A remorin protein interacts with symbiotic receptors and regulates bacterial infection

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Remorin proteins have been hypothesized to play important roles during cellular signal transduction processes. Induction of some members of this multigene family has been reported during biotic interactions. However, no roles during host-bacteria interactions have been assigned to remorin proteins until now. We used root nodule symbiosis between Medicago truncatula and Sinorhizobium melloti to study the roles of a remorin that is specifically induced during nodulation. Here we show that this oligomeric remorin protein attaches to the host plasma membrane surrounding the bacteria and controls infection and release of rhizobia into the host cytoplasm. It interacts with the core set of symbiotic receptors that are essential for perception of bacterial signaling molecules, and thus might represent a plant-specific scaffolding protein.

Results and Discussion

Root nodule symbiosis requires a complex molecular dialogue between the host and the bacteria during which the microbial symbionts remain surrounded by a host-derived plasma membrane (PM) forming an infection thread (IT) from which they are liberated at later stages into symbiosomes. Nod factors (NFs) are key signaling molecules secreted by rhizobia that are essential for triggering the first steps of infection and nodule organogenesis of legume plants, and several receptor-like kinases (RLKs) are involved in NF perception (1–6). In the model legume Medicago truncatula, perception of NFs produced by its rhizobial symbiont Sinorhizobium melloti has been shown to be mediated by the LysM-RLK NFP (1). NFP is necessary for all NF-dependent symbiotic responses and infection. Another LysM RLK, LYLK3 (3, 7), and an LRR RLK, DM2 (2, 8), are also involved in the control of bacterial entry and invasion. Recent data strongly suggest the importance of NFP, LYLK3, and DM2 in both early (root hair responses) and later (nodule infection and bacterial release) stages of symbiotic interactions, as all three RLKs are involved in IT initiation and progression (1, 3, 8) and are expressed specifically in a nodule zone where bacteria infect the host plant (1, 3, 8). Regulatory components such as scaffolding proteins with concordant localization have never been described.

Plant-specific remorin proteins were discovered almost two decades ago and, although they do not contain transmembrane domains, they have so far been almost exclusively detected in detergent-insoluble membrane fractions (commonly called lipid rafts) prepared from PM extracts (9). Interestingly, several members of the remorin family (10) were found to be differentially regulated during plant-microbe interactions, indicating functions during microbial infection and plant signaling processes (11–14). Indeed, a remorin protein (MtREM1.3) that was shown to be associated with the PM and to be phosphorylated upon treatment of potato leaves with polygalacturonic acid (15) has been suggested to play roles in cell-to-cell signaling and plant defense (16). This group of remorins was shown to bind oligogalacturonides and other polyanionic molecules, form oligomeric filamentous structures in vitro, and share structural similarities to viral movement proteins (15–17). Involvement of this group of remorins in controlling viral spreading in leaves has recently been demonstrated (18). Pivotal roles of remorin proteins in plant-microbe interactions are also supported by a strong induction of another protein family member in nodulated roots of the model legumes M. truncatula and Lotus japonicus (19, 20).

Roles of these plant-specific remorins remain unclear. We have chosen root nodule symbiosis as an inducible, biologically highly relevant yet dispensable system to explore the role of a remorin protein in detail. We show that a remorin is required for plant–bacteria interactions and can interact with several RLKs. Our findings imply the existence of specific signaling complexes involved in NF perception in PM subdomains.

Remorin protein interacts with symbiotic receptors

B. L., T. T., J. C., P. G., and T. O. analyzed data; and B. L. and T. O. wrote the paper.


Database deposition: MtSYMREM1 (BG580614) and MtLRR1.1 (EU849167) at GenBank.

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that has lost the ability to elicit initial morphological changes of roots with wild-type rhizobia, whereas no induction was in infection. To assess a role during the very first steps of bacterial perception by the host plant, we applied purified S. meliloti NFs to M. truncatula roots and measured transcript levels in a time course experiment. From these data, we hypothesize that altered infection in MtSYMREM1-RNAi roots led to the development of fewer or no nodules, the latter possibly due to mild infection conditions in pouches, whereas plants attempted to compensate for this effect by initiation of more infection threads.

