Mildew Locus O facilitates colonization by arbuscular mycorrhiza in angiosperms

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Summary

- Loss of barley Mildew Resistance Locus O (MLO) is known to confer durable and robust resistance to powdery mildew (Blumeria graminis), a biotrophic fungal leaf pathogen. Based on the increased expression of MLO in mycorrhizal roots and its presence in a clade of the MLO family that is specific to mycorrhizal-host species, we investigated the potential role of MLO in arbuscular mycorrhizal interactions.
- Using mutants from barley, wheat, and Medicago truncatula, we demonstrate a role for MLO in colonization by the arbuscular mycorrhizal fungi Rhizophagus irregularis.
- Early mycorrhizal colonization was reduced in mlo mutants of barley, wheat and Medicago truncatula, and this was accompanied by a pronounced decrease in the expression of many of the key genes required for intracellular accommodation of arbuscular mycorrhizal fungi.
These findings suggest that the primary role of MLO in angiosperms is in the establishment of symbiotic associations with beneficial fungi, which has been appropriated by powdery mildew.

Introduction

Plants have coevolved with microbes, which is evident in the elaborate strategies that plants use to both promote beneficial symbioses and to restrict pathogenesis. For example, to establish beneficial endosymbioses like arbuscular mycorrhization, plants have dedicated host signaling pathways involving hundreds of genes (Bonfante & Genre, 2010; Oldroyd, 2013; Bravo et al., 2016). Similarly, to keep pace with pathogens, components of the plant immune system are rapidly evolving and expanding (Chisholm et al., 2006; Jones & Dangl, 2006). Pathogens employ strategies to take advantage of host genes—so-called ‘susceptibility factors’—to cause disease (Panstruga, 2003; O’Connell & Panstruga, 2006). One example of a susceptibility factor is the barley \textit{Mildew resistance locus O} (MLO) gene—referred to here as MLO1 to distinguish it from other family members—which is required for successful colonization by powdery mildew fungi, \textit{Erysipheles} (Jørgensen, 1992). However, the extent to which host susceptibility factors are required for beneficial symbioses is unknown.

MLO encodes a plasma membrane protein that possesses seven transmembrane helices and a C-terminal calmodulin-binding domain (Devoto et al., 1999; Kim et al., 2002). Most research on MLO1 has been on its role in susceptibility to powdery mildew. Knock-out mutants of MLO1 in barley have been successfully deployed to provide broad-spectrum, durable resistance to powdery mildew since 1942 (Freisleben & Lein, 1942; Jørgensen, 1992). Despite considerable progress on the characterization of the powdery mildew resistance phenotype and its underlying molecular mechanism (Kim et al., 2002; Piffanelli et al., 2002; Consonni et al., 2010), MLO1’s biological role remains unclear. Notably, orthologs of MLO1 are only present in those plant species that can host arbuscular mycorrhizal fungi (Bravo et al., 2016). The study of other MLO family members has revealed their involvement in diverse plant processes, including pollen tube reception (AtMLO7/NORTIA) and root thigmomorphogenesis (Kessler et al., 2010; Bidzinski et al., 2014). The ancestral function of MLO proteins is unknown, but their presence in green algae suggests that the so-far characterized roles are derivative (Takamatsu, 2004; Jiao et al., 2011). In \textit{Arabidopsis thaliana}, loss of MLO family members
belonging to a separate clade from barley MLO1 confer resistance to powdery mildew in a redundant fashion (Kusch et al., 2016; Kuhn et al., 2017). Interestingly, one of these can complement the Atmlo7 pollen tube reception phenotype, suggesting a conserved biochemical function of MLO proteins (Jones et al., 2017).

