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Plant lysin motif extracellular proteins are required for arbuscular mycorrhizal symbiosis

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Arbuscular mycorrhizal fungi (AMF) can form a mutually beneficial symbiotic relationship with most land plants. They are known to secrete lysin motif (LysM) effectors into host root cells for successful colonization. Intriguingly, plants secrete similar types of LysM proteins; however, their role in plant–microbe interactions is unknown. Here, we show that *Medicago truncatula* deploys LysM extracellular (LysMe) proteins to facilitate symbiosis with AMF. Promoter analyses demonstrated that three *M. truncatula LysMe* genes *MtLysMe1/2/3*, are expressed in arbuscule-containing cells and those adjacent to intercellular hyphae. Localization studies showed that these proteins are targeted to the periarbuscular space between the periarbuscular membrane and the fungal cell wall of the branched arbuscule. *M. truncatula* mutants in which *MtLysMe2* was knocked out via CRISPR/Cas9-targeted mutagenesis exhibited a significant reduction in AMF colonization and arbuscule formation, whereas genetically complemented transgenic plants restored wild-type level AMF colonization. In addition, knocking out the ortholog of *MtLysMe2* in tomato resulted in a similar defect in AMF colonization. In vitro binding affinity precipitation assays suggested binding of MtLysMe1/2/3 with chitin and chitosan, while microscale thermophoresis (MST) assays revealed weak binding of these proteins with chitooligosaccharides. Moreover, application of purified MtLysMe proteins to root segments could suppress chitooctaose (CO8)-induced reactive oxygen species production and expression of reporter genes of the immune response without impairing chitotetraose (CO4)-triggered symbiotic responses. Taken together, our results reveal that plants, like their fungal partners, also secrete LysM proteins to facilitate symbiosis establishment.

arbuscular mycorrhizal symbiosis | immune response | LysMe proteins | chitin | *Medicago truncatula*

Significance

Proteins containing lysin motifs (LysM) play important roles in plant–microbe interactions. We previously revealed that AMF could secrete LysM effectors to promote mycorrhizal symbiosis. In this study, we further demonstrated that plants could secrete LysM extracellular (LysMe) proteins to the extracellular space between the peri-arbuscular membrane and the fungal cell wall where these proteins may bind chitin-derived molecules and play a role in the establishment of AM symbiosis. Our results suggest that, like their partner AMF, host plants also deploy secreted LysM proteins as susceptibility factors to facilitate AM symbiosis.

Most terrestrial plant species can interact with arbuscular mycorrhizal fungi (AMF) to form a mutualistic endosymbiosis which has existed for over 400 My on the earth (1). AMF colonization not only improves the uptake of soil nutrients such as phosphorus and nitrogen by the host plants but also enhances plant tolerance to biotic/abiotic stresses (2, 3). In return, the host plants provide sugars and lipids for AMF to maintain the symbiosis (4, 5).

Similar to pathogenic fungi, AMF also possess common fungal cell wall components such as chitin, which is one of the so-called microbial-associated molecular patterns (MAMPs) that trigger immune responses (6). MAMPs are recognized by plasma membrane–localized pattern recognition receptors (PRRs) (7–9). Lysin motif (LysM) receptor proteins/kinases are well-known PRRs that perceive chitin-derived molecules, and they play vital roles in oneself recognition and signaling during plant interaction with pathogenic as well as beneficial microbes (10, 11).

The LysM domain is around 40 amino acids in length and represents an ancient protein domain found in a variety of extracellular proteins and transmembrane receptors (12, 13). Plant LysM domain–containing proteins can be divided into four groups based on their structural characteristics and subcellular localization. These include (I) LysM receptor kinases, which can be further divided into two clades (LYKs, LysM receptor kinases and LYRs, LYK-related) based on the features of their kinase domains; (II) membrane-anchored LysM proteins without additional intracellular domains (LYPs); (III) intracellular non-secretory LysM proteins (LysMn); and (IV) LysM extracellular proteins (LysMe) (14, 15). Previous studies have shown that most plant type I LysM receptor kinases are involved in immune activation and/or symbiotic signaling by recognizing ligands containing N-acetylglucosamine (GlcNAc) structures. For example, the rice LysM protein OsCERK1 (*Oryza sativa* chitin elicitor receptor kinase 1) can interact with OsCEBiP (*O. sativa* chitin oligosaccharide elicitor-binding protein, a receptor containing extracellular LysM domains that lacks a recognizable intracellular signaling domain) to form heterodimers that mediate chitin signaling during fungal infection (16). Another rice LysM protein OsMYR1 (*O. sativa* Myc factor receptor 1) can directly bind CO4 (a short-chain chitooligosaccharide) and subsequently interacts with OsCERK1 to promote the establishment of AM symbiosis (17). Similarly, two type I LysM proteins from *Lotus japonicus*,

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NFR1 (LyK nod factor receptor 1) and NFR5 (LYR nod factor receptor 5), and their respective orthologs LYK3 (LysM receptor-like kinase 3) and NFP (Nod factor receptor perception) from *Medicago truncatula*, are crucial for the initiation of early plant responses to rhizobial infection upon the perception of rhizobial lipochitin-oligosaccharide signals (9, 14). In these legumes, MtLYK9 and LjLys9/MtLYR4 (which are homologs of OsCERK1 and OsMYR1) are known to control chitin-triggered immune responses (18). Two type II LysM proteins from *Arabidopsis thaliana* (i.e., AtLYM1 and AtLYM3 which sequence similarity to OsCEBiP) act as receptors for peptidoglycan (PGN) secreted by bacteria (19). However, the function of type III plant LysM proteins has not been reported (20).

The type IV LysM extracellular (LysMe) proteins in plants possess a relatively simple structure consisting of a signal peptide and a single LysM domain. To date, only one plant LysMe protein, OsEMSA1 (*O. sativa* EMBRYO SAC 1), was reported to play a role in rice sexual reproduction by affecting embryo sac development (21). However, whether the LysMe proteins are involved in plant–microbe interactions is unknown. It is interesting to note that fungal LysMe-like proteins with 1 to 3 LysM domains commonly occur in plant pathogenic fungi. These fungal proteins are "effectors" secreted into host tissues for subverting plant immune responses (22). For example, the LysM effector Ecp6 from fungus *Cladosporium fulvum* and its homologs from *Mycosphaerella graminicola* can sequester fungal cell wall–derived chitooligosaccharides (COs) to protect fungal hyphae from decomposition by plant chitinases and/or suppress chitin-triggered immune responses (23, 24). Likewise, the LysM effector protein Slp1 (secreted LysM protein 1) from the rice blast fungus *Magnaporthe oryzae* can directly bind chitin to prevent immune activation upon chitin recognition by cognate receptors in rice, thereby facilitating rapid spread of the fungus within host plant (25). Notably, we previously discovered that the AMF *Rhizophagus irregularis* also secreted a LysM effector RiSLM (*R. irregularis* SECRETED LYSM) that can both protect fungal hyphae from chitinase and subvert chitin-triggered plant immunity by binding COs to favor more symbiotic responses (26). Considering that AM host plants such as *M. truncatula* (Medicago), tomato, rice, and citrus possess conserved LysMe proteins that are strongly expressed in arbuscule-containing cells (27–30), we hypothesized that plant LysMe proteins may play important roles in facilitating AMF colonization.

To test this hypothesis, we focused on three *M. truncatula* LysMe genes (*MtLysMe1, MtLysMe2,* and *MtLysMe3*) that are highly expressed in root cells containing arbuscules. We found that knocking out *LysMe2* in *M. truncatula* or tomato resulted in impaired arbuscule formation. We also revealed specific localization of the MtLysMe proteins in the extracellular space between the plant-derived peri-arbuscular membrane and the fungal cell wall. In vitro affinity precipitation assays demonstrated binding of purified MtLysMe1/2/3 proteins with chitin and chitosan, while root treatment with MtLysMe1/2/3 resulted in suppression of CO8-induced reactive oxygen species (ROS) production and expression of reporter genes of the immune response. Altogether, these results suggest that host plants also secrete LysM proteins to facilitate AM symbiosis.

Results

Three *MtLysMe* Genes Are Strongly Expressed in Root Cortical Cells Containing Arbuscules. We previously found that the expression of three *M. truncatula LysMe* genes (Medtr4g091000, here named as *MtLysMe1*; Medtr4g091010, *MtLysMe2*; Medtr4g091020, *MtLysMe3*) were highly expressed especially in root cortical cells containing arbuscules and cortical cells adjacent to hyphae, which were isolated by laser microdissection (27) (*SI Appendix*, Fig. S1). Similarly, based on "Noble MtGEA V3 by LIPME" resource on the Expression Atlas (https://lipm-browsers.toulouse.inra.fr/pub/expressionAtlas/app/mtgeav3/), we found that the three genes were only expressed in roots colonized with AMF, with no detectable expression in other tissues or roots without AMF colonization (*SI Appendix*, Fig. S2).