In parallel, two stable Medicago RNAi lines were obtained by Agrobacterium tumefaciens-mediated gene transfer. To assess the phenotype of these plants, several cuttings from RNAi lines and from control plants carrying the empty vector were grown aeroponically and examined 32 dpi (Fig. 2A–F). In one of the transgenic lines (line 2), abnormal nodules were observed (Fig. 2D–E). Nodules were spherical instead of elongated, possibly due to a smaller meristematic zone (Fig. 2D and E). Using electron microscopy, we showed that in RNAi line 2, ITs in zone II were about 3-fold larger, indicating retarded bacterial release (Fig. 2F).

To assess whether MtSYMREM1 is required for bacterial infection and release, we generated a specific RNA interference (RNAi) construct targeting the 5′ noncoding region of MtSYMREM1 transcripts and obtained transgenic roots of M. truncatula by Agrobacterium rhizogenes-mediated gene transfer. Plants were grown vertically in pouches and subsequently inoculated with S. meliloti carrying a β-galactosidase gene (LacZ) for visualization. MtSYMREM1-RNAi roots developed 36.5% fewer nodules at 2 weeks postinoculation (wpi) [Student’s t test \( P = 0.0001; n = 134 \) (control) and \( n = 134 \) (MtSYMREM1-RNAi)]. Among these, 31.3% of the MtSYMREM1-RNAi plants did not nodulate under these conditions, whereas only 10.4% of the empty vector were not nodulated (Fig. S3A). Some MtSYMREM1-RNAi plants were kept for 6 wpi and analyzed in more detail. The examination of 15 control and 26 MtSYMREM1-RNAi plants revealed that about 31% of all transgenic plants did not develop any nodules, whereas in 35% of plants, nodules were abnormal and remained small and white. In contrast, 93% of all roots transformed with the vector control developed nodules that were not altered in their morphology (Fig. S3B). Such a spectrum of phenotypes (no nodules, and abnormal and normal nodules) likely depends on the level of silencing in the different RNAi plants, and has also been reported in studies on the symbiotic RLKs NFP, LYK3, and DMI2, implying that all these genes are required throughout infection and nodule organogenesis (1, 3, 8).

To examine the impact of MtSYMREM1 on infection, rhizobia were visualized by LacZ staining inside host roots and nodules. RNAi roots of A. rhizogenes-transformed composite plants showed about 3-fold more ITs (Fig. S3C) than the control, with most of them being aborted or highly branched in outer cell layers of the root (Fig. S3D). In addition, we occasionally observed uncontrolled release of bacteria into epidermal cells (Fig. S3D). An effect at this early step of IT formation in root hairs is coherent with the induction of MtSYMREM1 transcription 24 h after NF addition. Formation of sac-like structures and high degrees of branching of the IT also indicate a loss in IT stability and proliferation and demonstrate roles of MtSYMREM1 during bacterial infection. From these data, we hypothesize that altered infection in MtSYMREM1-RNAi roots led to the development of fewer or no nodules, the latter possibly due to mild infection conditions in pouches, whereas plants attempted to compensate for this effect by initiation of more infection threads.
in homozygous plants, whereas heterozygous and wild-type plants expressed the protein (Fig. 2J). Analysis of microscopic sections of wild-type and NF4432 mutant nodules at 3 wpi revealed that nodules of this line were stunted and more spherical compared to wild-type nodules (Fig. 2F). Infection threads in zone II appeared to be greatly enlarged and highly branched (Fig. 3G), confirming the RNAi-mediated phenotype described above. In contrast to RNAi plants, bacterial liberation from ITs was greatly reduced or completely absent (reflected by the lack of symbiosome) in these MtSYMREM1 knockout nodules (Fig. S3G). All together, knockdown and knockout plants show that MtSYMREM1 is important for rhizobial infection and regulates progression of infection structures.

