressergues et al., 2012, 2015). It contains seven members: miR171a–miR171f and miR171h (http://www.mirbase.org/). Bioinformatics searches have allowed us

to identify *LOM1* (Medicago: MTGI9-TC114268) and *LOM2* (Medicago: MTGI9-TC120850) as putative target genes of the *M. truncatula* miR171a–miR171f (Figure 1A). *LOM1* and *LOM2* share 40%–44% homology with *LOM1–LOM3* genes of *A. thaliana*.

In *M. truncatula*, we observed that RNAi-mediated silencing of *LOM1* strongly decreased root colonization and the formation of arbuscules by the mycorrhizal fungus *Rhizophagus irregularis* (Figures S1A and S1B, available online), suggesting that *LOM1* is a positive regulator of AM symbiosis. Moreover, expression (under the control of its own promoter) of a mutant of *LOM1*, in which the mRNA sequence could not be recognized by the miR171 family but in which the amino acid sequence of the encoded protein was unchanged, resulted in increased AM colonization and arbuscule formation compared to control roots (Figure S1C). Altogether, these results suggest that the miR171 family downregulates *LOM1* and so inhibits AM symbiosis. Arbuscule shape was never affected, suggesting a role of LOM1 in the control of root colonization and arbuscule abundance, rather than in the control of arbuscule differentiation.

We confirmed by RACE (rapid amplification of cDNA ends) PCR analyses that in *M. truncatula*, *LOM1* is cleaved by members of the miR171 family at positions 13–14 (Figure 1A), as already reported for the miR171 members of *A. thaliana* (Llave et al., 2002). Interestingly, alignment of the various miR171 members revealed that miR171b contains a mismatch at the identified cleavage site (Figure 1A). Plant miRNA sequences, and especially their cleavage sites, are generally well conserved, so this mismatch was surprising. We hypothesized that this mismatch could inhibit the cleavage activity of miR171b (Liu et al., 2014; Mallory et al., 2004; Schwab et al., 2005) and perhaps create a new cleavage site of another potential target. We found no such corresponding target genes in the *M. truncatula* genome, however. We compared the sequences of the miR171 families in ten plant species and found miR171b homologs with a mismatch in the cleavage site only in species that form AM symbiosis, namely *Lotus japonicus*, *Oryza sativa*, *Zea mays*, and *Solanum lycopersicum* (Figure 1B), suggesting that miR171b might be particularly important for the mycorrhizal process. In the genomes containing these miR171b homologs, we also found no putative target genes with an miR171b cleavage site.

To determine if miR171b has a distinct regulatory role from the other miR171 members, we analyzed the function of all these miRNAs by using synthetic miPEPs (Lauressergues et al., 2015; Couzigou et al., 2016). The sequence of these peptides is highly variable even within the same miRNA family. Contrary to the miRNA overexpression approach using the 35S promoter, which leads to ectopic expression, miPEP treatments upregulate miRNA synthesis specifically in cells where the corresponding pri-miRNAs are expressed, allowing fine analysis of the separate role of each miRNA of a same family (Lauressergues et al., 2015; Couzigou et al., 2016). Following these treatments, we scored *LOM1* expression and mycorrhization rate (Figures 2A and 2B). *LOM1* expression and mycorrhizal colonization decreased significantly in response to all the miPEPs except to that corresponding to miR171b, which activated both expression of *LOM1* and mycorrhization (Figures 2A and 2B). To confirm this stimulation of mycorrhization, we overexpressed miPEP171b in transformed roots of *M. truncatula* and found a rate of mycorrhizal colonization higher than in control roots (Figure S1D). In addition, we identified the miPEPs corresponding to the miR171b homologs in *Lotus japonicus*, tomato, and rice, and treated these plants with their specific miPEP171b. In all three plant species, miPEP171b treatments increased miR171b homolog expression and mycorrhization (Figure S2).