A time-course study showed that the expression of the *MtLysMe1/2/3* genes sharply increased in *M.truncatula* roots after inoculation with *R. irregularis* (Fig. 1*A*). The pattern of induced expression of *MtLysMe1/2/3* appears to coincide with that of *R. irregularis elongation factor 1*α (*RiEF1α,* a fungal housekeeping gene representing AMF abundance in roots) and the *M. truncatula phosphate transporter 4* gene (*MtPT4*, a marker gene for AM symbiosis) (31) (Fig. 1*B*), indicating that the induced expression of *MtLysMe1/2/3* correlates with increased AMF colonization.

To investigate spatial expression characteristics of *MtLysMe1/2/3*, each of the three corresponding promoters was amplified and fused with the β-glucoronidase (GUS) reporter gene. The resultant DNA constructs were expressed in Medicago roots by *Agrobacterium rhizogenes*–mediated transformation. While we did not detect any GUS activity in roots without *R. irregularis* inoculation (Fig. 1 *C*, *E*, and *G*), we observed strong GUS activity in root cortical cells containing arbuscules and cells adjacent to intercellular hyphae (Fig. 1 *D*, *F*, and *H*).

Structural and Evolutionary Analysis of the Deduced MtLysMe Proteins. The deduced MtLysMe1/2/3 proteins contain an N-terminal signal peptide followed by a single Lysin Motif (LysM) (*SI Appendix*, Fig. S3*A*). These three genes are clustered in a tandem array, suggesting that they are the result of recent duplications, which is further supported by subsequent phylogenetic analyses.

Given that the lysin motif proteins commonly play roles in plant–microbe interactions via binding PGN (from bacteria) or chitin (from fungi) (10), we predicted and analyzed the structures of MtLysMe proteins on the basis of structural characteristics of the fungal effector Ecp6 (an effector containing LysM domain that bind chitin) (32) by using GENEDOC. We identified four cysteine residues predicted to be able to form two pairs of disulfide bonds (1-1, 2-2, indicated by red numbers) and two potential chitin-binding motifs (indicated by blue lines) with 1-3 amino acid differences among the three MtLysMe2/3 proteins (*SI Appendix*, Fig. S3*A*).

We then retrieved 304 homologous protein sequences from 50 plant species and performed phylogenetic analysis using MAFFT (*SI Appendix*, Table S1). Based on this analysis, plant LysMe proteins could be divided into two main branches, one of which is common in all plants, while the other only occurs in AM host plants such as Medicago (red font), citrus (purple font), rice (blue font) and tomato (green font) (*SI Appendix*, Fig. S3*B*). Notably, MtLysMe1/2/3 are in the clade that is exclusive for AM host plants, further implying a role of these proteins in AM symbiosis.

The MtLysMe Proteins Specifically Localize in the Periarbuscular Space. LysMe proteins were previously reported to be extracellular proteins as they possess signal peptides at N-terminal but have no transmembrane domains (12). To determine the subcellular localization of the three MtLysMe proteins, the coding sequence of each of *MtLysMe1/2/3* was amplified and translationally in-frame fused with the mCherry fluorescent protein in a binary vector to express MtLysMe1/2/3-mCherry fusion proteins from the *35S* promoter. Each of the three *35Spro:MtLysMe1/2/3-mCherry* constructs was coexpressed with *35Spro:SP-OsRAmy3A-GFP*, an apoplast marker (33), in *Nicotiana benthamiana* leaves

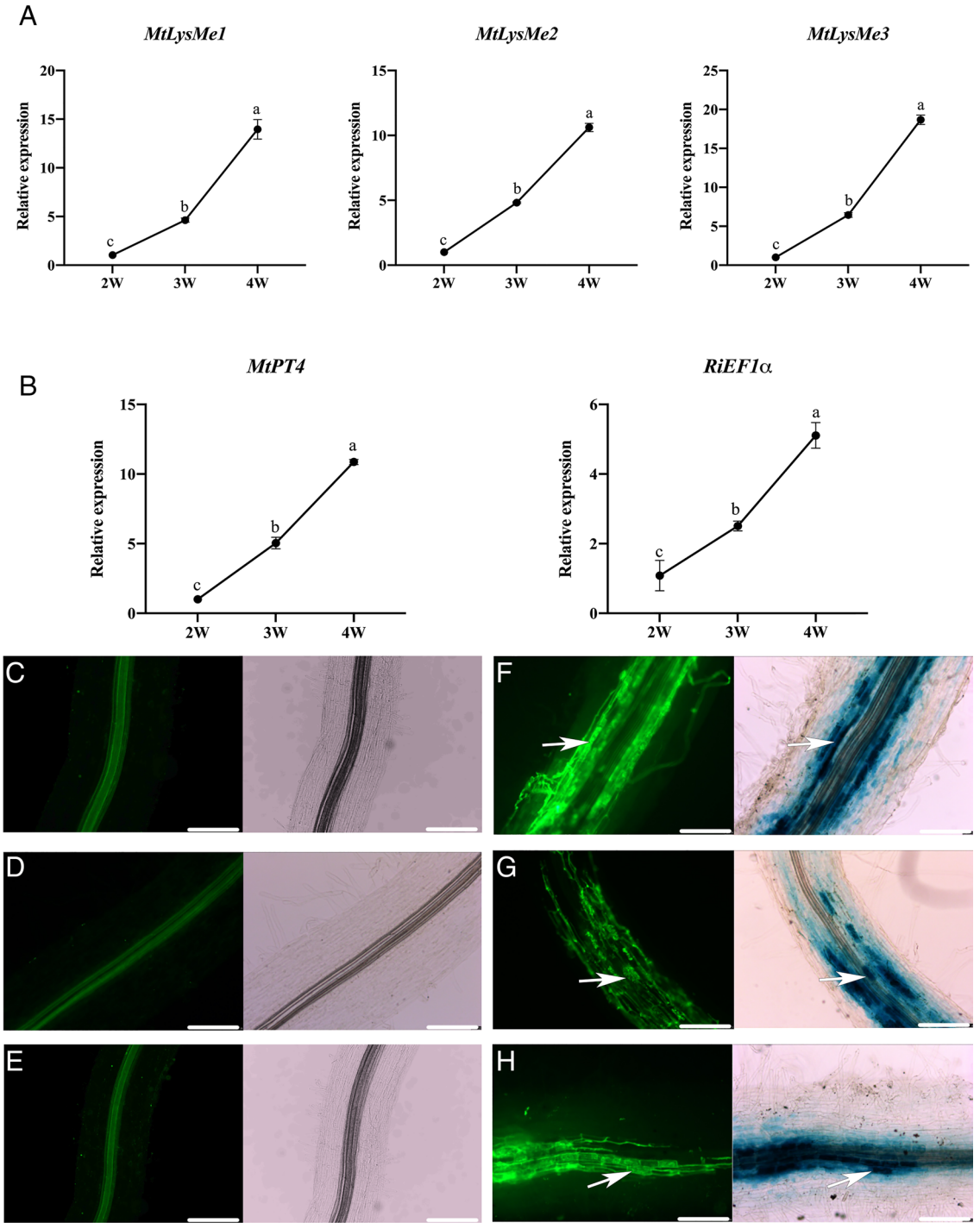

Fig. 1. Three *MtLysMe* genes are strongly expressed in cortical cells containing arbuscules. Transcript levels of *MtLysMe1/2/3* (*A*) and *MtPT4* and *RiEF1α* genes (*B*) in roots at different times after inoculation with *R. irregularis* as determined by qRT-PCR. Expression is normalized against *MtEF1α* using the $2^{-\triangle\triangle Ct}$ method. Error bars represent SEs for three biological replicates. Different letters indicate significant difference assessed by Student's *t* tests, $P < 0.05$. *MtLysMe1* (*C*), *MtLysMe2* (*D*), and *MtLysMe3* (*E*) *pro:GUS* showed no signal in Medicago roots without *R. irregularis* inoculation; *MtLysMe1* (*F*), *MtLysMe2* (*G*), and *MtLysMe3* (*H*) *pro:GUS* showed strong signals in arbuscule-containing cells as visualized with WGA488 staining for fungal structures and GUS staining for promoter activity. The white arrows point to arbuscule-containing cells (Scale bar, 100 μm). The experiment was repeated three times and showed similar results.

via *A. rhizogenes*–mediated transformation. As expected, we observed colocalization between each of the three MtLysMe1/2/3-mCherry fusion proteins and OsRAmy3A-GFP (*SI Appendix*, Fig. S4 *A–C*). Next, we coexpressed MtLysMe1/2/3-mCherry with CBLIN-GFP, a plasma membrane marker (34), in *N. benthamiana* leaves. Imaging after leaf plasmolysis induced by 800 mM mannitol confirmed the apoplastic localization of MtLysMe1/2/3-mCherry in *N. benthamiana* leaves (*SI Appendix*, Fig. S5 *A–C*).

