# *Medicago* plants control nodulation by regulating proteolysis of the receptor-like kinase DMI2

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Short title: Proteasome control of DMI2

One-sentence summary: The symbiotic receptor DMI2 in *Medicago* undergoes constitutive degradation by the proteasome, which is blocked upon rhizobia inoculation.

## Abstract

Plants use receptor-like kinases to monitor environmental changes and transduce signals into plant cells. The *Medicago truncatula* DOES NOT MAKE INFECTIONS 2 (DMI2) protein functions as a co-receptor of rhizobial signals to initiate nodule development and rhizobial infection during nitrogen-fixing symbiosis, but the mechanisms regulating DMI2 protein level and folding are still unknown. Here we report that DMI2 protein abundance changes during nitrogen-fixing symbiosis. DMI2 accumulates in the nodules and is induced by rhizobia treatment through a posttranscriptional process. However, DMI2 induction is independent of perception of Nod factor, a group of lipo-chitooligosaccharides secreted by rhizobia. The stability of the DMI2 protein is controlled by the proteasome pathway: in rhizobia-free environments, DMI2 is degraded by the proteasome, but during rhizobial infection, DMI2 is protected from the proteasome, resulting in protein accumulation. Furthermore, proteasome inhibitor-promoted accumulation of DMI2 protein in *Medicago* roots induces the expression of two early nodulation marker genes, supporting the hypothesis that DMI2 accumulation activates downstream symbiosis signaling. The extracellular region of DMI2 contains two Malectin-like domains (MLD) and a leucine-rich repeat. When conserved amino acids in the MLDs are mutated, DMI2 fails to restore nodule development in *dmi2* mutants, and point-mutated MLD proteins are degraded constitutively, suggesting that the MLD may be vital for the accumulation of DMI2. Our findings suggest that legumes control nodule development through modulating the protein level of DMI2, revealing a layer of regulation in the interaction between plants and rhizobia in nitrogen-fixing symbiosis.

## Introduction

Plants form symbiotic relationships with surrounding microbes to gain access to nutrients in natural environments. Most land plants form beneficial interactions with arbuscular mycorrhizal (AM) fungi, developing mycorrhized roots, which provide phosphorous and micronutrients to plants in exchange for fixed carbon. A subset of plants, including plants of the legume family, develop nitrogen-fixing symbioses in specialized organs, or root nodules. This sophisticated symbiosis with rhizobia provides legumes with nitrogen fixed by rhizobia hosted inside plant cells of the nodules (Oldroyd et al., 2011).

Plants use receptors to discriminate between symbiotic and pathogenic microbes. In both AM and nitrogen-fixing symbiosis, plant roots detect the existence of beneficial microbes by a group of receptor-like kinases (RLKs). In nitrogen-fixing symbiosis, rhizobia secrete a group of lipo-chitooligosaccharides, or Nod factors, to initiate nodule development and symbiosis. In *Medicago truncatula*, Nod factors are perceived by two RLKs: NFP (Nod Factor Perception) and LYK3 (LysM Domain-containing Receptor-like Kinase 3), which are named NFR1 (Nod Factor Receptor 1) and NFR5 in *Lotus japonicus*, respectively. The perception of Nod factors activates the common symbiosis signaling pathway, including root hair-associated calcium spiking, early nodulation gene activation and cortical cell division (Schauser et al., 1999). DMI2 (DOES NOT MAKE INFECTIONS 2, the name in *M. truncatula*)/SYMRK (the name in *L. japonicus*)/NORK (Nodulation Receptor Kinase, the name in *M. sativa*) is believed to interact with Nod factor receptors (Antolín-Llovera et al., 2014). Mutations in *DMI2* lead to the abortion of rhizobial infection at a very early stage (Endre et al., 2002; Stracke et al., 2002). DMI2 is also indispensable for AM symbiosis and plant–*Frankia* symbiosis, another independently-evolved nitrogen-fixing symbiosis between certain plants and *Frankia* bacteria (Endre et al., 2002; Stracke et al., 2002; Gherbi et al., 2008), indicating a conserved role of DMI2 throughout the evolution of plant–microbe symbioses.