To further investigate the role of miR171b in the mycorrhization process, we expressed a GUS reporter gene under the control of the miR171b gene promoter in roots of *M. truncatula* (Figure 3). The reporter gene was preferentially expressed in tissues colonized by *R. irregularis* (Figures 3C and 3D) and, in particular, in arbuscule-containing cells (Figures 3E and 3F). This is consistent with our previous micro-transcriptomic analysis showing that miR171b expression is increased during mycorrhization (Formey et al., 2014). Similar GUS analyses of the expression of the other miR171 family members were performed (Figure S3). Three distinct expression patterns were observed. miR171a and miR171c were expressed in all root tissues, irrespective of the mycorrhizal status (Figures S3E–S3L). miR171e and miR171f were expressed mainly in central cylinder, irrespective of the mycorrhizal status (Figures S3Q–S3X). The last expression pattern is represented by miR171d, which was mainly expressed in central cylinder and faintly in root parts colonized by the fungus (Figures S3M–S3P). The *LOM1* promoter drove expression of the GUS reporter gene in nearly all root tissues (Figures S3A–S3D), suggesting that LOM1 may be silenced by these miRNAs in most root tissues but protected from this silencing in the colonized root cells thanks to the expression of miR171b. In agreement with this, a laser capture microdissection (http://mtgea.noble.org/v3/) analysis showed a higher expression of *LOM1* in arbuscule-containing cells compared to adjacent cells or cortical cells of non-infected roots (Gaude et al., 2012).

To examine if miR171a, c, d, e, and f and miR171b have antagonistic regulatory functions, we first co-expressed *M. truncatula LOM1* in tobacco leaves with each of the *M. truncatula* miR171 members individually, and compared the expression of *LOM1* with and without any miR171 expression. Whereas expression of miR171a, c, d, e, or f repressed *LOM1* expression, the expression of miR171b did not; on the contrary, it slightly increased *LOM1* expression in this assay (Figure 4A, gray histogram). Then, to see whether miR171b would protect *LOM1* expression against silencing by the other miR171 members, we co-expressed *LOM1*, miR171b, and each of the miR171 members individually. In each case, except when miR171f was co-expressed, expression of miR171b increased expression of *LOM1* when compared to the control (Figure 4A, black histogram), indicating that miR171b protects *LOM1* from silencing by most of the other members of the miR171 family. The increased *LOM1* expression observed in tobacco leaves transformed only with miR171b might be the result of protection against endogenous tobacco miR171 activity. Finally, we mutated the cleavage site of *LOM1* to render it complementary to miR171b and expressed it together with each of the miR171 family members. This new allele of *LOM1* was then silenced by miR171b in the tobacco leaf assay, but not by the other members of the miR171 family (Figure 4B), indicating that miR171b can have a genuine function of gene silencing. This further shows that miR171b-mediated protection of *LOM1* is based upon this mismatch at the cleavage site.