We then tested whether MtSYMREM1 localizes to the PM and more specifically to lipid rafts, as accumulations in such putative microdomains has been shown for some group 1 remorins (9, 18, 22), and lipid rafts have been shown to be crucial to control microbial infection of host cells in animals (23). Expression of a CFP-MtSYMREM1 fusion protein in leaf epidermal cells of Nicotiana benthamiana revealed the protein to be localized at the PM (Fig. 3A). Protein immunoblotting detected the endogenous MtSYMREM1 protein in microsomal fractions of inoculated roots from M. truncatula shortly after inoculation (Fig. 3B). A weak but specific signal was also detected in the fraction containing cytosolic proteins, in coherence with MtSYMREM1 being a soluble protein (Fig. 3B). The slight band shift that was observed comparing soluble and microsomal fractions at 4 and 10 dpi suggests a secondary modification of a so far unknown nature. Phase partitioning of the microsomal fraction confirmed that MtSYMREM1 is almost exclusively present in the PM (Fig. 3C). In a second approach, we isolated detergent-insoluble PM fractions (lipid rafts) from hyper-nodulated roots (using the M. truncatula sun2-sickle double mutant) and found that the MtSYMREM1 protein was highly enriched in this fraction (Fig. 3D). This indicates that group 2 remorins are specific marker proteins for lipid rafts and also highlights the potential role of membrane subcompartments during symbiotic plant-microbe interactions. The importance of these putative microdomains has also been suggested during host-pathogen interactions (24, 25).

We then used in situ immunofluorescence to spatially localize the endogenous MtSYMREM1 protein in nodules. Highly distinct fluorescence signals were detected along the PM of ITs in zone II (Fig. 3E) and around the symbiosome membrane (zone III) of 10-day-old nodules (Fig. 3F). The protein strongly accumulated in distinct spots in proximal parts of zone II that we identified as infection droplets, where bacteria are released from the ITs into the host cytoplasm (Fig. 3E). Almost no labeling was found in the nodule periphery and the meristematic zone (Fig. S4). Immunogold labeling and transmission electron microscopy on high-pressure frozen and cryo-substituted sections of 10-day-old nodules confirmed the presence of MtSYMREM1 in the PM of nodular ITs (Fig. 3G and H) and on symbiosome membranes (Fig. 3F). Gold particles were predominantly found in distinct patches, indicating accumulation of the protein in defined sites within or near the PM (Fig. 3H), possibly reflecting enrichment of the protein in lipid rafts.

These data indicate a distinct and spatial localization of the protein in close physical proximity to invading rhizobia, and thus at the plant-microbe signaling interface. The accumulation and expansion into the cytoplasm of gold particles during immunolocalization experiments suggests oligomerization of MtSYMREM1 (Fig. 3H). This would be in agreement with in vitro cross-linking experiments that show filament formation by group I remorins from potato and tomato (17). We thus used bimolecular-fluorescence complementation (BiFC) to test oligomerization of MtSYMREM1 in plants. Split-YFP proteins (YFP<sup>+</sup>/YFP<sup>-</sup>) were fused individually to the N terminus of MtSYMREM1, and both fusion proteins were coexpressed in N. benthamiana leaves. Clear BiFC signals were found at 2 dpi with A. tumefaciens in the PM of leaf epidermal cells showing homooligomerization of MtSYMREM1 proteins (Fig. 4A).

Despite MtSYMREM1 not sharing sequence similarities with proteins outside the remorin family, its structural and spatial features suggest interesting analogies with animal caveolin proteins. Caveolins are small, oligomeric, soluble scaffolding pro-