To determine exactly where the MtLysMe proteins are localized in arbuscule-containing cells of Medicago roots, we expressed MtLysMe1/2/3-mCherry from their respective native promoters in Medicago roots by hairy root transformation. The positively transformed roots were then inoculated with *R. irregularis* and subjected to confocal imaging at 5 wk after inoculation. As shown in Fig. 2, fluorescent signals from the MtLysMe1/2/3-mCherry fusion proteins were clearly detectable in arbuscule-containing root cortical cells around the arbuscular trunk (indicated by yellow arrows) and

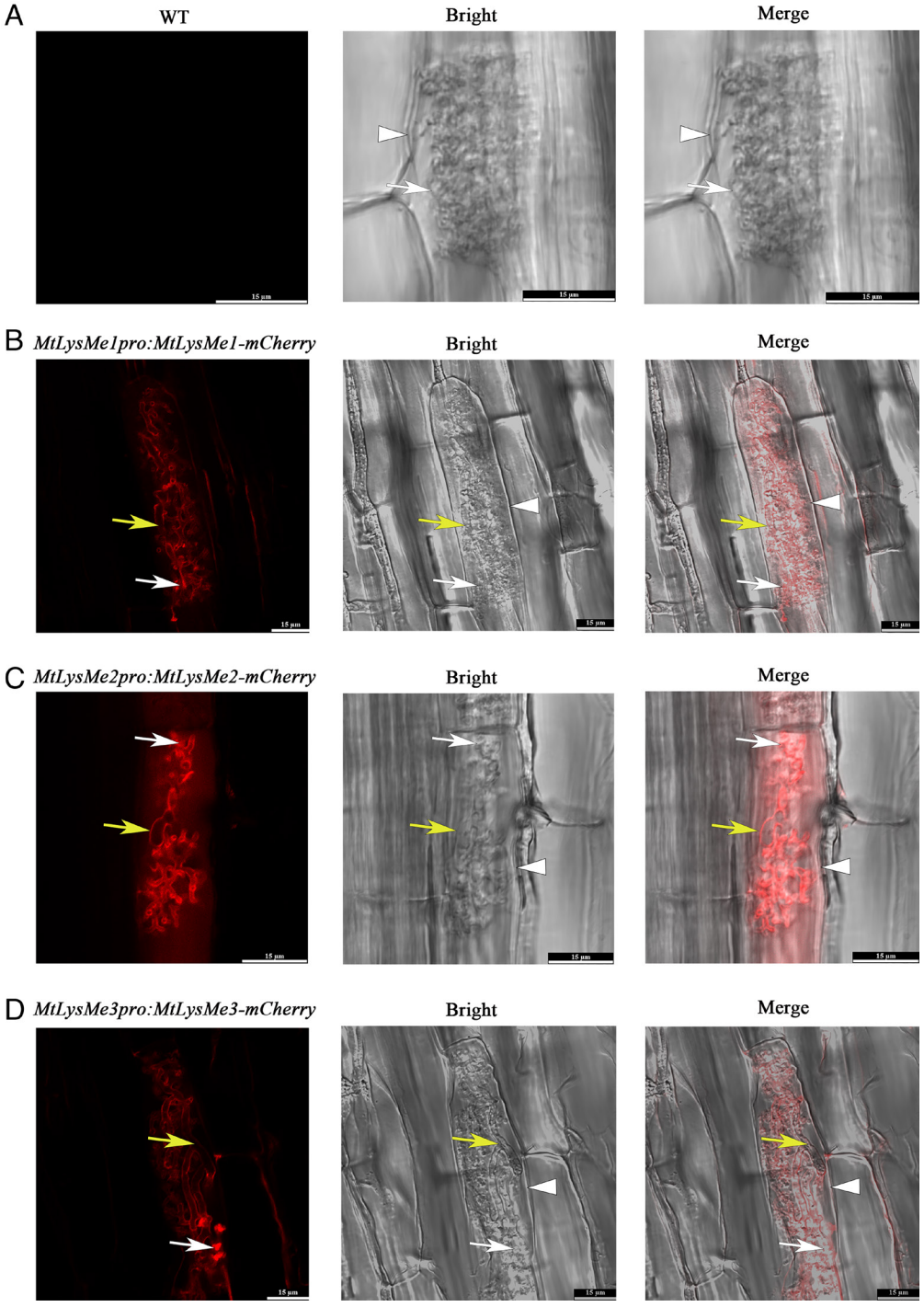

Fig. 2. Subcellular localization of MtLysMe proteins in mycorrhizal root cells of Medicago. Representative confocal images of arbuscule-containing cells of wild-type roots (*A*) and transgenic roots expressing *MtLysMe1pro:MtLysMe1-mCherry* (*B*), *MtLysMe2pro:MtLysMe2-mCherry* (*C*), or *MtLysMe3pro:MtLysMe3-mCherry* (*D*). Yellow and white arrows point to arbuscule trunks and arbuscule branches, respectively. White arrowheads point to the plasma membrane (Scale bar, 15 μm).

branches (indicated by white arrows). No signal was detectable around the plasma membrane (indicated by the white arrowhead) of the arbuscule-containing cells or anywhere in uninfected neighboring cells. The space surrounding the trunks and branches of the arbuscule, that is, the space between the peri-arbuscular membrane and the fungal cell wall, is called peri-arbuscular space (PAS) (35). Given that the promoters are active in cells not containing arbuscules, MtLysMe proteins are likely secreted by several cells, but are only stable/detectable in the PAS (Fig. 8).

Knocking Out *MtLysMe1/2* Reduced AMF Colonization and Arbuscule Abundance. To investigate whether MtLysMe proteins play any roles in AMF colonization including arbuscule formation, we employed CRISPR/Cas9-targeted mutagenesis to knock out *MtLysMe1/2/3* individually. While we successfully generated *mtlysme1* and *mtlysme2* single mutants (see below), we never obtained any *mtlysme3* mutants because positive calli transformed with the corresponding DNA construct targeting *MtLysMe3* failed to redifferentiate (*SI Appendix*, Fig. S6 *A–C*). DNA sequencing of the target genes identified one-nucleotide insertion in the CDS region of *MtLysMe1* in the *mtlysme1* mutant and one-nucleotide deletion in the CDS region of *MtLysMe2* in the *mtlysme2* mutant (*SI Appendix*, Fig. S7 *A* and *B*). We inoculated the roots of 2-wk-old *mtlysme1* and *mtlysme2* mutants with *R. irregularis* spores and assessed AMF colonization in 5 wk. Roots of the *mtlysme1* mutant showed a significantly lower arbuscular abundance (A%), while it had no difference in AMF colonization (F%, M%) as compared to WT (wild-type) (Fig. 3 *A*, *D*, and *E*). Notably, roots of the *mtlysme2*