Most land plants can form an endosymbiosis with members of the *Glomeromycota*, reflecting the ancient origins of this interaction (Remy et al., 1994; Parniske, 2008). This mutualistic relationship supplies water and nutrients—particularly phosphate, but also nitrogen and zinc—to the host plant (Cooper & Tinker, 1978; Lambert et al., 1979; Govindarajulu et al., 2005; Watts-Williams & Cavagnaro, 2012). Several key components of the signaling pathway required for establishing mycorrhizal symbioses (the common SYM pathway) are conserved in green algae, suggesting that land plant ancestors were preadapted for symbiosis (Delaux et al., 2013; Delaux et al., 2015). Furthermore, MLO1 was previously suggested to have a role in arbuscular mycorrhization (Ruiz-Lozano et al., 1999). However, the analysis was limited to a single *mlo* allele, which was evaluated at a single time point. Since then, an additional study found no evidence supporting a role for an MLO1 homolog in arbuscular mycorrhization of pea (Humphry et al., 2011).

Using mutants from barley, wheat, and *M. truncatula* and transcript profiling of *Hvmlo1* mycorrhizal roots, we reveal an unequivocal role for MLO in the early stages of arbuscular mycorrhizal colonization in angiosperms. The findings suggest that the primary role of MLO is in the establishment of fungal symbioses, which has been exploited by powdery mildew.

**Materials and methods**

**Phylogenetic analyses**

Sequences were aligned using MUSCLE (Edgar, 2004), and a maximum likelihood tree was constructed using MEGA X following a Jones-Taylor-Thornton (JTT) model with 100 bootstrap replications (Kumar et al., 2018). Protein sequences and references are in Methods S1.

**Barley, wheat and *M. truncatula* mutants**

Details of the barley and wheat *mlo* mutants used are shown in Methods S2. To identify homozygous *M. truncatula mlo8 Tnt1*-insertion mutants, DNA was extracted (DNeasy 96 Plant
Kit, Qiagen). Genotyping of segregating seedling populations was performed by PCR using primers listed in Methods S3. To detect the MtMLO8 cDNA, RNA was extracted from mycorrhizal M. truncatula roots (Plant RNeasy Kit, Qiagen). DNases were removed (TURBO DNA-free, Ambion), and 150 ng of RNA was retrotranscribed (SuperScript IV reverse transcriptase, Invitrogen). cDNA was amplified by RT-PCR (Phusion High Fidelity Polymerase kit, New England Biolabs) using primers listed in Methods S3.

**Plant growth conditions and mycorrhization and powdery mildew assays**

For mycorrhization experiments, barley, wheat, and M. truncatula were grown in pots containing 80-90% Terragreen/sand and 10-20% mycorrhizal inoculum (homogenized soil substrate containing Allium schoenoprasum roots exposed to Rhizophagus irregularis for 8 weeks). Full descriptions of seed sterilization, soils substrates, and growth conditions for mycorrhization and powdery mildew assays are provided in Methods S4.

**Agrobacterium rhizogenes-mediated gene transfer**

A. rhizogenes-mediated gene transfer (Boisson-Dernier et al., 2001), was performed using strain AR1193. The promoter-GUS construct pMtMLO8:GUS was generated using Gateway® technology (Primrose & Twyman, 2013): entry vector pDONR207; destination vector 243_pKGW-GGRR. The 1983 bp promoter region of MtMLO8 (Medtr3g115940) was amplified using primers with Gateway-compatible end sequences (Methods S3). The complementation construct pMtMLO8:MtMLO8 was generated using the Golden Gate system (Engler et al., 2009) using the same MtMLO8 promoter region fused to the 1656 bp coding region. Domesticated DNA parts were synthesized by GeneArt® (Life Technologies). pL2V-50507 was used for the L2 backbone, with pLjUBIQUITIN:DsRed:t53S placed in position 1, and pMtMLO8:MLO8:t35S in position 2.

**Fungal staining and quantification**

Staining (ink, GUS, and WGA-Alexa488) and visualization methods are summarized in Methods S5. Samples were blinded and mycorrhizal infection structures quantified using the gridline intersect method (Giovannetti and Mosse, 1980). Powdery mildew fungal structures were quantified by calculating the ratio of germinated spores: colony formation.
**Gene expression analyses**

RNAs were extracted from root tissue (Plant RNeasy Kit, Qiagen) and was treated with DNase (RNase-Free DNase Set, Qiagen). Methods for RT-qPCR and transcriptome analyses can be found in Methods S6.