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**Fig. 2.** MtSYMREM1 is required for nodulation and infection. Phenotypes were scored on roots transformed with an *MtSYMREM1*-RNAi construct by *A. tumefaciens* (A–F) -mediated gene transfer. (A–C) Control plants transformed with the empty vector developed normal nodules (A, B). (D–F) Abnormal nodules with altered meristem structure developed on plants with intermediate screening levels. Electron microscopy showed ITs (*) in zone II to be enlarged (F). A Tnt1 transposon insertion line (NF4432) was identified (H) and characterized (G–J). Segregation and the nodulation phenotype of this mutant were analyzed (G); wt*, wild-type nodules; wt; nodules from mutant lines that were genotyped as “wild-type” in the *MtSYMREM1* locus; HET, heterozygous; HOM, homozygous; seg., segregation; av., average; stdev, SD; *P* values were obtained from a Student’s *t* test (significance level *P* ≤ 0.01). (I) Western blot analysis on proteins from isolated nodules probed with α-MtSYMREM1 antibodies. MtSYMREM1 protein is not detectable in the homozygous line NF4432. (J) Nodules (3 wpi) from NF4432 were embedded, sectioned, and stained with toluidine blue. [Scale bars, 1 mm (A and D), 5 μm (C and F), 100 μm (B, E, and J).]
MtSYMREM1 protein was also found on symbiosome membranes in zone III showing clustered accumulation of gold particles at and near the PM. (F indicate accumulations of gold labels. (F) Immunogold labeling of endogenous MtSYMREM1 in non-infected cells (F, stars). (G) Immunogold labeling of endogenous MtSYMREM1 in nodule IT membranes (F and inset) and bacterial release sites in zone II (F, arrows) and on the symbiosome membrane in zone III (F) in 10-day-old nodules. Growth and differentiation of Medicago roots using specific antibodies. Subcellular fractionation (B), phase partitioning (C), and isolation of detergent-insoluble membrane (D) show predominant accumulation of MtSYMREM1 in membranes, at the PM, and in lipid raft subdomains, respectively, membrane proteins; SN, non-membrane proteins; PM, plasma membrane proteins; others, non-PM proteins; DIM, detergent-insoluble membrane proteins. Native MtSYMREM1 localizes specifically to IT membranes (E and inset) and bacterial release sites in zone II (E, arrows) and on the symbiosome membrane in zone III (F) in 10-day-old nodules. Plant and rhizobial DNA were stained with DAPI (blue), and secreted bacterial proteins were detected using a colicin-A-specific antibody. (E) Immunogold labeling of endogenous MtSYMREM1 in a nodular IT using transmission electron microscopy. Arrows indicate accumulations of gold labels. (H) Close-up of upper left part of the IT showing clustered accumulation of gold particles at and near the PM. (I) MtSYMREM1 protein was also found on symbiosome membranes in zone III (arrows). (J) Scale bars, 20 μm (A, E, and F), 1 μm (E, inset, G, and I), 100 nm (H, I).

Fig. 3. MtSYMREM1 is located at the plasma membrane close to the symbionts. (A) CFP-MtSYMREM1 fusion proteins were expressed in leaves of N. benthamiana. The construct was driven by the constitutive CaMV 35S promoter. (B–F) Immunoblotting and immunolocalization of MtSYMREM1 on Medicago roots using specific antibodies. Subcellular fractionation (B), phase partitioning (C), and isolation of detergent-insoluble membrane (D) show predominant accumulation of MtSYMREM1 in membranes, at the PM, and in lipid raft subdomains, respectively, membrane proteins; SN, non-membrane proteins; PM, plasma membrane proteins; others, non-PM proteins; DIM, detergent-insoluble membrane proteins. Native MtSYMREM1 localizes specifically to IT membranes (E and inset) and bacterial release sites in zone II (E, arrows) and on the symbiosome membrane in zone III (F) in 10-day-old nodules. Plant and rhizobial DNA were stained with DAPI (blue), and secondary antibodies were coupled to Alexa488. No labeling was found in uninfected cells (F, stars). (G) Immunogold labeling of endogenous MtSYMREM1 in a nodular IT using transmission electron microscopy. Arrows indicate accumulations of gold labels. (H) Close-up of upper left part of the IT showing clustered accumulation of gold particles at and near the PM. (I) MtSYMREM1 protein was also found on symbiosome membranes in zone III (arrows). (J) Scale bars, 20 μm (A, E, and F), 1 μm (E, inset, G, and I), 100 nm (H, I).

Proteins that form filamentous structures, localized in PM caveolae, which are particular lipid rafts. These proteins interact with several receptors and signaling proteins, among them the insulin receptor IR3 (26, 27). Cavelin 1 was shown to recruit proteins to the caveolae microdomains and to play important roles in regulation of signaling components (27).

Considering the localization and oligomerization data presented here, we decided to test the hypothesis that MtSYMREM1 may play an analogous role to caveolins by testing for interactions between MtSYMREM1 and three RLKs playing essential roles in Nod factor perception, signaling, and rhizobial infection, NFP, LYK3, and DMI2. In a first step, we tested direct interactions between the proteins using a split-ubiquitin yeast two-hybrid assay. Yeast growth was sustained on selective media when bait constructs of either NFP, LYK3, or DMI2 were coexpressed with the prey construct MtSYMREM1, indicating interaction of MtSYMREM1 with all three symbiotic RLKs. To test specificity of the approach, we also cloned a so far uncharacterized LRR RLK from M. truncatula (MtLRRII.1) that is not implicated in root nodule symbiosis. No interaction between MtLRRII.1 and MtSYMREM1 was detected (Fig. 4B). In addition, no interaction of any of the four RLKs was detected with the originally described group 1 remorin SfREM1.3 (Fig. S5A). These data strongly suggest that MtSYMREM1 specifically interacts with the symbiotic RLKs NFP, LYK3, and DMI2 in yeast.