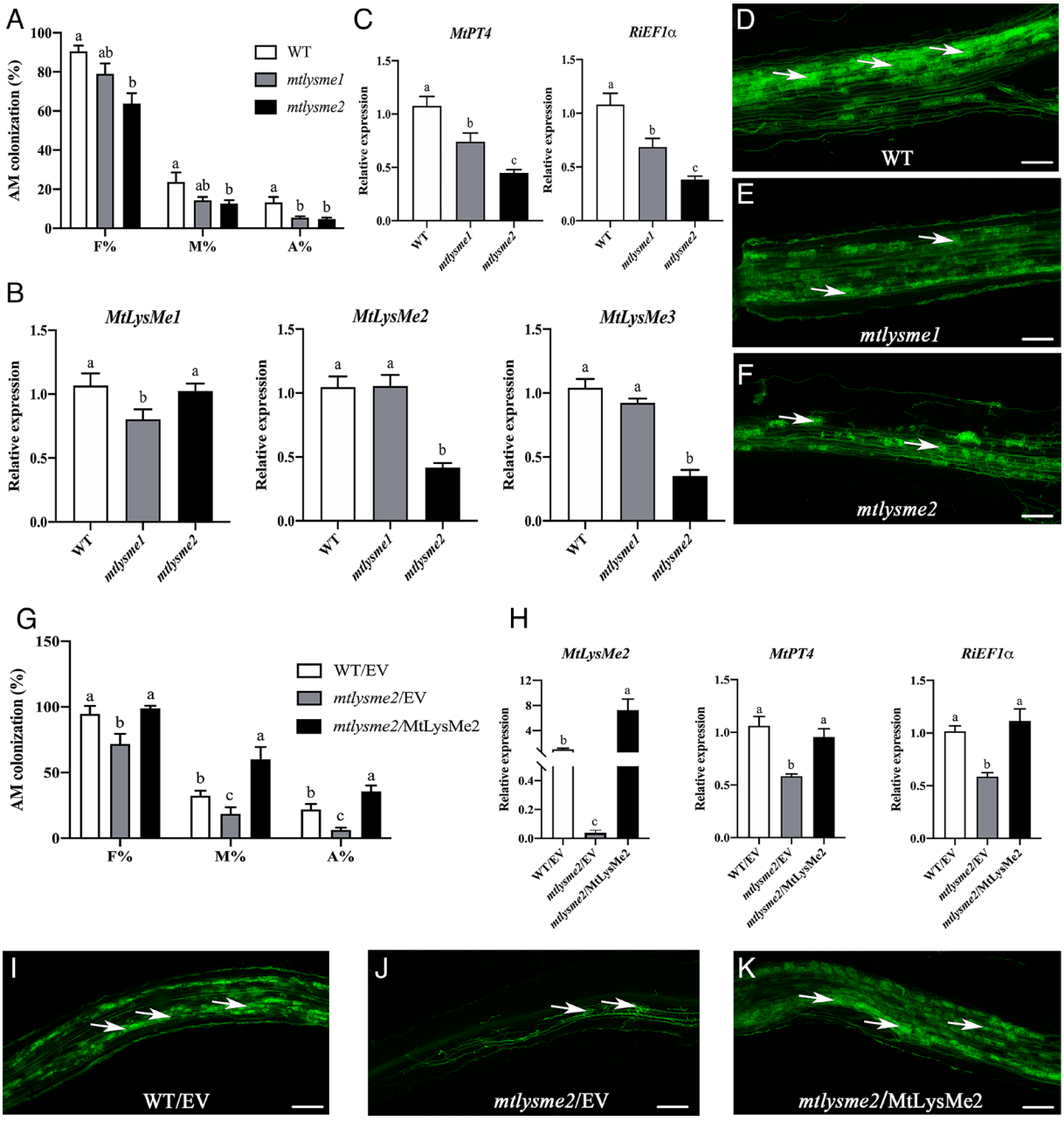

Fig. 3. The *MtLysMe* genes are indispensable for the establishment of mycorrhizal symbiosis in Medicago. (*A*) Quantification of AMF colonization levels in *mtlysme1* (n = 7), *mtlysme2* (n = 7) mutants and WT (n = 10). F%, frequency of analyzed root fragments that are mycorrhized; M%, intensity of infection in the whole roots; A%, intensity of arbuscule abundance in the whole roots. (*B* and *C*), Expression levels of *MtLysMe1*, *MtLysMe2*, *MtLysMe3, MtPT4* and *RiEF1α* in *mtlysme1*, *mtlysme2* mutants and WT. (*D–F*), WGA-alexa488 staining of mycorrhization in roots of WT, *mtlysme1* and *mtlysme2* plants. (*G*) AMF colonization in roots of WT/EV (n = 6), *mtlysme2*/EV (n = 4) and *mtlysme2*/MtLysMe2 (n = 4). (*H*) Expression levels of *MtLysMe2, MtPT4* and *RiEF1α* in roots of the same genotypes as in (*G*). (*I–K*), WGA-alexa488 staining of mycorrhization in roots of WT/EV, *mtlysme2*/EV and *mtlysme2*/MtLysMe2 plants. Different letters indicate significant difference (Student's *t* tests, $P < 0.05$). White arrows point to root cells containing arbuscules. This experiment was repeated twice (Scale bar, 100 μm).

mutant exhibited significantly lower levels of both AMF colonization (F%, M%) and arbuscule abundance (A%) when compared to WT (Fig. 3 *A, D*, and *F*). Consistently, transcript levels of *MtPT4* and *RiEF1α* (AM marker genes) were significantly lower in *R. irregularis* inoculated roots of the two mutants when compared to that of WT, with a larger reduction in *mtlysme2* than in *mtlysme1* (Fig. 3*C*). To determine whether the decrease of arbuscule abundance in the mutants is due to increased arbuscule degradation, we examined the expression levels of two marker genes (*MtCP3* and *MtCHITINASE*) for arbuscule degradation (36). We found no significant changes in the expression of these two marker genes (*SI Appendix*, Fig. S8 *A* and *B*), suggesting that the reduced arbuscule abundance in the two mutants is probably a result of reduced arbuscule formation.

Because the three *MtLysMe* paralogous genes are highly homologous to each other, there may be functional redundancy among them, and if so, there may be compensatory expression of the other members if one of them is knocked out. Our gene expression analyses showed that the transcription of neither *MtLysMe2* nor *MtLysMe3* was changed in the mycorrhizal roots of the *mtlysme1* mutant as compared to that of WT (Fig. 3*B*). Unexpectedly, we found that the expression levels of *MtLysMe1* and *MtLysMe3* were respectively slightly and significantly decreased in the mycorrhizal roots of *mtlysme2* relative to those of WT (Fig. 3*B*). These observations indicate no transcriptional compensation from other paralogs when *MtLysMe1* or *MtLysMe2* is mutated. Instead, these results support that *MtLysMe2* plays a major role in accommodating AMF, thus explaining both

the severer mycorrhization phenotype of *mtlysme2* and lower AMF-induced expression *MtLysMe1* and *MtLysMe3* in the absence of *MtLysMe2*.

Next, we introduced a construct containing the full-length CDS of *MtLysMe2* under control of the *MtBCP1* promoter (which possesses strong and constitutive activity in arbuscule-containing cells) (37) in *mtlysme2* roots via hairy root transformation. Wild-type (WT/EV) and *mtlysme2* (*mtlysme2*/EV) roots transformed with an empty vector were used as controls. The transgenic roots were inoculated with *R. irregularis* spores and subjected to RT-PCR and microscopy analyses 5 wk after inoculation. As expected, the *MtLysMe2* transgene was highly expressed in the *mtlysme2*/MtLysMe2 transgenic roots (Fig. 3*H*). Interestingly, while the AMF colonization frequency (F%) of *mtlysme2*/*MtLysMe2* transgenic roots was restored to that of WT/EV roots, the AMF colonization intensity (M%) and arbuscular abundance (A%) of the former were significantly higher than those of the latter (Fig. 3 *G, I–K*). These AM phenotypes also correlated with the expression of AM marker genes (*MtPT4 and RiEF1α*) in the respective transgenic roots (Fig. 3*H*).

Taken together, the above genetic data support an essential role of *MtLysMe2* and *MtLysMe1* to a lesser extent in facilitating AM symbiosis.

Knocking Out *SlLysMe2* Reduced AMF Colonization and Arbuscule Abundance in Tomato. To test whether the role of *LysMe* genes in AMF colonization is conserved in other plant species, we knocked out the probable *MtLysMe2* orthologous gene, S*lLysMe2,* in tomato (*Solanum lycopersicum*) via CRISPR/Cas9-targeted mutagenesis. The *sllysme2* mutant line obtained contains a two-nucleotide deletion in the CDS region of S*lLysMe2* (*SI Appendix*, Fig. S7*C*). Importantly, AMF colonization and arbuscule abundance were also significantly reduced in roots of the *sllysme2* mutant as compared to those of WT plants (Fig. 4 *A–D*), indicating that the *SlLysMe2* gene also contributes to the establishment of AM symbiosis.

Expression of Immune Reporter Genes in *MtLysMe* Mutants. Our previous studies indicated that fungal LysM proteins are involved in plant immune response (26). Therefore, we hypothesized that the *MtLysMe* proteins may play similar roles. To this end, we measured the expression of three defense marker genes (*MtEPI:* NAD-dependent epimerase/dehydratase, *MtPAL:* phenylalanine ammonia lyase, and *MtTHA*: thaumatin) in the roots of the *mtlysme1* and *mtlysme2* mutants inoculated with *R. irregularis*. Our RT-PCR analyses showed that the expression of these three defense marker genes was only slightly increased in *mtlysme1* but significantly enhanced in *mtlysme2* when compared to that in wild-type plants (Fig. 5 *A–C*). By contrast, the expression of those genes was significantly decreased in the roots of *mtlysme2*/MtLysMe2 (in which *MtLysMe2* is overexpressed) relative to that in *mtlysme2*/EV (Fig. 5 *D–F*). Therefore, there appears to exist a negative correlation between the expression of *MtLysMe2* and the expression of defense marker genes, suggesting that the role of MtLysMe2 in facilitating AM symbiosis might be realized via attenuating plant immune response.