The DMI2 protein contains an intracellular kinase domain, a transmembrane domain, and the

extracellular portion, including a region with three leucine rich repeats (LRRs) and a Malectin-like domain (MLD). In human cells, the single-domain protein Malectin functions in the endoplasmic reticulum (ER) lumen in protein quality control by binding to di-glucosylated Glc2Man9GlcNAc2, a glycan composed of three glucoses, nine mannoses, and two N-acetylglucosamines (Schallus et al., 2008). Utilizing its carbohydrate binding activity, Malectin directly interacts with misfolded glycoproteins and inhibits their secretion (Qin et al., 2012).

While Malectin-like sequences are widespread among biological kingdoms, two features make MLD-containing proteins in plants unique: (1) their gene families are greatly expanded in plants, and (2) MLDs mostly occur in the extracellular portion of receptor-like kinases. A few MLD-containing RLKs have been shown to play vital roles in plant development, male-female interaction, disease resistance, and plant-microbe symbiosis (Endre et al., 2002; Boisson-Dernier et al., 2011; Hok et al., 2011; Haruta et al., 2014). Although the functions of MLDs have yet to be revealed, the position of MLDs in the extracellular portions of proteins points to the possibility that MLDs may be necessary for activating or deactivating the intracellular kinase domain through binding extracellular ligands. Interestingly, it has been reported that the MLD of SYMRK/DMI2 is cleaved constitutively, with or without rhizobia, and the MLD-cleaved SYMRK/DMI2 protein outcompetes the full length SYMRK/DMI2 in the interaction with Nod factor receptors (Antolín-Llovera et al., 2014).

During nitrogen-fixing symbiosis, the host is required to provide nutrients to the rhizobia (Oldroyd et al., 2011). This burden on the plant has promoted the evolution of a sophisticated regulatory network controlling the scale and timing of nodule development (Oldroyd et al., 2011). Several reports show that overexpressing the full-length SYMRK/DMI2 or the intracellular kinase domain of SYMRK/DMI2 leads to spontaneous nodule formation even in the absence of rhizobia (Ried et al., 2014; Saha et al., 2014), suggesting the protein level of DMI2 needs to be regulated precisely. SYMRK/DMI2 has also been reported to interact with two E3 ligases: SINA4 (SEVEN IN ABSENTIA 4) and SIE3 (SYMRK-INTERACTING E3 UBIQUITIN LIGASE) (Den Herder et al., 2012; Yuan et al., 2012). However, direct genetic

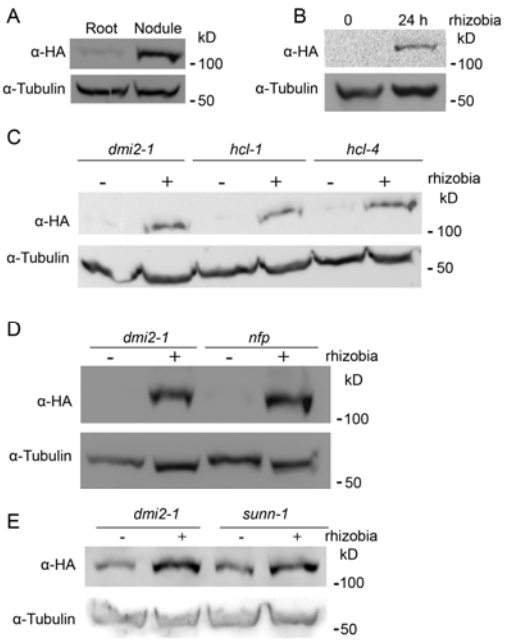
97 evidence illustrating these two E3 ligases affect the level of SYMRK/DMI2 *in planta* is still
98 missing, and the dynamics of DMI2 protein levels during nitrogen-fixing symbiosis remain
99 unknown.
100
101 Here we report that DMI2 protein levels are tightly regulated by legume hosts to properly
102 respond to rhizobia infection. We find that, without rhizobia infection, DMI2 protein is
103 constitutively degraded through the proteasome apparatus; during rhizobia infection, the
104 DMI2 protein level is induced by blocking proteasome-mediated degradation. Meanwhile if
105 key amino acid residues in the DMI2-MLD are mutated, DMI2 is degraded constitutively,
106 suggesting a key role of MLD in the regulation of DMI2 protein homeostasis. Taken together
107 with the report that overexpression of the DMI2/SYMRK kinase domain causes spontaneous
108 nodulation (Ried et al., 2014; Saha et al., 2014), fine-tuning the protein level of DMI2 is
109 critical for legumes to maximize the profit of nitrogen-fixing symbiosis at the lowest cost.
110
111