To confirm these data by an independent approach in planta, we cloned these genes into plant expression vectors and expressed them heterologously in N. benthamiana leaves. All RLK-YFP fusion proteins localized to the PM, as shown by confocal laser-scanning microscopy (Fig. S5B). In a next step, we generated YFP-C-fusion proteins of the three symbiotic RLKs and MtLRRII.1 and coexpressed them with YFP-N-MtSYMREM1 in N. benthamiana leaves. Clear BiFC signals at the PM were obtained for NFP, LYK3, and DMI2 and no signal at all for MtLRRII.1 when coexpressed with MtSYMREM1 (Fig. 4C). These data confirm in planta specific interactions of MtSYMREM1 with the three symbiotic RLKs. Unfortunately, the very low expression levels hindering visualization in M. truncatula of the symbiotic RLKs as fluorescent fusion proteins or by immunofluorescence restrict this analysis currently to heterologous systems. However, interactions of this remorin protein with the RLKs on IT membranes is genetically supported because impaired expression of MtSYMREM1 and of any of the three receptors alters or abolishes IT growth and bacterial infection.

Data presented here show that MtSYMREM1 is a symbiotic receptor-binding protein. Expression patterns of MtSYMREM1 and the phenotypes of loss-of-function mutants indicate a role of this gene in bacterial signal perception during initial stages of infection and throughout nodule development and, more precisely, a role in IT growth. Based on the data presented here and by analogy to scaffolding proteins from animals, we hypothesize that MtSYMREM1 functions as a scaffolding protein that mediates spatial regulation of signaling complexes during symbiotic plant-microbe interactions. As such, MtSYMREM1 might be required for preinfection, polar growth of infection structures, and bacterial release through regulation of receptor proteins in functional PM subdomains.

Materials and Methods

Plant Growth and Transformation and Genotyping of Tnt1 Insertion Lines. For qRT-PCR, Western blotting, and fractionation, M. truncatula (Jemalong A17) was grown on vertical plates. For PM and lipid raft preparation and qRT PCR after NF treatment, hypernodulated TR122 M. truncatula mutants (21) were grown aeroponically for 3 weeks. Details have been described in ref. 28. Transgenic roots were generated using A. rhizogenes-mediated gene transfer using Arqua1 strain on M. truncatula Jemalong A17 as described in ref. 29 and grown for a total of 8 weeks on vertical plates and in pouches (28). Successful transformation was detected by the presence of dsRED fluorescence that was used as a visual marker. Transgenic plants were obtained by Agrobacterium tumefaciens transformation using the AGL1 strain on M. truncatula Jemalong 2HA as described in ref. 28. Seed from the R1 generation of M. truncatula R108 Tnt1 transposon insertion lines was obtained from The Samuel Roberts Noble Foundation after screening the mutant population by PCR using the following primers: REMtnStScreen: 5′-TTGTGGAGTGG-CAGGGTGT-3′ and Tnt1r: 5′-CAATGAACAGGACAACTGCT-3′. All comparative experiments were done using the corresponding genotype as control.

Leaves of N. benthamiana were infiltrated with A. tumefaciens strain C58 GV3101-pMP90 containing the indicated constructs always in combination with strain C58 GV301 for expression of the silencing inhibitor P19 (30).