The MtLysMe Proteins Bind Chitin/Chitosan/Cellulose In Vitro. The deduced MtLysMe1/2/3 proteins contain two chitin-binding regions (*SI Appendix*, Fig. S3*A*), suggesting that the MtLysMe proteins may bind chitin, thereby reducing chitin-induced immune response during AMF colonization. To test this speculation, we first tried to make recombinant MtLysMe proteins using the baculovirus-insect cell expression system. The *MtLysMe1/2* genes were translationally fused with a C-terminal 10×His-tag and an N-terminal Myc-tag (*SI Appendix*, Fig. S9 *A* and *B*), while *MtLysMe3* was translationally fused with an N-terminal 6×His-SUMO-tag (*SI Appendix*, Fig. S9*C*) because the 10×His-MtLysMe3-Myc fusion construct failed to be expressed. We obtained purified recombinant proteins of ~15 kDa for the tagged MtLysMe1/2 proteins and ~24 kDa for the tagged MtLysMe3 after affinity purification

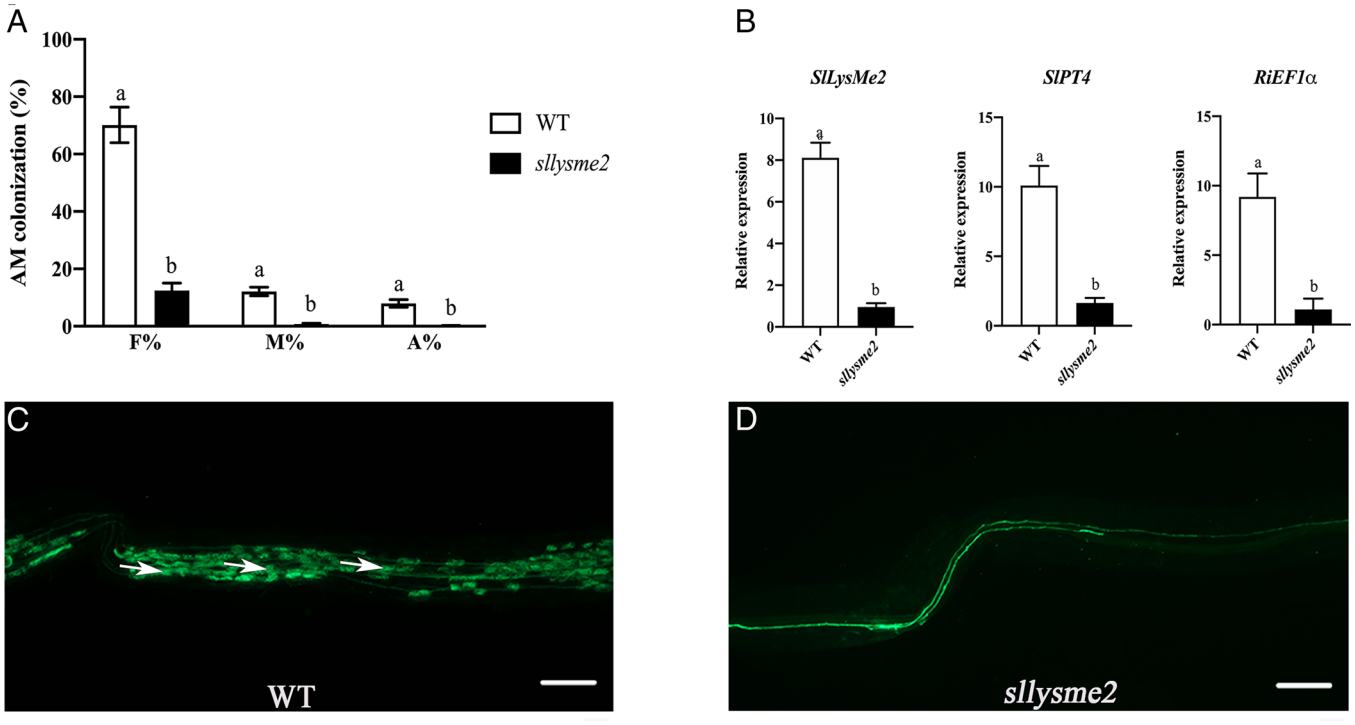

Fig. 4. The *MtLysMe2* homologous gene *SlLysMe2* is indispensable for the establishment of mycorrhizal symbiosis in tomato. (*A*) AMF colonization in roots of the *sllysme2* mutant (n = 4) and WT (n = 6) plants. (*B*) Expression levels of *SlLysMe2, SlPT4,* and *RiEF1α* in the same plants shown in (*A*). (*C* and *D*), WGA-alexa488 staining of mycorrhization in the *sllysme2* mutant and WT plants. White arrows point to root cells containing arbuscule. This experiment was repeated three times with similar results. Letters indicate significant difference ($P < 0.05$) assessed by Student's *t* tests (Scale bar, 100 μm).

(*SI Appendix*, Fig. S9). Second, we incubated each of the three MtLysMe recombinant proteins with insoluble carbohydrates including chitin, chitosan (deacytelated chitin), cellulose, and xylan in a chitin-binding buffer at pH8.0 or pH5.2 (the lower pH may better match the slightly acidic subcellular environment of the PAS) (38). The results showed that i) all the three MtLysMe proteins could coprecipitate with insoluble chitin, chitosan, and cellulose, although the MtLysMe1 showed a weaker binding with insoluble chitosan and ii) none of the MtLysMe proteins coprecipitated with xylan (*SI Appendix*, Fig. S10 and Fig. 6).

MtLysMe's Binding with Chitin May Contribute to the Suppression of Plant ROS Production and Immune Reporter Gene Expression. Chitooligosaccharide chitooctaose (CO8), a breakdown product of chitin, has been shown to be a potent elicitor of chitin-triggered immunity in plants (39). We thus further tested whether the MtLysMe proteins bind CO8 by using microscale thermophoresis (MST) to measure the binding affinity. The Kd values of MtLysMe1/2/3 with CO8 were respectively 971.41 μM, 1,382.3 μM, and 1,522.3 μM at pH8 buffer and 151.39 μM, 250.89 μM, and 170.62 μM at pH5.2 buffer (*SI Appendix*, Fig. S11), indicating rather low binding affinity between the MtLysMe recombinant proteins and CO8.

Next, we tested whether application of MtLysMe recombinant proteins might interfere with chitin-induced plant immune responses during AMF colonization. We first measured CO8-induced ROS production with or without MtLysMe proteins, by using a chemiluminescence assay. Medicago roots showed a clear ROS burst in response to 1 μM CO8 with or without TE buffer, while the MtLysMe1/2/3 proteins alone without CO8 did not induce ROS (*SI Appendix*, Fig. S12). Importantly, although cotreatment of Medicago roots with 1 μM CO8 and 1 μM MtLysMe proteins did not significantly suppress CO8-induced ROS production, increasing the amount of the MtLysMe proteins by five times relative to the amount of CO8 resulted in significant reduction of CO8-induced ROS production, especially in the case of MtLysMe2 (Fig. 7 *A* and *B*). In addition, we also tested whether the MtLysMe proteins could inhibit flg22-induced ROS production. Our results showed no inhibition of an ROS burst induced by 100 nM flg22 in *Medicago* roots when cotreated with any of the three MtLysMe proteins at 100 nM or 500 nM (Fig. 7 *C* and *D*).

Then, we examined whether the MtLysMe recombinant proteins could suppress expression of defense marker genes (*MtEPI*, *MtPAL,* and *MtTHA*) induced by treatment of *Medicago* roots with 100 nM CO8 (*SI Appendix*, Fig. S13 *A–C*). Interestingly, similar to that of ROS experiment, expression of the marker genes was significantly suppressed by addition of 500 nM but not 100 nM (equal molar concentration as that of CO8) MtLysMe proteins (Fig. 7 *E–G*). As controls, treatments with any of the three MtLysMe proteins alone did not alter expression of the defense markers genes (*SI Appendix*, Fig. S13 *A–C*). It is worth pointing out that expression of *MtEPI* and/or *MtPAL* was slightly higher when 100 nM MtLysMe1/3 was added, but this was not seen with addition of 100 nM MtLysMe2 (Fig. 7 *E* and *F*). These results suggest that sufficiently high levels of the MtLysMe proteins could inhibit CO8-induced ROS production and expression of reporter genes of the immune response.