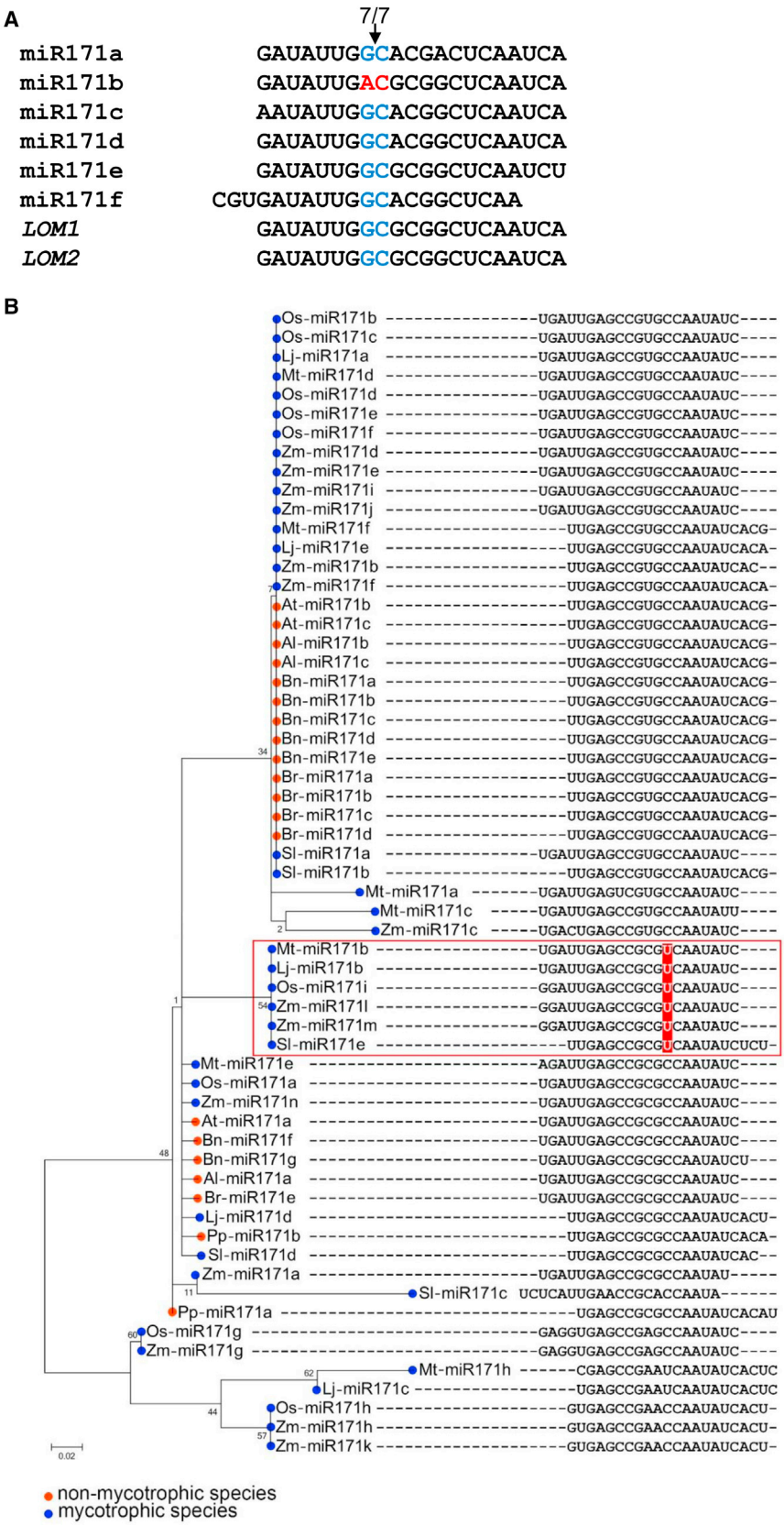

Figure 1. Cleavage Sites and Relation Tree of the miR171 Family

(A) Sequence alignment of members of the miR171 family (reverse complement of miRNA sequences) and of *LOM1* and *LOM2* from *M. truncatula*. The cleavage site of *LOM1* is shown in blue, as determined by 5′ RACE PCR analysis (the number of 5′ RACE PCR clones corresponding to the cleaving site is indicated by arrow).

(B) Sequence comparison and alignment of mature miR171 sequences in ten species: *Arabidopsis lyrata* (Al), *Arabidopsis thaliana* (At), *Brassica napus* (Bn), *Brassica rapa* (Br), *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), *Oryza sativa* (Os), *Physcomitrella patens* (Pp), *Solanum lycopersicum* (Sl), and *Zea mays* (Zm). The red dots indicate non-mycotrophic species, and the blue dots indicate mycotrophic species. The clade of miR171b orthologs is framed in red.

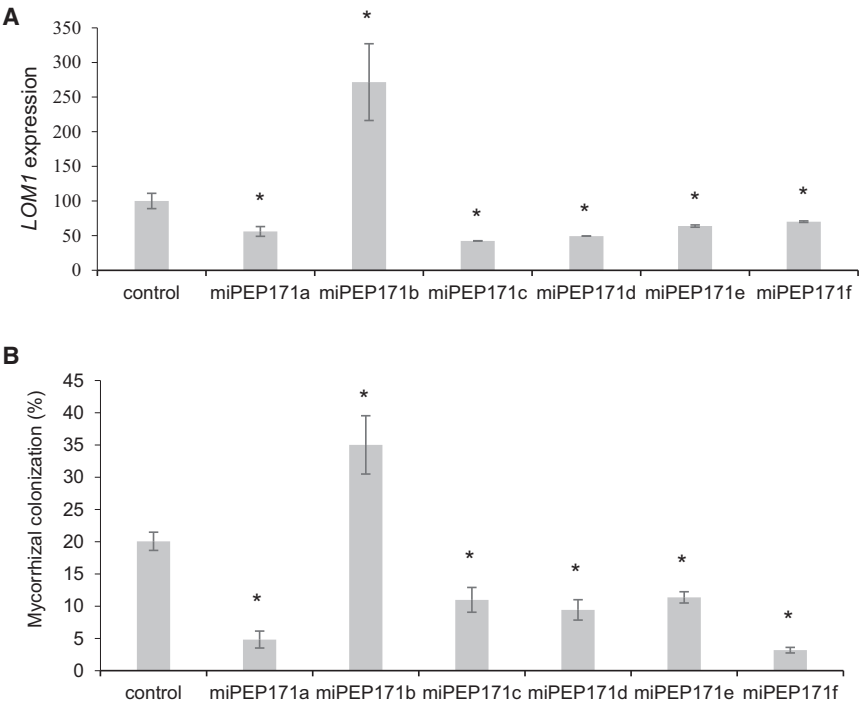

Figure 2. The Effect of miPEPs of the miR171 Family on *LOM1* Expression and Mycorrhization in *M. truncatula*

(A) Quantification by qRT-PCR of *LOM1* expression in mycorrhizal roots treated with solvent (control) or with 0.1 μM miPEP, as indicated.