**Results**

To assess a potential function of MLO1 in mycorrhization, we compared expression levels of barley *MLO1* (*HvMLO1*) in mycorrhizal and non-mycorrhizal roots (Fig. 1a). At 22 days post-inoculation (dpi) with *R. irregularis*, RT-qPCR analysis indicated that *HvMLO1* was induced in mycorrhizal roots. We then evaluated mycorrhizal phenotypes of barley *Hvmlo1* mutants by quantification of mycorrhizal fungal infection structures over a time-course. Arbuscules and vesicles were quantified at 22, 29, and 36 dpi with *R. irregularis* (20% inoculum) in barley cv. Ingrid wild type, *Hvmlo1-1*, and *Hvmlo1-5* roots (Fig. 1b). We found a reduction in arbuscule and vesicle occurrence in *Hvmlo1-1* and *Hvmlo1-5* compared to wild type at 22 dpi. This difference was maintained in *Hvmlo1-5* at 29 dpi, whereas *Hvmlo1-1* showed a similar phenotype to wild type. By 36 dpi, no significant difference in mycorrhization between the lines was detectable. Wild type roots appeared to have established full mycorrhization between 22 and 29 dpi, whereas the *Hvmlo1* mutants established full colonization between 29 and 36 dpi. Overall, reduced mycorrhization was more evident at early time points, suggesting that mycorrhizal development in *Hvmlo1* is delayed, but later recovers to wild type levels.

To further assess the mycorrhizal phenotype of *Hvmlo1* during the early stages of mycorrhization, we performed a detailed analysis of symbiotic structures (hyphopodia, intraradical hyphae, arbuscules and vesicles) at early time points using less *R. irregularis* inoculum (10% inoculum) (Fig. 1c,d). At 17 dpi, *Hvmlo1-5* showed a statistically significant reduction in the occurrence of hyphopodia, arbuscules, and vesicles compared to wild type. Similar to the previous experiment, by 26 dpi, *Hvmlo1-5* showed a statistically significant reduction in only arbuscule occurrence compared to wild type. A similar phenotype was observed between wild type and *Hvmlo1-5* in a different genetic background, barley cv. Pallas, in two separate experiments (Fig. S1). We observed no differences in the physical structure of hyphopodia or arbuscules in *Hvmlo1-5* mutants (Fig. 1c).
To gain insight into the nature of the delayed infection in *Hvml01-5*, we carried out comparative transcriptomic analyses at 17 dpi and 26 dpi with *R. irregularis* (Fig. 1e). At 17 dpi, there were considerably fewer differentially regulated genes (DEGs) in *Hvml01-5* than wild type, with 86% (3171/3672) of all wild type DEGs not responding in *Hvml01-5*. Whereas by 26 dpi, despite the recovery of observable colonization in the mutant, there was still a substantial (~30%) reduction of the number of DEGs in *Hvml01-5* relative to wild type, suggesting persistent perturbation of the mycorrhizal interaction.
Fig. 1 Mycorrhizal colonization in barley and wheat mlo1 mutants. (a) Relative expression of HvMLO1 in wild type barley cv. Ingrid roots without (-) and with R. irregularis (+AM) at 22 dpi. Relative expression levels were measured by RT-qPCR and normalized to HvEF1alpha (barley). Statistical comparisons were made relative to non-mycorrhized root samples. Bars represent means of 8 biological replicates ± SEM (Student’s t-test: ***P <0.001).

(b, d, f) Quantification of arbuscular mycorrhizal infection structures in barley cv. Ingrid and wheat cv. KN1999 wild type and mlo1 roots after inoculation with R. irregularis: hyphopodia (H), intraradical hyphae (IH), arbuscules (A), and vesicles (V). The binomial occurrences of mycorrhizal structures are shown as a percentage of the total number of root sections assessed. Statistical comparisons have been made to the wild type. Values
are the mean of 12 biological replicates ±SEM (error bars) (General Linear Model with a logit link function; ANOVA; *, P < 0.05; **, P < 0.01; ***, P < 0.001). (c) Appearance of hyphopodia (H) and arbuscules (A) in wild type and \textit{Hvmlo1-5} roots at 17 dpi with \textit{R. irregularis}. Mycorrhizal fungal structures were stained using Alexa Fluor 488 wheat germ agglutinin. Photos indicate representative examples from 40 observations. Scale bars = 50 \mu m.