## Results

### Rhizobia induce DMI2 protein at the posttranscriptional level

To determine the dynamics of DMI2 protein levels during nitrogen-fixing symbiosis, a stable transgenic *Medicago dmi2-1* line was used. This line expresses the *DMI2* genomic sequence driven by its native promoter; it is also fused to a dual affinity tag containing three copies of the hemagglutinin epitope (HA) and a single StrepII (ST); therefore, the resulting protein is named DMI2-HAST. The *gDMI2:HAST* construct complements the phenotype of *dmi2* mutants and the protein is easy to detect (Riely et al., 2013). After inoculating the transgenic lines with *Sinorhizobium meliloti* strain ABS7, we compared the protein accumulation of DMI2-HAST between nodules and untreated roots. The result showed that before rhizobia treatment, the DMI2-HAST protein level in the roots was very low (Figure 1A), which is consistent with previous reports that DMI2-HAST protein is almost undetectable (Riely et al., 2013). In nodules, the protein level of DMI2-HAST was much higher compared with rhizobia-free roots (Figure 1A). Furthermore, by analyzing the expression of *DMI2* in nodules and un-inoculated roots in the *M. truncatula* gene expression atlas database (Benedito et al., 2008), we found that the transcription of *DMI2* is not highly activated in whole nodules (Supplemental Figure S1A and B), which is also consistent with a previous report using Northern blot assays (Bersoult et al., 2005). These results suggest that the DMI2-HAST protein accumulates in the nodules through posttranscriptional regulation.

To further investigate the protein level variation of DMI2 during rhizobia inoculation, we treated *dmi2-1 gDMI2:HAST* plant roots with rhizobia strain ABS7 and checked protein abundance during rhizobia infection at the earliest stage. Twenty-four hours post ABS7 strain treatment, the protein level of DMI2-HAST increased dramatically compared with untreated roots (Figure 1B). Treating *dmi2-1 gDMI2:HAST* plants with 1/2 Basic Nodulation Medium (BNM), the liquid medium for rhizobia inoculation, *Agrobacterium rhizogenes* strain Arqua1, *S. meliloti* strain Rm1021 and ABS7 showed that DMI2-HAST protein level increased only during rhizobia inoculation (Supplemental Figure S2A), indicating that the DMI2-HAST




induction effect is specific to rhizobia. To establish how quickly the protein accumulates, we analyzed the protein level of DMI2-HAST at different time points post rhizobia treatment, and found that as early as 3 hours after ABS7 inoculation, DMI2-HAST protein level was already induced (Supplemental Figure S2B). To rule out the possibility that the accumulation of DMI2-HAST was a result of transcriptional activation, we checked the expression level of *DMI2* by reverse transcription quantitative PCR (RT-qPCR), as well as by browsing the *Medicago truncatula* Gene Expression Atlas and Mt 4.0 database (Benedito et al., 2008; Krishnakumar et al., 2014). The RT-qPCR assay showed that transcription of *DMI2* was not subsantially induced at 3, 6, 12, 24 hours after rhizobia inoculation, with microarray and RNA-Seq data showing similar results (Supplemental Figure S3A, B and C), which is consistent with previous reports that *DMI2* transcripts are not induced significantly by rhizobia treatment in a Northern blot assay (Mirabella et al., 2005). Taking these results together, we conclude that rhizobia treatment induces the abundance of DMI2 protein by affecting posttranscriptional regulation at a very early stage of symbiosis.

It is reported that DMI2/SYMRK interacts directly with the Nod factor receptors to perceive Nod factors (Antolín-Llovera et al., 2014). To determine whether the accumulation of DMI2-HAST protein in the presence of rhizobia is dependent on Nod factor receptors, we

expressed *gDMI2:HAST* in *hcl-1* and *hcl-4* (two mutant alleles of the *Medicago* Nod factor receptor gene *LYK3*) (Smit et al., 2007), as well as in *dmi2-1*. Similar to *dmi2-1*, 24 hours post rhizobia treatment, the protein level of DMI2-HAST in *hcl-1* and *hcl-4* mutants accumulated to a much higher level compared with the control (Figure 1C). We also performed this experiment with the mutant of another Nod factor receptor, *NFP* and found similar results (Figure 1D). In various experiments detecting the protein level changes of DMI2-HAST, the intensity of the DMI2-HAST bands in un-inoculated *Medicago* roots ranged from very weak to hard to detect. It has been reported that in *hcl-1*, *hcl-4* and *nfp* mutants, the expression of *DMI2* transcripts was identical to wild type (Mirabella et al., 2005), indicating that the accumulation of DMI2-HAST protein in *hcl-1*, *hcl-4* and *nfp* is also post-transcriptionally controlled.