NF Treatment and Rhizobia Inoculation. Roots were treated with the major N. melloti NF: NodSm-IV, Ac, S, C16:2 at 10−8 M as described in ref. 21.
Yeast Two-Hybrid Experiments. Plasmids coding for ALGSNubG and ALGSNubL cloning vectors, and transformation protocols were provided by Dualsystem Biotech (DUALmembrane kit 2). The coding sequences of MtLRRII.1, LYK3, NFP, and DM2 were cloned in the pCCW-SUC bait plasmids (LEU2), leading to addition of Cub-lexaVP16 to the C terminus of the proteins. Their respective signal peptides (predicted by Psort) were replaced by the yeast SUCl signal peptide. MTSYMREM1 coding sequence was cloned in the pD3L prey plasmid (TRP1) leading to addition of NubG to the N terminus of the protein. The NMYF2 yeast strain [leu2, trp1, his3, LexA VP16-lexH31] was first transformed with the bait vectors and a single colony was subsequently transformed with the different prey vectors. A minimum of 50 double-transformed colonies were mixed and tested for interaction on SD-WLH medium supplemented with 5 mM 3AT.

Antibodies, Protein Extraction, and Western Blotting. Polyclonal antibodies were raised against two peptides (SYKLEKEPGNESC and AAGTDTEDRDAV) located in the N-terminal region of the MtSYMREM1 protein (Eurogentec). Antibodies were subsequently purified against these peptides and used in a 1:1000 dilution for western blotting. Microsomal, PM, and detergent-insoluble membrane fractions were prepared as previously described in ref. 22. After centrifugation of the microsomal fraction, the supernatant was precipitated with 10% TCA. All fractions were resuspended in the same volume, and equal volumes were loaded onto a 10% SDS/PAGE. For protein detection in M. truncatula root nodules, 200 mg fresh weight of tissue was used, and one quarter of the volume of solubilized proteins was separated on SDS/PAGE. Proteins were visualized by using HRP-coupled secondary anti-rabbit antibodies (Santa Cruz Biotechnology) together with Immobilon staining solutions (Millipore).

Histological Analysis and Microscopy. Fluorescence images were acquired using a Leica SP2-SE confocal microscope or a Zeiss Axiohot2 microscope equipped with a Plan-Apochromat 100×/1.40 Oil immersion objective (Zeiss) together with Immobilon staining solutions (Millipore). The same labeling protocol was used for electron microscopy. Samples were placed in a Zeiss Axioplan2 and a Leica DMI 6000B. For immunolocalization, samples were treated before the immunolocalization procedure. For electron microscopy, samples were placed in a flat specimen carrier, 400 μm in depth, filled with sucrose phosphate buffer. The carrier was quickly transferred to the Leica EM PACT high-pressure freezer (Leica Microsystems) and immediately frozen at 2,000 bars. Samples in carriers were stored in liquid nitrogen until transfer to the Leica AFS (automatic freeze substitution) in freeze substitution medium (0.25% uranyl acetate in anhydrous acetone) previously cooled at −90°C, followed by gradual freeze substitution at −45°C for 36 h, −60°C for 8 h, and −30°C for 14 h before being transferred to −20°C. Here the FS medium was replaced by rectapure acetone (two rinses of 10 min each). Samples were pulled off the carriers and individually placed in plastic capsules filled with acetone. Acetone was progressively replaced by London Resin (LR) gold resin during an embedding step of 48 h before plastic capsules were finally transferred into galantine capsules filled with LR gold. Polymerization was carried out under UV light at −20°C for 48 h and then at +20°C for 36 h. Ultrathin sections (70–80 nm) were prepared with a Reichert Ultracut microtome and collected on nickel carbon-coated grids and processed for electron microscopy immunolabeling. Remorin immunolocalization was performed on sections by treating with a polyclonal anti-remorin antibody 1/250 in PBS buffer with 0.2% gelatin and 0.2% glycerol (pH 7.4) (PBSGG) overnight at 4°C. Sections were rinsed three times with PBSGG buffer and incubated with goat anti-rabbit Alexa488 (Molecular Probes) secondary antibodies (1/250 in PBSGG) for 2 h at room temperature. After staining, autofluorescence was quenched using Evans blue (Sigma; 0.5% in PBS, 5 min at room temperature), and the sections were mounted in Mowiol 4-88 (Calbiochem) containing 1,4 diazabicyclo(2.2.2) octane (Sigma) as anti-lysing agent and 4,6-diamino-2-phenylindole (Sigma) as nuclear stain. The same labeling protocol was used for electron microscopy on ultrathin sections with secondary goat anti-rabbit antibodies labelled with 15 nm gold grains. (1/20 in PBSGG) for 2 h at room temperature. Observations were done on a JEOL 120 kV electron microscope.
Structural and ultrastructural studies on nodules were done from material fixed with 2% glutaraldehyde and embedded in Technovit 7100 (Kulzer) for light microscopy and Epon for electron microscopy.