(e) Venn diagrams showing the number of differentially regulated genes (DEGs) in mycorrhizal roots relative to uninoculated roots in wild type and the proportion of genes similarly responding in \textit{Hvmlo1-5} using a cut-off of fold change > 2; FDR-corrected P-value < 0.05.

To further investigate the nature of the differences in \textit{Hvmlo1-5}, we identified the sequences of potential barley orthologues of known mycorrhizal genes previously identified in other species and examined their expression in our data. Amongst the genes induced more than 2-fold in wild type—but not in \textit{Hvmlo1-5}—at 17 dpi, were genes required for early infection and components of the common signalling pathway required for nodulation and mycorrhization, \textit{VAPYRIN}, \textit{NSP1}, \textit{NSP2}, \textit{DMI2}, \textit{IPD3} and \textit{NOPE1} homologues (Catoira \textit{et al.}, 2000; Ané \textit{et al.}, 2002; Lévy \textit{et al.}, 2004; Messinese \textit{et al.}, 2007; Pumplin \textit{et al.}, 2010; Murray \textit{et al.}, 2011; Nadal \textit{et al.}, 2017) (\textbf{Table 1}, \textbf{Table S1-S3}, \textbf{Fig. S2}). This suggests that \textit{MLO1} is required for timely or full-activation of early processes of mycorrhizal colonization. In addition, potential orthologues of several genes involved in arbuscule development and function were affected in \textit{Hvmlo1-5}, including \textit{RAM1}, \textit{RAD1}, \textit{RAM2}, \textit{EXO70I}, \textit{STR}, \textit{STR2 AMT2-3}, \textit{AMT3-4} and \textit{PT4} homologues (Harrison \textit{et al.}, 2002; Zhang \textit{et al.}, 2010; Gobbato \textit{et al.}, 2012; Wang \textit{et al.}, 2012; Breuillin-Sessoms \textit{et al.}, 2015; Xue \textit{et al.}, 2015; Zhang \textit{et al.}, 2015). Generally, these genes were induced to a lesser extent than wild type at 17 dpi, and partly or fully recovered by 26 dpi, which mirrors the initial delay and later recovery of the colonization phenotype. Together, these phenotypic and transcriptomic results indicate a role for MLO1 in the early stage of mycorrhization.
Table 1: Counts per million (CPM) values of potential orthologues of previously described mycorrhizal genes in wild type and Hvmlo1-5 non-mycorrhizal and mycorrhizal roots: significant fold changes between wt and Hvmlo1-5 at corresponding time points and treatments are indicated (FDR-corrected P-value; *, P < 0.05; **, P < 0.01; ***, P < 0.001).

| Mt gene | Mt identifier | Hv identifier | 17 dpi | 26 dpi |
|---------|--------------|---------------|--------|--------|
|         |              |               | non-mycorrhizal | mycorrhizal |
|         |              |               | wt       | Hvml1-5  |
|         |              |               | mycorrhizal  | wt       |
|         |              |               | Hvml1-5   | Hvml1-5  |