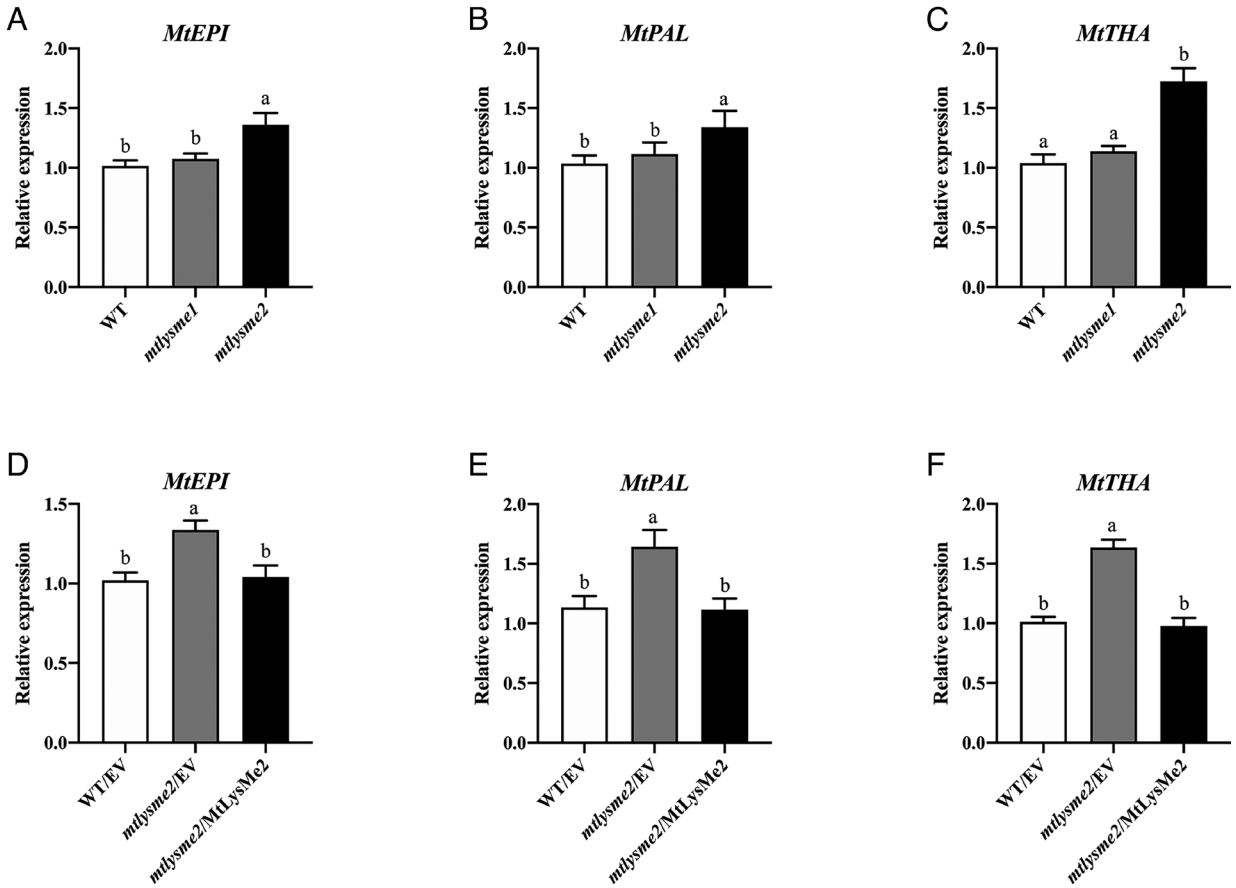

Fig. 5. Expression of immune reporter genes in *MtLysMe2* mutants. (*A–C*), Expression levels of *MtEPI* (*A*), *MtPAL* (*B*), and *MtTHA* (*C*) in roots of *mtlysme1* and *mtlysme2* mutants inoculated with *R. irregularis*, as determined by qRT-PCR analysis. (*D–F*), Expression levels of *MtEPI* (*D*), *MtPAL* (*E*), and *MtTHA* (*F*) in roots of WT/EV, *mtlysme2*/MtLysMe2, and *mtlysme2*/EV, as determined by qRT-PCR analysis. This experiment was repeated three times with similar results. Expression is normalized against *MtEF1α* using the $2^{-\triangle\triangle Ct}$ method. Error bars represent SEs from three biological replicates. Different letters indicate significant difference ($P < 0.05$) assessed by Student's *t* tests.

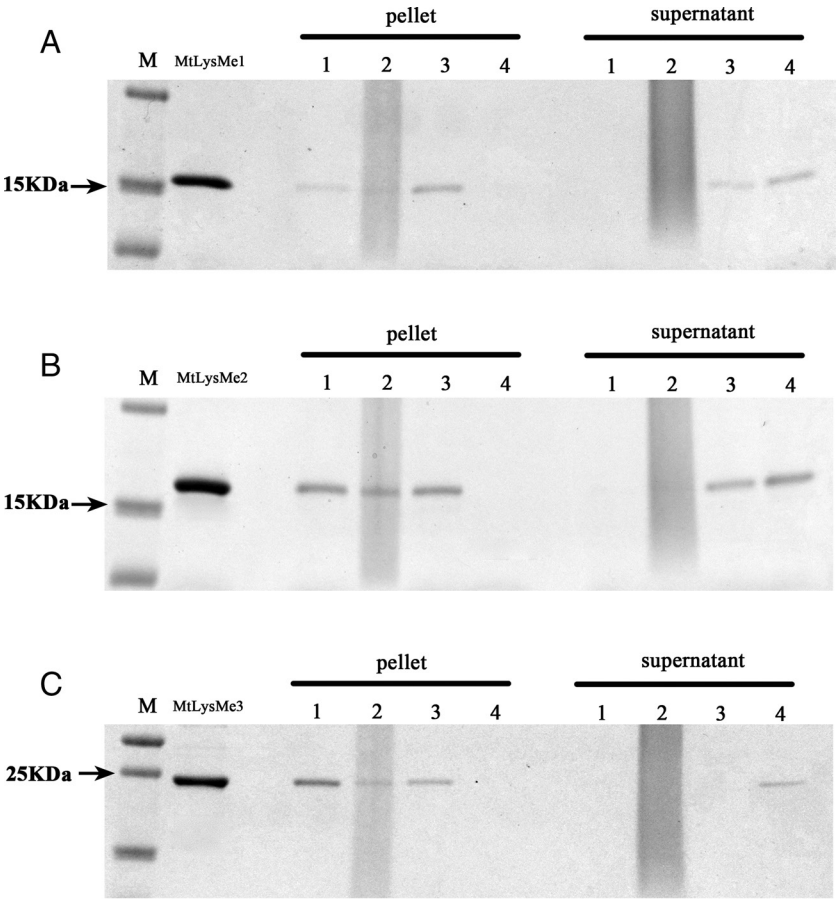

Fig. 6. The MtLysMe proteins bind chitin/chitosan/cellulose in vitro at pH5.2. The binding affinity between the three purified MtLysMe recombinant proteins and insoluble chitinchitosan, cellulose, and xylan was determined by affinity precipitation assays (for details, see *SI Appendix*, *Materials and Methods*). Protein coprecipitated with the insoluble polymers was subjected to SDS-PAGE analyses and visualized by Coomassie Brilliant Blue R-250. (*A*), MtLysMe1; (*B*), MtLysMe2; (*C*) MtLysMe3. The first lane "M" is the protein marker. The experiment was repeated three times with similar results.

The MtLysMe Proteins Do Not Bind CO4 to Block CO4-Triggered Symbiotic Responses. As chitotetraose (CO4) is a key Myc factor to activate the signaling pathway for AM symbiosis (40), we also investigated whether the MtLysMe proteins could bind CO4 and impact CO4-triggered symbiotic responses. We first tested the affinity between the three MtLysMe proteins and CO4 by MST and found that none of the three MtLysMe proteins showed a binding to CO4 at a pH of 8 or 5.2 binding buffer (*SI Appendix*, Fig. S14). We then checked the expression levels of symbiotic marker genes (*MtPUB1*, *MtTUBB1*, and *MtVAPYRIN*) upon CO4 treatment with or without the MtLysMe proteins, by using the same experimental setup as for the CO8 treatment mentioned above. The results showed that CO4 induced the expression of all the three marker genes with or without TE buffer, while the three MtLysMe proteins alone treatment did not (*SI Appendix*, Fig. S13 *D–F*). The expression of *MtPUB1* induced by CO4 was not affected, whereas the expression of *MtTUBB1* and *MtVAPYRIN* generally were upregulated by adding the MtLysMe proteins (*SI Appendix*, Fig. S15 *A–C*).

Combining the results from the CO8 treatment experiments, we propose that the endogenous MtLysMe proteins may suppress chitin-triggered ROS production and expression of reporter genes of the immune response while still allowing CO4-mediated signaling for AM symbiosis.

The MtLysMe Proteins Cannot Protect Fungal Hyphae Against Chitinase. Previous studies revealed that fungal LysM effectors either suppress chitin-triggered immunity and/or protect fungal cell walls against chitinase (23, 26). We also wondered whether the MtLysMe proteins also have the ability to protect the fungal cell wall against chitinase. To test this, germinated *Trichoderma viride* spores were incubated for 24 h with chitinase in the presence or absence of the MtLysMe proteins. We found that the fungal hyphae treated with 10 μM or even 50 μM MtLysMe proteins were still susceptible to chitinase, similar to those treated with buffer (*SI Appendix*, Fig. S16). This result indicates that the MtLysMe proteins are unable to protect the cell wall of fungal hyphae against chitinase.

Discussion

Chitin is a major component of the cell wall in both symbiotic and pathogenic fungi, and it is recognized by plants as an MAMP for immune activation during plant–fungal interaction (6). Despite having chitin in their cell walls, AMF can establish a symbiotic association with plants without inducing an intense defense response (41). Correspondingly, we previously discovered a LysM domain–containing effector RiLSM from *R. irregularis* that can bind chitin and can suppress plant immune responses to allow successful AMF colonization (26). Based on the results of this study, we propose that AM host plants also secrete LysMe proteins as "susceptibility factors" to facilitate AM symbiosis establishment.