(B) Quantification of mycorrhizal colonization in *M. truncatula* roots treated with solvent (control) or with 0.1 μM miPEP, as indicated. Error bars represent SEMs; asterisks indicate a significant difference between the test condition and the control according to Student's t test or the Kruskal-Wallis test (n = 6 for expression and n = 30 roots for mycorrhization, $p < 0.05$).

We show here that the use of miPEPs, together with GUS analysis, is powerful to really discriminate the exact role of each of the members of an miRNA family in a biological process. By this way, we described the antagonistic roles of members of an miRNA family during the mycorrhization process. Up to now, few miRNAs have been functionally shown to regulate the AM symbiosis (Lauressergues et al., 2012; Bazin et al., 2013; Etemadi et al., 2014). We identified in this study five additional ones (miR171a, c, d, e, and f) with a negative effect on the establishment of AM symbiosis, and we identified an miRNA (miR171b) with positive effect on this symbiosis.

Whereas the biogenesis of miRNAs is now well described (Reis et al., 2015), regulation of miRNA activity remains poorly understood. Here, we have identified a mechanism of regulation of miRNA activity in which one member of an miRNA

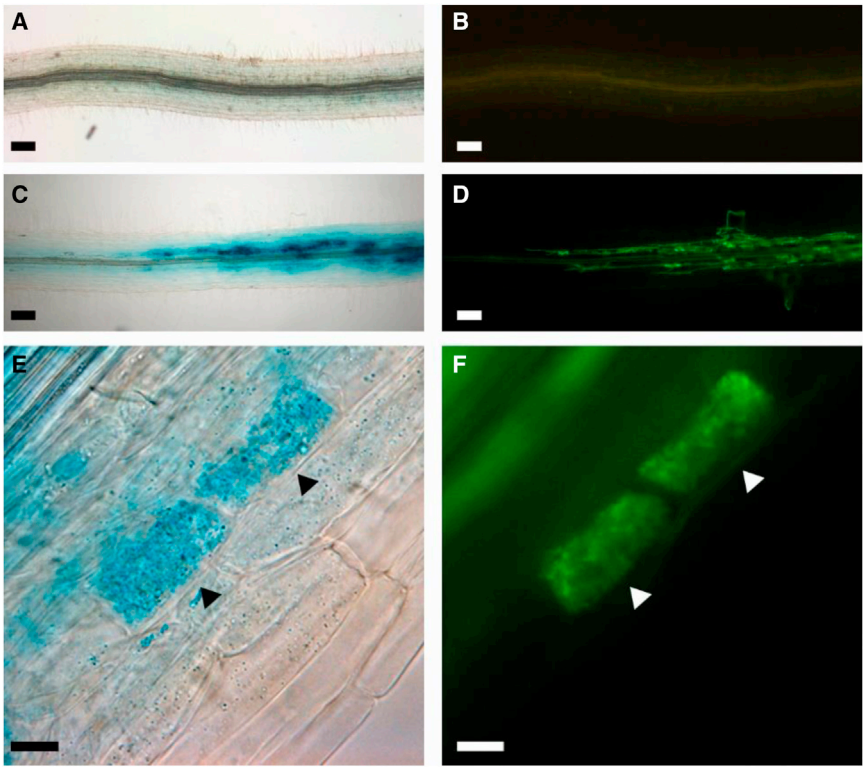

Figure 3. Expression of *miR171b* in *M. truncatula* Roots

GUS reporter gene expression (blue) driven by the miR171b promoter in roots that are not colonized (A) or colonized (C and E) by the AM fungus *Rhizophagus irregularis*, as observed by bright field microscopy. Corresponding fluorescence microscopy (B, D, and F) to reveal the presence of the fungus stained with WGA-FITC (green). Black and white arrowheads indicate two cells containing arbuscules. Scale bars, 100 (A–D) and 10 μM (E and F).