|         | mean normalized CPMs |                 |        |        |
|---------|----------------------|-----------------|--------|--------|
|         |                      |                 |        |        |
| VAPYRN  | Medtr6g027840        | HORVU4Hr1G002180| 14.3   | 11.0   |
|         |                      |                 | 32.2   | 16.2   ***|
|         |                      |                 | 17.0   | 18.8   |
|         |                      |                 | 62.4   | 63.6   |
| NSP1    | Medtr8g020840        | HORVU2Hr1G104160| 1.0    | 2.6    |
|         |                      |                 | 4.0    | 2.2    *|
|         |                      |                 | 6.0    | 6.9    |
|         |                      |                 | 8.9    | 6.6    |
| NSP2    | Medtr3g072710        | HORVU4Hr1G061310| 2.7    | 3.2    |
|         |                      |                 | 11.2   | 2.5    ***|
|         |                      |                 | 4.5    | 5.8    |
|         |                      |                 | 47.2   | 57.3   |
| DMI1    | Medtr2g005870        | HORVU5Hr1G120340| 28.3   | 33.3   |
|         |                      |                 | 44.2   | 33.2   **|
|         |                      |                 | 61.1   | 62.1   |
|         |                      |                 | 80.6   | 74.6   |
| DMI2    | Medtr5g030920        | HORVU2Hr1G058820| 11.1   | 17.6   |
|         |                      |                 | 72.8   | 29.2   ***|
|         |                      |                 | 57.0   | 71.3   |
|         |                      |                 | 181.0  | 195.0  |
| DMI3    | Medtr8g043970        | HORVU1Hr1G068660| 14.0   | 17.0   |
|         |                      |                 | 23.8   | 14.9   **|
|         |                      |                 | 38.4   | 35.2   |
|         |                      |                 | 52.3   | 48.6   |
| IPD3    | Medtr5g026850        | HORVU7Hr1G008420| 12.6   | 19.8   |
|         |                      |                 | 56.4   | 22.9   ***|
|         |                      |                 | 54.7   | 56.7   |
|         |                      |                 | 158.8  | 138.8  |
| NOPE1   | Medtr3g093270        | HORVU2Hr1G005470| 9.1    | 10.1   |
|         |                      |                 | 53.5   | 18.5   ***|
|         |                      |                 | 18.8   | 15.9   |
|         |                      |                 | 121.8  | 149.8  |
| RAD1    | Medtr4g104020        | HORVU3Hr1G088780| 0.3    | 0.5    |
|         |                      |                 | 7.6    | 0.4    ***|
|         |                      |                 | 1.5    | 1.6    |
|         |                      |                 | 16.9   | 15.6   |
| RAM2    | Medtr1g040500        | HORVU4Hr1G011110| 5.3    | 4.7    |
|         |                      |                 | 30.6   | 5.5    ***|
|         |                      |                 | 7.7    | 8.5    |
|         |                      |                 | 77.7   | 68.1   |
| EXO70I  | Medtr1g017910        | HORVU7Hr1G052100| 0.1    | 0.1    |
|         |                      |                 | 11.6   | 2.2    ***|
|         |                      |                 | 0.1    | 0.1    |
|         |                      |                 | 34.0   | 37.4   |
| STR     | Medtr8g107450        | HORVU1Hr1G060690| 1.3    | 0.5    |
|         |                      |                 | 14.5   | 1.7    ***|
|         |                      |                 | 0.7    | 0.4    |
|         |                      |                 | 56.1   | 50.4   |
| STR2    | Medtr5g030910        | HORVU3Hr1G066220| 0.0    | 0.0    |
|         |                      |                 | 26.2   | 2.5    ***|
|         |                      |                 | 0.1    | 0.2    |
|         |                      |                 | 83.3   | 84.2   |
| AMT2-3  | Medtr8g074750        | HORVU3Hr1G082610| 0.0    | 0.0    |
|         |                      |                 | 14.3   | 0.7    ***|
|         |                      |                 | 0.0    | 0.0    |
|         |                      |                 | 53.8   | 45.5   |
| AMT2-4  | Medtr7g115050        | HORVU5Hr1G095030| 0.0    | 0.0    |
|         |                      |                 | 3.0    | 0.5    ***|
|         |                      |                 | 0.0    | 0.0    |
|         |                      |                 | 9.8    | 12.0   |
| PT4     | Medtr8g074750        | HORVU6Hr1G058690| 0.0    | 0.0    |
|         |                      |                 | 3.9    | 1.0    |
|         |                      |                 | 0.0    | 0.0    |
|         |                      |                 | 16.6   | 21.0   |
To investigate the conservation of function for this MLO, we used phylogenetic analysis to identify MLO proteins which may be functionally analogous to barley HvMLO1 (Fig. 2). The analysis included mycorrhizal host species: basal angiosperm *Amborella trichopoda* (Atr); dicots *Glycine max* (Gm), *M. truncatula* (Mt), *Pisum sativum* (Ps) and *Solanum lycopersicum* (Sl); and monocots *H. vulgare* (Hv), *Oryza sativa* (Os) and *Triticum aestivum* (Ta), as well as non-mycorrhizal host species: moss *Physcomitrella patens* (Pp); and dicots *A. thaliana* (At), *Beta vulgaris* (Bv), *Dianthus caryophyllus* (Dc) and *Lupinus angustifolius* (La). As previously shown (Kusch et al., 2016), the phylogenetic analysis supported the view that embryophyte MLO proteins diverge into seven clades. HvMLO1 groups in clade IV, which is comprised only of species that are mycorrhizal-hosts, while powdery mildew susceptibility factors identified in monocots and dicots are found in clades IV and V, respectively.