LysMe proteins contain a signal peptide and one lysin domain and thus they are considered the simplest type of LysM proteins in plants (12). A previous phylogenetic study revealed that LysMe proteins as well as their lysin motifs are conserved in all organism kingdoms and that these proteins form a clade that is distinguishable from other clades of LysM domain–containing proteins (42). Our phylogenetic analyses in this study further revealed the existence of a LysMe orthogroup, including MtLysMe1/2/3 and SlLysMe2, in AM host plants but not in plants that have lost the ability to support AM symbiosis (*SI Appendix*, Fig. S3*B*). Consistently, symbiosis-related

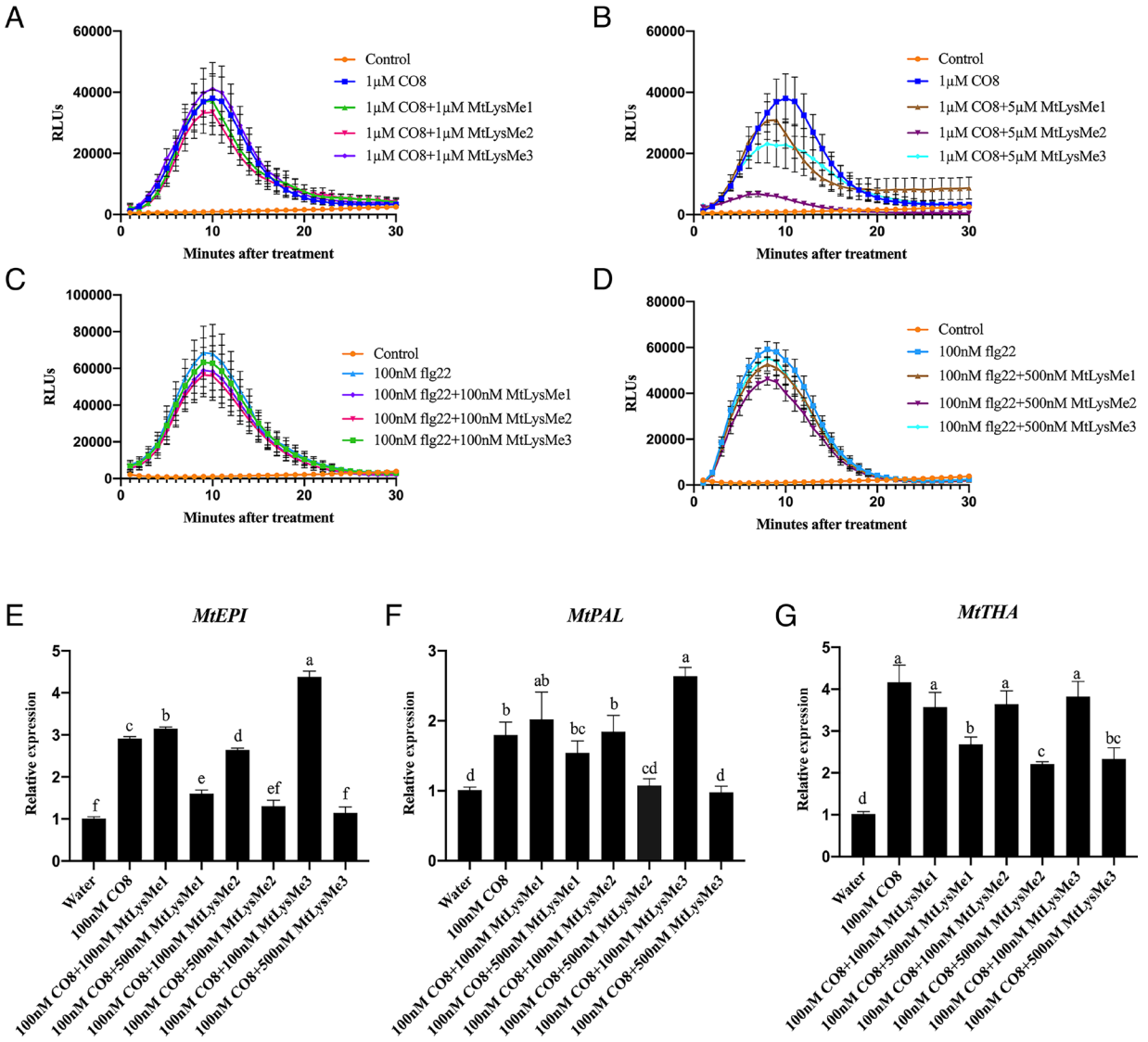

Fig. 7. MtLysMe proteins suppress CO8-induced ROS production and immune reporter gene expression. (*A–D*), The MtLysMe proteins suppress CO8-triggered ROS burst in Medicago roots. Root pieces were treated with 1 μM CO8 with or without 1 μM (*A*) or 5 μM (*B*) of MtLysMe proteins. Error bars represent SEs from six biological replicates. Root pieces were treated with 100 nM flg22 with or without 100 nM (*C*) or 500 nM (*D*) of MtLysMe proteins. Error bars represent SEs from six biological replicates. (*E–G*) MtLysMe proteins suppress CO8-triggered induction of three defense marker genes (*E*, *MtEPI*; *F*, *MtPAL*; *G*, *MtTHA*) in *Medicago* roots cotreated with 100 nM CO8 and 500 nM MtLysMe proteins for 1 h. Expression is normalized against *MtEF1α* using the $2^{-\triangle\triangle Ct}$ method. Error bars represent SEs from three biological replicates. Different letters indicate significant difference ($P < 0.05$) assessed by Student's *t* tests. This experiment was repeated three times with similar results.

LysMe genes have also been shown to be highly expressed in arbuscule-containing cells in AM host plants such as Medicago, tomato, rice, and citrus (27–30). Hence, the in silico and expression analyses strongly suggest that these AMF-inducible *LysMe* genes are implicated in the establishment of AM symbiosis.

Where do plant LysMe proteins work? Based on their N-terminal signal peptide, plant LysMe proteins are predicted to be secreted into the extracellular space (12). As an initial finding of this study, the MtLysMe1/2/3-mCherry fusion proteins were indeed observed around the arbuscular branches (Fig. 2 *A–C*). As these fusion proteins were confirmed to be secreted into the extracellular space (i.e., the apoplast) in *N. benthamiana* leaves (*SI Appendix*, Figs. S4 and S5), we inferred that the MtLysMe proteins must be secreted into the periarbuscular space (PAS) between fungal cell wall and peri-arbuscular membrane (Fig. 8), which is the interface where the host plant and the fungal partner exchange signals and nutrients (38). It is interesting that although the MtLysMe proteins were almost exclusively located in arbuscule-containing cells, the *MtLysMe1/2/3pro:GUS* activity was detected in both arbuscule-containing cortical cells and those adjacent to intercellular hyphae. These results suggest that while the transcription of the *MtLysMe* genes can be induced in cortical cells containing arbuscules and/or adjacent to intercellular hyphae, only the MtLysMe proteins targeted to the PAS (where they may play a role in arbuscule formation and/or accommodation, thereby facilitating the establishment of AM symbiosis) can accumulate above a threshold level that is detectable. This implies that the MtLysMe proteins in cells lacking an arbuscule either cannot accumulate above the threshold level and/or may be degraded. As a corollary, the PAS may provide a subcellular compartment with physiological conditions good for the accumulation of MtlysMe proteins, which may ensure a sufficient amount of MtlysMe proteins in the PAS to perform their cellular functions.

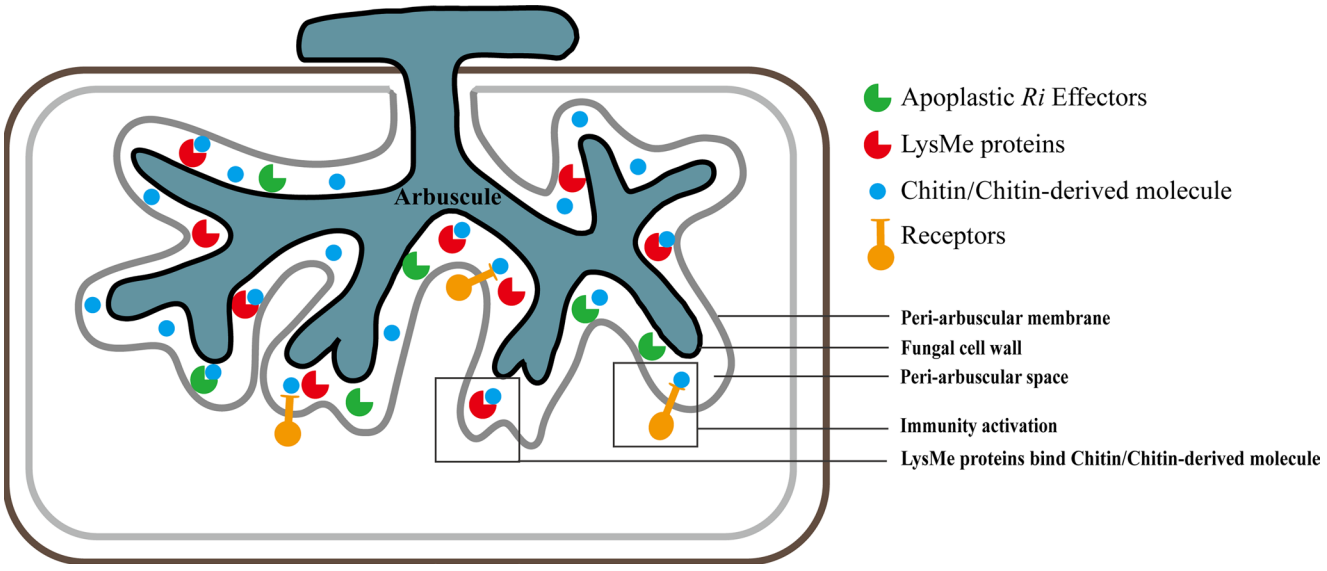

Fig. 8. A working model for how MtLysMe proteins facilitate AM symbiosis. The MtLysMe proteins are induced upon AMF inoculation and secreted into the periarbuscular space (PAS) between the fungal cell wall and the plant-derived periarbuscular membrane (PAM). There they accumulate at high levels and bind chitin and/or chitin derivatives such as CO8 to suppress chitin-triggered plant immunity, thereby promoting AMF symbiosis.