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Supporting Information

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Fig. S1. qRT PCR analysis of gene expression of all known remorins in different plant organs of M. truncatula. (A) MtREM2.2 (MtSYMREM1) shows a distinct expression pattern, with an exclusive expression in nodules. Error bars indicate the SD obtained from three independent biological replicates. (B) The listed primers were used for expression analysis of different remorin genes. Nomenclature was used according to ref. 1.


Fig. S2. MtSYMREM1 is differentially expressed upon inoculation of roots with different S. meliloti strains. Induction patterns at 10 dpi depend on the infection phenotype of the symbiont. Phenotypes of these mutants are listed in the panel below: Nod, nodulation; Inf, infection; Fix, nitrogen fixation. Data were derived from microarray experiments.
Fig. S3. Analysis of MtSYMREM1-RNAi and transposon insertion lines. (A) Significantly more transgenic roots, obtained by A. rhizogenes transformation, were impaired in nodulation at 2 weeks postinoculation (wpi) when being transformed with the MtSYMREM1-RNAi construct (χ² test: P value = 2.5 × 10⁻⁵; n = 134 per genotype. Three independent experiments were performed, and standard deviation represents the variability between the experiments. (B) The majority of MtSYMREM1-RNAi roots developed no or abnormal nodules on plants grown in pouches for 6 wpi after transformation with A. rhizogenes. The total number of plants is indicated within the histogram bars. (C) Increased numbers of ITs were found upon knockdown of MtSYMREM1; n = 5 per genotype. (D) Normal IT in root hairs from control roots carrying the empty vector and sac-like structures, and highly branched IT on MtSYMREM1-RNAi roots. Uncontrolled rhizobial release into epidermal cells (stars) was occasionally observed in MtSYMREM1-RNAi roots (Lower Right). Arrows mark ITs. (Scale bars, 20 μm.) Rhizobia carrying a lacZ reporter gene are stained in blue. (E) In plants transformed by A. tumefaciens with empty vector or MtSYMREM1-RNAi construct, transcript levels of MtSYMREM1 were reduced in RNAi line 2 compared to RNAi line 1 and empty vector control plants. Error bars indicate the SD obtained from three independent biological replicates. (F) Expression levels of all remorin genes that were analyzed before (Fig. S1) were tested in nodules of MtSYMREM-RNAi line 2 and control plants. As indicated, transcript levels are reduced by about 50% for MtSYMREM1, whereas the other genes were not silenced in these plants. (G) Microtome sections of 3-wpi nodules of insertion line NF4432 and wild-type (wt*) plants were analyzed. Infection threads (*) were enlarged and highly branched in the mutant line compared with wild-type nodules. (Scale bars, 30 μm.)
Fig. S4. Immunolocalization of MtSYMREM in a 10-day-old nodule. (A) Bright-field image of a 10-day-old nodule and indication of the meristematic and infection zones. (B) Immunolocalization of MtSYMREM1 using specific MtSYMREM1 antibodies. Plant and rhizobial DNA were stained with DAPI (blue) and secondary antibodies were coupled to Alexa488. (C) Evans blue was used to visualize plasma membranes. (Scale bars, 100 μm.)

Fig. S5. LYK3, NFP, and DMI2 fusion proteins localized to the plasma membrane interact with MtSYMREM1 but not with StREM1.3. (A) The split-ubiquitin system (Cub-NubG) was used to study interactions in a yeast two-hybrid assay. Yeast expressing interacting pairs was able to grow on -WLH supplemented with 5 mM 3AT. MtSYMREM1 interacts with LYK3, NFP, and DMI2 but not with MtLRRII.1. All receptor-like kinases are expressed and can interact with the positive control, the yeast membrane protein ALG5 fused to Nubl, whereas no interaction was detected with the negative controls ALG5-NubG and StREM1.3. (B) The indicated constructs were expressed in N. benthamiana, and YFP fluorescence was imaged by confocal laser-scanning microscopy. (Scale bars, 20 μm.)