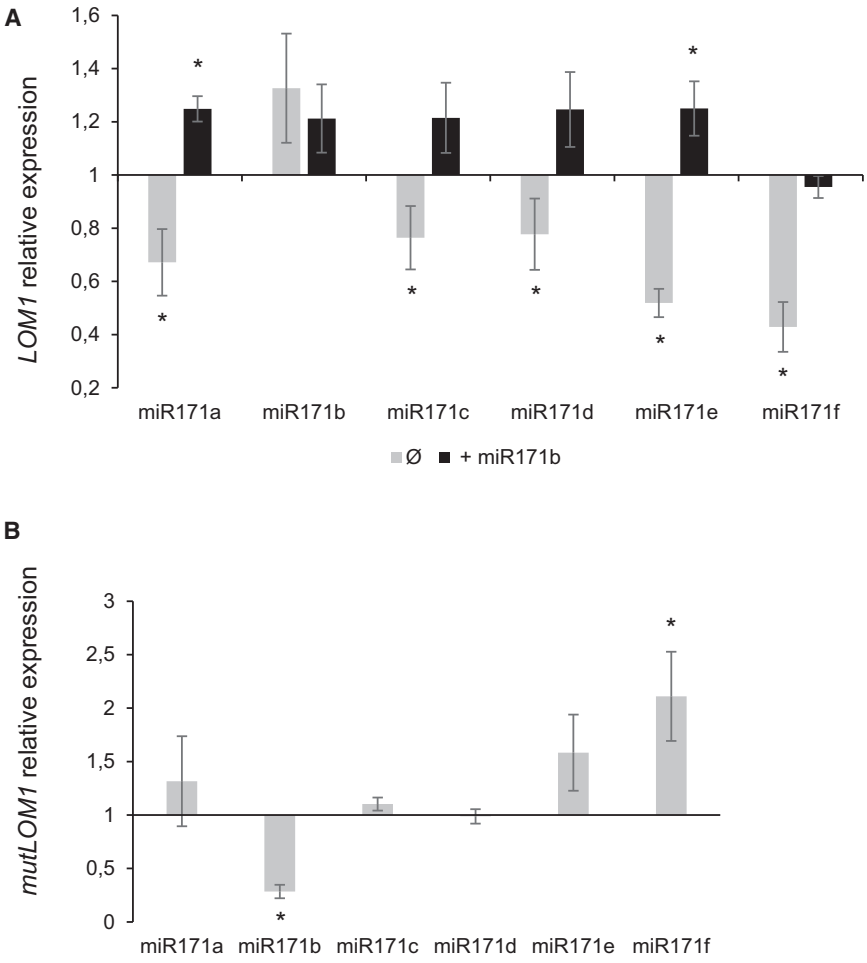

Figure 4. Effect of miR171b and Other Members of the miR171 Family on *LOM1* Expression in Tobacco Leaves

(A) Quantification of *LOM1* expression by qRT-PCR co-expressing *LOM1* and one member of the miR171 family in the absence (gray bars) or presence (black bars) of a third vector expressing miR171b. Expression level for the control (*LOM1* expression and empty vector [gray bars] and *LOM1* and miR171b expression [black bars]) was set at 1.

(B) Quantification of *LOM1* expression by qRT-PCR co-expressing *LOM1* with a mutated cleavage site complementary to that of miR171b (*mut LOM1*), and one member of the miR171 family. Values are expressed as a ratio of *LOM1* expression in the presence of the miR171 vector to *LOM1* expression in the presence of an empty control vector. Error bars represent SEMs; asterisks indicate a significant difference between the test condition and the control according to the Kruskal-Wallis test ($n = 8$, $p < 0.05$).

family has a mutation in its canonical cleavage site that apparently protects the target gene against silencing by the other family members. We hypothesize that this miRNA interacts with the target mRNA but does not cleave it; thus, this miRNA competes with the other members of its family to prevent their activity. A symmetrical phenomenon has been reported in which an miRNA is neutralized by long RNA targets containing a sequence complementary to the miRNA but with a mismatched loop at the cleavage site. In this process, called "target mimicry" (Franco-Zorrilla et al., 2007), the target RNA is not cleaved but it sequesters the miRNA and prevents its activity. In the case described here, by contrast, the target gene transcript, rather than the miRNA, would be sequestered. We have identified at least one other miRNA family, the miR156 family, in which some members have a mismatch at the cleavage site for which there are, apparently, no corresponding target genes (Figure S4). Interestingly, we identified the presence of this putative protective miRNA in several species of the green lineage, suggesting a conservation of the mechanism. Also, a point mutation in the cleavage site of miR534b in *Physcomitrella patens* has been reported, although no functional analyses were performed (Saleh et al., 2011). These findings suggest that protective miRNAs may exist in other plant miRNA families and that they may be part of a widespread mechanism for regulating gene silencing by miRNAs.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Plasmids were constructed by using the Golden Gate cloning strategy (Lauressergues et al., 2015). For promoter-GUS reporter gene analyses, the sites of transcription initiation were identified by using 5′ RACE PCR, and 3 kb of promoter were cloned upstream of the GUS open reading frame. To express the miR-resistant *LOM1* gene, we amplified the full-length *LOM1* gene under the control of 3 kb of its promoting region and mutated the miRNA cleavage site while conserving the wild-type amino acid sequence.