To investigate a possible role of MtLysMe proteins in AM symbiosis, we knocked out *MtLysMe1* or *MtLysMe2* by CRISPR/Cas9-targeted mutagenesis and observed a profound negative effect on AMF colonization level and arbuscular abundance caused by ablation of either gene, particularly *MtLysMe2* (Fig. 3 *A–F*). Moreover, overexpression of *MtLysMe2* as a transgene in *mtlysme2* not only rescued *mtlysme2*'s defects in AM establishment but also significantly enhanced AM symbiosis (Fig. 3 *G*, *I–H*). Hence, we have provided strong genetic evidence for an important role of *MtLysMe1* and *MtLysMe2* in particular in facilitating AM symbiosis.

To understand how the MtLysMe proteins may promote AM symbiosis, we did gene expression analysis with the mutant lines and found that the expression of three defense marker genes was significantly increased in *mtlysme2* but only slightly ($P > 0.05$) increased in *mtlysme1* plants inoculated with *R. irregularis* (Fig. 5 *A–C*). Interestingly, the transcription of the three defense markers genes was found to be upregulated in non-colonized *mtlysme1*, while there were no significant transcriptional changes for the same three genes in non-colonized *mtlysme2* compared with the WT (*SI Appendix*, Fig. S17 *A–C*). These observations suggest that while *MtlysMe1* may help prevent autoactivation of defenses in non-colonized roots, *MtlysMe2* may play a role in dampening AMF-induced defenses in mycorrhizal roots (Fig. 3*B*).

LysM domains are reported to bind fungal chitin or bacterial PGN to stimulate plant immune response (16, 19). We thus focused our efforts on testing whether the three MtLysMe proteins could bind chitin or other carbohydrates to suppress immune responses in Medicago roots. We showed that all the three MtLysMe recombinant proteins could bind chitin and chitosan in vitro (*SI Appendix*, Fig. S10 and Fig. 6). Both chitin and chitosan are strong immune triggers in plants (6). Our subsequent microscale thermophoresis experiments revealed low binding affinity between the MtLysMe proteins with CO8 as a breakdown product of chitin (*SI Appendix*, Fig. S11), with their Kd values being much higher than those of the chitin-sequestering LysM proteins ECP6 (3.7 μM) (23) and Slp1 (2.4 nM) (25). Consistent with this, we found that a five times higher molar concentration relative to that of CO8 was required for these MtLysMe recombinant to suppress CO8-triggered ROS and defense gene expression (Fig. 7). This low-affinity binding may also explain why these MtLysMe proteins are highly enriched in the PAS, with the assumption that they bind chitin or chitin-derived molecules such as CO8 to attenuate chitin-induced immune responses. Not surprisingly, the three MtLysMe recombinant proteins also showed some differences in suppressing ROS production and expression of reporter genes of the immune response (Fig. 7), which may be attributable to the 1-3 amino acid differences between these proteins in their LysM domain (*SI Appendix*, Fig. 3*A*). This reasoning is based on the notion that LysM receptor–ligand binding requires precise protein sequences (43) and/or post-translational modifications of the LysMe domain (26). Indeed, we found that compared with MtLysMe1, MtLysMe2 did have a stronger ability to suppress CO8-triggered ROS production and expression of reporter genes of the immune response (Fig. 7). Given the high similarity in gene induction, protein sequence, and chitin binding among the three MtLysMe proteins (*SI Appendix*, Figs. S1 and S3*A* and Fig. 7), we expected MtLysMe3 to also play a role in AM symbiosis. Unfortunately, we did not obtain a single mutant line for *MtLysMe3*, despite over 10 transformation attempts. We noticed that the callus transformed with the CRISPR/Cas9 construct targeting *MtLysMe3* never turned green and thus failed to regenerate transgenic plantlets (*SI Appendix*, Fig. S6). This implies that knocking out *MtLysMe3* may result in programmed cell death due to autoactivation of immunity and/or failure in cell differentiation.

The LysM receptor LjEPR3 was reported to bind exopolysaccharides by a unique extracellular domain structure (44), Interestingly, recent structural analyses revealed that the first two LysM-like domains M1 and M2 of LjEPR3 present non-canonical (M1: βαββ; M2: βαβ) LysM folds, while the third domain represents a classical LysM domain (βααβ). Such a structural signature is thought to be shared by a ubiquitous class of receptors in the plant kingdom and is responsible for the alternative substrates of LjEPR3 (44). To assess whether the MtLysMe proteins may also possess such a structural signature for binding similar molecules, we used AlphaFold (https://github.com/deepmind/alphafold) to predict the structures of the LysM domains of the MtLysMe proteins and found that they all possess the classical LysM domain βααβ (*SI Appendix*, Fig. S18 *A–C*). In addition, we tested the binding affinity between the MtLysMe

proteins and laminarihexaose (*SI Appendix*, Fig. S18*D*) or laminarin (*SI Appendix*, Fig. S18*E*) by MST assays and confirmed that the MtLysMe proteins did not bind to either molecule. Whether the MtLysMe proteins could bind other unknown chitin-derived molecules remains to be further investigated. For example, the LysM receptor AtCERK1 was thought to act as a coreceptor for the recognition of laminarihexaose (1,3-β-D-$(Glc)_6$) and activation of immune responses, even though there is no direct interaction between 1,3-β-D-glucans and LysM domains based on in silico analyses and isothermal titration calorimetry binding assays (45).

Relevant to their positive role in AM symbiosis, we showed that the MtLysMe proteins did not bind CO4 based on MST assays (*SI Appendix*, Fig. S14), which agrees with our observations that the MtLysMe proteins did not affect or even enhanced CO4-induced expression of symbiotic genes (*SI Appendix*, Fig. S15). A similar observation was also made for the fungal RiSLM effector (26). Another previous study reported that the rice CO4 receptor OsMYR1 could suppress CO8-mediated immune response by competitively binding with OsCERK1 against OsCEBiP to inhibit the formation of immune receptor complexes (46). Together, these findings reinforce the importance of suppressing the immune signaling to allow symbiosis signaling during AMF colonization. In a preliminary analysis, we tested whether MtLysMe proteins would interact with the LysM receptor LYK3 (which in Medicago is closely related to CERK1) or NFP (which in Medicago is involved in CO4 and LCO perception) (39). Using yeast two-hybrid and bimolecular fluorescent complimentary (BIFC) assays, we did not find an interaction between MtLysMe and MtLYK3 or MtNFP (*SI Appendix*, Figs. S19 and S20). However, future studies should reveal whether MtLysMe may interact with other (LysM or other) immune receptors.

In summary, results from this study support the following working model. Upon detection of AMF infection, host plants produce LysMe proteins and secreted them into the PAS where these proteins accumulate and bind chitin and/or chitin-derived molecules to attenuate chitin-induced initiation of immune signaling at the periarbuscular membrane, thereby facilitating the establishment of AM symbiosis (Fig. 8).

Materials and Methods

The materials and methods are described in detail in *SI Appendix*, Material and Methods, including Plant materials, growth conditions and Rhizophagus irregularis inoculation; Phylogenetic analyses; Vector construction and plant transformation; GUS and WGA staining; Transient protein expression in Nicotiana benthamiana; Protein expression and purification; Chitin binding affinity precipitation assays; Measurement of ROS; CO8 and CO4 treatments for immune and symbiotic response detection; Microscale thermophoresis assay; Chitinase protection assay; RNA extraction and gene expression detection; Root staining and AMF colonization analysis; Yeast two-hybrid assays; BiFC assays.

Data, Materials, and Software Availability. Sequence data from this article can be found in the GenBank/EMBL (https://www.ncbi.nlm.nih.gov/) libraries and Phytozome (https://phytozome-next.jgi.doe.gov/). The details of these gene sequences were listed in Dataset S1. All data are included in the manuscript and/or supporting information.

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