To further confirm that the induction of DMI2 protein level by rhizobia was independent of Nod factor perception, we inoculated *Medicago* plants with wild-type *S. meliloti* RM1021 and two rhizobium mutant strains, RJW14 and JT210, which have defects in Nod factor synthesis (Wais et al., 2002). The protein level of DMI2 was induced to a similar level by RJW14 and JT210 compared with the wild-type strain (Supplemental Figure S4A and B). These results show that DMI2 protein accumulation induced by rhizobia is independent of Nod factor perception, alluding to the existence of another as-yet-unknown rhizobia signal.

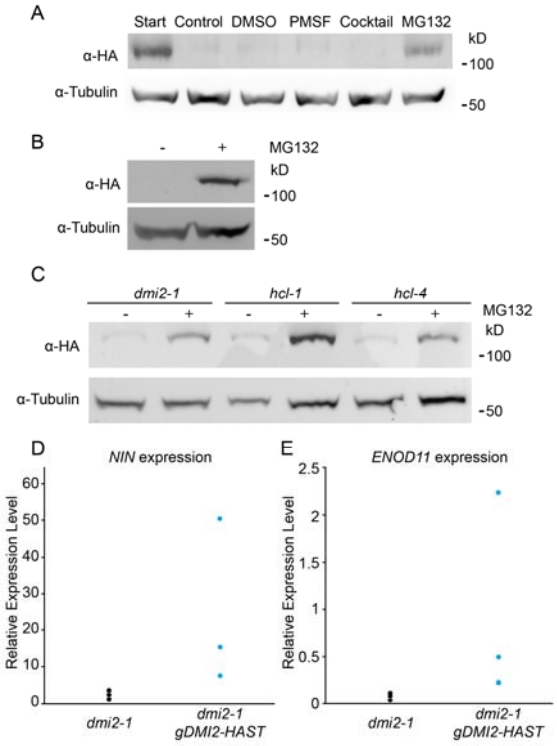
SUNN (SUPER NUMERIC NODULES) is an LRR receptor kinase, which functions in the shoot to regulate nodule numbers, and *sunn* mutants display a super-nodulation phenotype (Penmetsa et al., 2003; Elise et al., 2005). Surprisingly, it was reported that overexpressing the DMI2/SYMRK kinase domain in *sunn* mutants decreases the number of nodules formed in these super-nodulation mutants (Saha and DasGupta, 2015). To analyze whether *SUNN* could affect the induction of DMI2-HAST protein by rhizobia, we transformed *gDMI2-HAST* into *dmi2-1* and *sunn-1* backgrounds and checked the protein level of DMI2-HAST during ABS7 strain infection. The result showed that DMI2-HAST was induced to a similar level in *sunn-1* and *dmi2-1* mutant backgrounds (Figure 1E), suggesting the induction of DMI2 protein by rhizobia is also independent of *SUNN*.

**DMI2 protein is constitutively degraded in un-inoculated *Medicago* roots**

The accumulation of DMI2 protein by rhizobia is regulated at the posttranscriptional level, indicating protein stability regulation may play a role. To test this hypothesis, we performed a cell-free degradation assay using protein samples from *dmi2-1 gDMI2-HAST* roots grown in a sterile environment. DMI2-HAST protein from un-inoculated roots was completely degraded within 2 hours (Figure 2A). To study which mechanism was responsible for the degradation of DMI2-HAST, several different proteolysis inhibitors were tested. While the protease inhibitor PMSF (phenylmethyl sulfonyl fluoride) and a plant-specific protease inhibitor cocktail failed to rescue the protein level of DMI2, the proteasome inhibitor MG132 largely prevented DMI2-HAST protein from degradation (Figure 2A), showing that in un-inoculated roots DMI2-HAST may be degraded in a proteasome-dependent manner.