![Fig. 2 MLO phylogeny](image)

*Fig. 2 MLO phylogeny.* A phylogenetic tree showing the relationship between MLO proteins from *A. trichopoda, A. thaliana, B. vulgaris, D. caryophyllus, G. max, H. vulgare, L. angustifolius, M. truncatula, O. sativa, P. sativum, P. patens, S. lycopersicum and T. aestivum.* The *T. aestivum* MLO protein dataset often included near-identical homoeoalleles (A, B, and D copies); therefore, for simplicity, only one homoeoallele was included in the phylogenetic analysis (A homoeoallele where possible). The tree was generated using MEGA X from an amino acid alignment. The phylogenetic tree was calculated via the maximum likelihood method, using JTT modeling with 100 bootstrap replications. Bootstrap values > 0.5 are shown. The scale bar indicates the evolutionary distance based on the amino acid substitution rate. Circles indicate collapsed clades; known powdery mildew susceptibility factors are labeled in bold, and candidate MLO proteins for a role in mycorrhization are highlighted.
The wheat functional orthologue of HvMLO1, TaMLO1, is a powdery mildew host susceptibility factor (Wang et al., 2014). Tamlo1-abd contains TALEN-induced mutations in all three homoeoalleles (A, B, and D) (Wang et al., 2014). To assess the mycorrhizal phenotype of Tamlo1-abd during the early stages of mycorrhization, we performed a detailed analysis of mycorrhizal structures (hyphopodia, intraradical hyphae, arbuscules, and vesicles) at early time points similar to those used for barley (Fig. 1f). At 16 dpi with R. irregularis, Tamlo1-abd showed a significant reduction in arbuscule occurrence compared to wild type. However, consistent with our findings in barley, by 23 dpi Tamlo1-abd colonization levels were similar to wild type. Wheat mlo1 mutants exhibited the delayed mycorrhizal phenotype in two separate experiments, suggesting a conserved role for MLO1 in mycorrhization in cereals.

To determine whether MLO’s role in mycorrhization extends to dicots, we investigated M. truncatula MtMLO8, the apparent orthologue of HvMLO1, which was previously found to be conserved exclusively in mycorrhizal host plants (Bravo et al., 2016). We first tested its expression in mycorrhizal roots, and we found that like HvMLO1, MtMLO8 was induced relative to non-mycorrhizal roots (Fig. 3a, Fig. S3a), consistent with data from several studies available in the public database (Benedito et al., 2008; Breakspear et al., 2014; Luginbuehl et al., 2017). To establish in which cell types MtMLO8 is expressed, we studied its expression in M. truncatula roots using the MtMLO8 promoter to drive GUS expression using the Agrobacterium rhizogenes mediated hairy root transformation system. MtMLO8 promoter activity was strongly associated with cortical cells containing arbuscules (Fig. 3a, S3b).