Plant Transformation

Tobacco leaf infiltration and *M. truncatula* root transformation were performed according to Combier et al. (2008).

Plant Cultivation

Plants were cultivated according to Lauressergues et al. (2012). To test the effects of miPEPs on mycorrhization, plants were watered three times per week with 0.1 μM miPEP.

miPEP Sequences

Peptides were synthesized by Smartox (https://www.smartox-biotech.com/) and dissolved at 10 mM according to the manufacturer's recommendations. They are as follows: MtmiPEP171a (MKKFEFPSAF), MtmiPEP171b (MLLHRLSKFCKIERDIVYIS), MtmiPEP171c (MVNLYFV), MtmiPEP171d (MHMYLK), MtmiPEP171e (MMVFGKPKKAMLVRFNPKTDLHV), MtmiPEP171f (MSHID), LjmiPEP171b (MYHRSKAKLCQTDGDDGGGSDM), OsmiPEP171i (MIARYIEREMTSKLGRGRKRAARLVAVFLLG), and SlmiPEP171e (MKLGNIEGTYFIICLGRYI).

Phenotypic Analyses

For root mycorrhizal phenotyping, roots were cleared in 10% w/v KOH and rinsed in sterile water; treated for 30 min with fluorescein-conjugated wheat germ agglutinin (WGA-FITC) (Invitrogen), which binds fungal chitin; then washed three times for 10 min in PBS and observed using an inverted light

microscope or a confocal microscope (Leica). Alternatively, they were stained with Schaeffer black ink as described by Vierheilig et al. (1998). The percentage of mycorrhization was established by using the grid intersect method described by Giovannetti and Mosse (1980). More precise quantification of mycorrhizal colonization was also performed as described by Trouvelot et al. (1986): the frequency (F) of mycorrhiza in the root system and the arbuscule abundance (a) (percentage) were calculated in the colonized root sections by using Mycocalc software (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

5′ RACE PCR

5′ RACE PCR was conducted according to the manufacturer's recommendations (FirstChoise RLM-RACE kit-Ambion) on RNA extracted directly from roots with QIAGEN RNeasy kit.

Sequence Comparison Analysis

The evolutionary history of miRNAs was inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories [+*G*, parameter = 0.8653]). The analysis involved 60 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 15 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Analysis of miR156 homologs was performed using miRBase database (http://www.mirbase.org/) for the identification of miRNAs and Blast search against the different genomes (https://phytozome.jgi.doe.gov/pz/portal.html#!search) for the identification of target genes.

Expression Analyses

qRT-PCR analyses were performed as described in Lauressergues et al., (2015). Expression levels for the controls were set at 100.

Statistical Analyses

The mean values of relative gene expression or mycorrhization were compared by using the Kruskal-Wallis test ($n < 30$) or Student's t test ($n > 30$). Each experiment was repeated at least three times. The error bars represent the SEM. The asterisks indicate significant differences ($p < 0.05$).

Imaging

GUS staining was performed as described in Combier et al. (2006) and images were acquired using an Axiozoom V16 macroscope (Zeiss).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.12.001.

AUTHOR CONTRIBUTIONS

J.-P.C. designed the research; J.-P.C., J.-M.C., B.G., O.A., C.G., and D.L. performed the experiments; and J.-P.C., J.-M.C., and G.B. wrote the paper.

ACKNOWLEDGMENTS

This work was funded by the French ANR project miRcorrhiza (ANR-12-JSV7-0002-01). We thank J.M. Prospéri (UMR AGAP 1334) for *M. truncatula* seeds and members of the Imagery center (TRI Genotoul, FRAIB), particularly A. Le Ru, for helpful assistance and advice. C.G. was supported by the Emmy Noether program of the DFG (GU1423/1-1). This work was also supported by Toulouse Tech Transfer (http://www.toulouse-tech-transfer.com) for valorization and transfer.

Received: May 27, 2016
Revised: September 1, 2016
Accepted: November 16, 2016
Published: December 29, 2016

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