The *in planta* protein degradation of DMI2-HAST was then examined by treating *dmi2-1 gDMI2:HAST* roots with MG132. Four hours post MG132 treatment, the accumulation of DMI2-HAST protein was significantly enhanced (Figure 2B), showing that the inhibition of proteasome activity mimics the effect of rhizobia treatment. When treating *hcl-1* and *hcl-4* with MG132, the protein level of DMI2-HAST was similar to *dmi2-1* plants, suggesting the accumulation of DMI2 protein by MG132 treatment is also independent of Nod factor receptors (Figure 2C). To rule out that MG132 treatment promoted the transcriptional activation of *DMI2*, we analyzed the dynamics of *DMI2* transcripts during MG132 treatment by RT-qPCR. The transcript level of *gDMI2:HAST* was indistinguishable before and after MG132 treatment (Supplemental Figure S5). These data show that MG132 can block the degradation of DMI2-HAST protein in un-inoculated *Medicago* roots, suggesting that the DMI2-HAST protein is degraded through a proteasome apparatus in the absence of rhizobia.

Recently there have been several reports showing that overexpressing the kinase domain or the full-length DMI2/SYMRK protein can induce a spontaneous nodulation phenotype in legume plants (Ried et al., 2014; Saha et al., 2014). To evaluate whether MG132-induced




DMI2 protein accumulation carries any biological significance, we checked the expression of *ENOD11 (EARLY NODULIN 11)* and *NIN (NODULE INCEPTION PROTEIN)*, two marker genes of early nodule development (Schauser et al., 1999), in *dmi2-1* and *dmi2-1 gDMI2:HAST* roots after MG132 treatment. As shown in Figure 2D and E, 4 hours after MG132 treatment in *dmi2-1 gDMI2:HAST* roots, the expression of *NIN* and *ENOD11* was induced to a higher level compared to *dmi2-1* mutants. We conclude that DMI2 protein accumulation may be able to partially activate downstream nodulation signaling.

**DMI2 protein is protected from degradation in inoculated roots**

To further test the effect of MG132 on DMI2 protein in inoculated *Medicago* roots, *S. meliloti* ABS7-inoculated *dmi2-1 gDMI2-HAST* roots were treated with MG132 for 4 hours. Compared to untreated roots, the protein level of DMI2-HAST was not further increased (Supplemental Figure S6), showing that MG132 has little impact on DMI2 protein level in the presence of rhizobia, which indicates that in inoculated *Medicago* roots, proteasome-mediated degradation of DMI2 has already been blocked (Figure 2B). Thus, during nitrogen-fixing

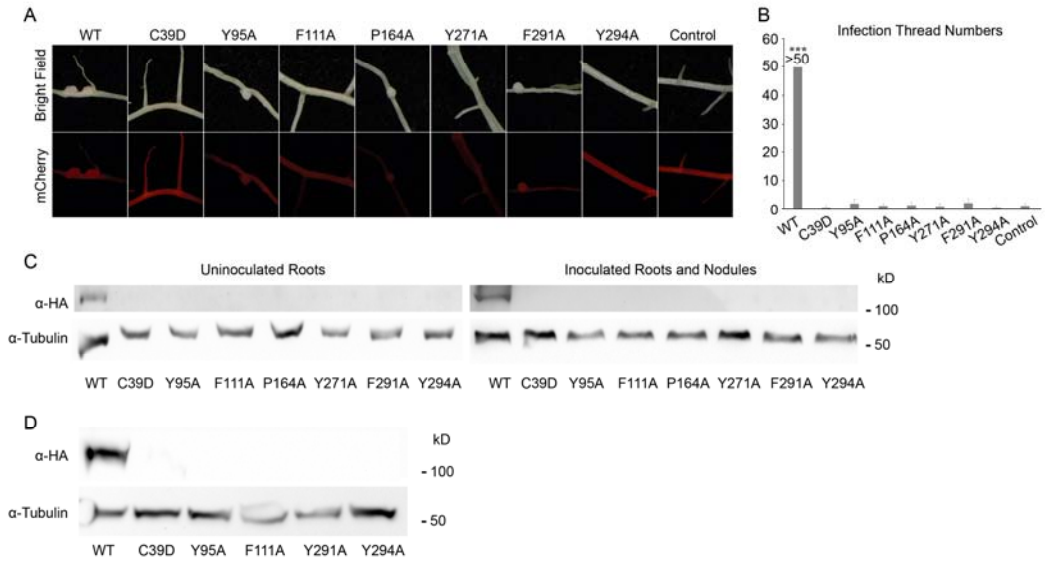
symbiosis, DMI2 protein is protected from proteasome-mediated degradation.