To investigate whether MtMLO8 functions during mycorrhization, seeds from two independent M. truncatula cv. R108 lines carrying Tnt1 insertions in the coding sequence of these genes were obtained, and homozygous mutants were identified (Fig. 3c, d) (Tadege et al., 2008). We performed a detailed analysis of mycorrhizal structures (hyphopodia, intraradical hyphae, arbuscules, and vesicles) at multiple time points in wild type, Mtmlo8-1 and Mtmlo8-2. Compared to wild type, there was a significant reduction in arbuscules and vesicles at 16 dpi in both alleles of Mtmlo8 (Fig. 3e), consistent with our results using the orthologous mutants in barley and wheat. By 22 dpi—and at later time points (Fig. S4a)—there was no difference between the lines. We observed no impairment in the physical structure of mycorrhizal structures, hyphopodia and arbuscules in Mtmlo8 mutants (Fig. S4b). To validate that
mycorrhizal phenotype observed was due to mutations in *MtMLO8*, and not a consequence of additional *Tnt1* insertions or other mutations in the mutant backgrounds, we expressed *MtMLO8* from its native promoter in *Mtmlo8-1* mutant roots by transformation with *A. rhizogenes*. When arbuscule occurrence in roots of wild type plants transformed with the empty vector reached approximately 30%—corresponding to when the *Mtmlo8* phenotype could be observed—mycorrhizal structures (hyphopodia, intraradical hyphae, arbuscules, and vesicles) were quantified in the transgenic roots. The expression of *MtMLO8* under the control of its native promoter successfully complemented the *Mtmlo8-1* mutant phenotype (Fig. 3f). In summary, these results suggest that mutations in *MtMLO8* affect the early stages of mycorrhization and delay arbuscule development.

To assess whether, like HvMLO1 and TaMLO1, MtMLO8 might also have a role in powdery mildew susceptibility, we inoculated wild type and *Mtmlo8* mutant leaves with an isolate of powdery mildew that can infect *M. truncatula*, *Erysiphe pisi*. We observed a small but statistically significant reduction in the percentage of successful host cell entry (ratio of germinated spores: colony formation) in *Mtmlo8* compared to wild type (Fig. 3g). This result indicates that *MtMLO8* is required not only for early mycorrhizal infection but also for powdery mildew colonization.
Fig. 3 MtMLO8 is involved in mycorrhizal colonization of *M. truncatula*. (a) Relative expression of *MtMLO8* in wild type *M. truncatula* cv. R108 without (-) and with *R. irregularis* (+AM) at 22 dpi. Relative expression levels...
were measured by RT-qPCR and normalized to the geometric mean of MtUBIQUITIN and MtPTB. Statistical comparisons were made relative to non-mycorrhized root samples. Bars represent means of 8 biological replicates ± SEM (error bars) (Student’s t-test: ***P <0.001). (b) Activity of the MtMLO8 promoter in mycorrhized and non-mycorrhized wild type roots at 21 dpi with R. irregularis, assessed using a promoter-GUS fusion. Bright-field and corresponding green fluorescence images of M. truncatula hairy roots expressing the β-glucuronidase (GUS) gene under the control of the MtMLO8 promoter. Mycorrhizal fungal structures were visualized using Alexa Fluor 488 wheat germ agglutinin. Solid arrowheads indicate cells containing arbuscules and empty arrowheads indicate hyphopodia. Scale bars = 100 μm. (c) Gene structure of M. truncatula MtMLO8. Arrows indicate the Tobacco retrotransposon 1 (Tnt1) insertion sites in the Mtmlo8 mutants. (d) RT-PCR was used to detect the accumulation of the MtMLO8 transcript in wild type, Mtmlo8-1, and Mtmlo8-2 mutant roots. MtUBIQUITIN was used as a constitutive control. (e) Quantification of mycorrhizal structures in M. truncatula cv. R108 wild type (wt), Mtmlo8-1 and Mtmlo8-2 roots. (f) Complementation of the Mtmlo8-1 mutants and quantification of mycorrhizal structures in hairy roots. Mtmlo8-1 was transformed with pMtMLO8:MtMLO8 or empty vector control (EV), and wild type were transformed with EV control. The binominal occurrences of mycorrhizal structures, hyphopodia (H), intraradical hyphae (IH), arbuscules (A) and vesicles (V), are shown as a percentage of the total number of root systems assessed. (g) Quantification of powdery mildew infection on wild type, Mtmlo8-1 and Mtmlo8-2 leaves at 72 hpi with Erysiphe pisi. The binominal occurrences of successful host cell entry are shown as a percentage of the total number of spores observed. For figures e, f, g statistical comparisons have been made to the wild type. Bars represent the mean of 15 (d), 10 (e) and 10 (f) biological replicates ± SEM (error bars) (General Linear Model with a logit link function; ANOVA, post hoc pairwise; *, P < 0.05; **, P < 0.01; ***, P < 0.001).

**Discussion**

Since MLO’s function in facilitating powdery mildew infection is disadvantageous to the host, it follows that it must also fulfill some other positive role that explains its conservation. The phylogenetic analyses, mutant phenotypes, and gene expression studies presented here point to a symbiotic role for members of the MLO clade IV and suggest that—at least in part—selection to maintain this gene is related to its function in mycorrhizal interactions. A common thread between the established roles of MLO in fungal interactions, pollen-tube reception, and thigmotropism is touch-sensing. Indeed, MLO proteins have been reported to accumulate at the site of powdery mildew penetration (Bhat et al., 2005), as well as at the contact point between the pollen tube and synergid cell (Kessler et al., 2010). These responses could result from a generic response to mechanical stimuli. Notably, a role for mechanosensing has been proposed for the receptor kinase Feronia (Hamant & Haswell, 2017), which was shown to be required for proper re-localization of MLO7/NORTIA to the site of pollen tube contact in the synergid cell (Kessler et al., 2010; Jones et al., 2017). However, other cues may activate MLO, for example, both Blumeria graminis and R. irregularis produce effectors that could target MLO (Kloppholz et al., 2011; Requena et al., 2018; Pennington et al., 2019; Saur et al., 2019).
Regardless of how it is activated, it will be of interest to study the localization of MLO1 or its orthologues during hyphopodium formation.

The mycorrhizal phenotype of the mlo mutants examined was not severe, but may be expected to have measurable consequences on fitness in a natural setting with competition for limited resources. Furthermore, global gene expression was still affected at the later time point, so a detailed study of growth and nutrient uptake characteristics of the mutant may show differences. In addition, the increased expression of other MLOs during mycorrhization (Fig. S3a) in M. truncatula suggests that—as for other mlo phenotypes such as powdery mildew resistance (Kuhn et al., 2017) and synergid cell reception (Jones & Kessler, 2017)—MLOs act redundantly, with different MLOs potentially contributing unequally across different cell types and developmental stages. It is, therefore, possible that higher-order mutants will have increasingly stronger arbuscular mycorrhizal phenotypes. The powdery mildew phenotype of Mtmlo8 was relatively minor. It is possible that multiple MLO genes contribute to powdery mildew susceptibility as is the case in A. thaliana, for example, MtMLO1—the apparent orthologue of PsMLO—which is involved in powdery mildew susceptibility in pea (Humphry et al., 2011; Pavan et al., 2011). Notably, the narrow time window for mycorrhizal phenotype definition and potential functional redundancy with gene family members could explain why MLO has not so far been identified in mutagenesis screens.

Despite having small yield penalties, mutants for MLO have been deployed in agriculture for decades. Understanding the role of MLO in symbiotic interactions with mycorrhizal fungi is therefore vital information for sustainable agriculture (Martin et al., 2018). Our detailed phenotypic and transcriptomic analyses in both cereals and M. truncatula provide the basis for further elucidation of MLO function in mycorrhization and how plants balance this beneficial association with susceptibility to parasitic powdery mildews. As mycorrhizal and powdery mildew respectively infect root and shoot, it may be possible to generate genotypes that could fully support mycorrhiza while remaining non-hosts for powdery mildew.

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**Author contribution**

CNJ performed experimental work and data analyses. MC, JDM, and CJR supervised and co-directed the research. CNJ, JDM, CJR and MC wrote the manuscript.

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