**MLD is vital for DMI2 function**

In the extracellular region, DMI2 has a LRR and a MLD. To gain more insights about the MLD, we aligned the MLD with human Malectin protein using a homology modeling method (http://swissmodel.expasy.org/) (Biasini et al., 2014). While originally DMI2-MLD was described as a Malectin-like sequence, we found that it actually contains two tandem matches to the human Malectin-A domain, and each match has about 140 amino acids (Supplemental Figure S7A and B). The human Malectin protein is reported to bind Glc2Man9GlcNAc2 and regulate protein folding (Schallus et al., 2008). However very little is known about the function of DMI2-MLD or any MLD in plants.

To study the function of DMI2-MLD, we aligned the amino acid sequence of DMI2-MLD with its close homologs in the plant kingdom. The results show that DMI2-MLD is conserved among its homologs in dicots (Supplemental Figure S8), suggesting MLD may have a conserved role. To gain further insights into the function of DMI2-MLD, we performed site-directed mutagenesis. We analyzed the following point mutations: $DMI2^{C39D}$, $DMI2^{Y95A}$, $DMI2^{F111A}$, $DMI2^{Y164A}$, $DMI2^{F271A}$, $DMI2^{Y291A}$, and $DMI2^{F294A}$, where the numbers indicate the location of these residues in the full-length DMI2 protein including the signal peptide sequence (Supplemental Figure S9A and B). We targeted these amino acids because they were conserved among the homologs included in the alignment (Supplemental Figure S8), pointing to a greater possibility that these point mutations may affect the function of DMI2-MLD. Among them, Y95A, Y164A, and Y291A may be in the predicted ligand binding pockets (Figure S9A) (Schallus et al., 2008).

To investigate whether mutating the conserved amino acids in MLD would affect the function of the DMI2 protein, we introduced wild-type *gDMI2-HAST* and the 7 MLD point-mutated versions into *dmi2-1* mutant plants using the hairy root transformation method. As shown in Table 1, 14 days after rhizobia inoculation, *dmi2-1* plants expressing wild-type *gDMI2-HAST*




generated many nodules, while *dmi2-1* roots expressing MLD point mutants had few nodules, and even fewer of which were pink, suggesting a failure in nitrogen fixation (Figure 3A). Some point mutations, such as DMI2$^{C39D}$, had no nodules at all (Table 1). These results show that regarding to sufficient nodule development, MLD is vital for the proper function of DMI2.

To enter legume roots, rhizobia normally have to penetrate plant cells through a plant-derived tubular structure at infected root hair cells, known as the infection thread (Oldroyd et al., 2011). It has been shown that, in *dmi2/symrk* mutants, the development of the infection thread is blocked (Endre et al., 2002; Stracke et al., 2002). To find out whether blocking the function of MLD could affect the role of DMI2 in infection thread formation, we checked the infection thread phenotype of *dmi2-1* mutants transformed with wild type and the 7 MLD point mutation versions of *gDMI2-HAST*, respectively. Three days after rhizobia inoculation, infection threads could be seen under the microscope in *dmi2-1* mutants transformed with wild-type *gDMI2-HAST.* In contrast, there were few infection thread-like structures in the plants transformed with *gDMI2-HAST* containing point mutations in MLD (Supplemental Figure S10). Counting the numbers of infection threads, we found that *dmi2-1* roots transformed with wild-type *gDMI2-HAST* could produce large numbers of infection threads, but the roots transformed with *gDMI2-HAST* with point mutations in MLD had very few infection threads (Figure 3B). These results show that MLD is necessary for DMI2 protein to

function properly in infection thread formation. Taken together, the proper function of MLD is necessary for DMI2 protein to play a fundamental role in nodule development at the very early stage.

**MLD is required for the homeostasis of DMI2**

Since the protein level of DMI2 is important for its proper function in nodule development, we studied whether the amino acid substitutions in MLD would affect the protein level of DMI2. In *Medicago* roots expressing either wild type or MLD point mutations of *gDMI2-HAST*, the protein could be detected in plants transformed with wild type sequence, but the plants transformed with *gDMI2-HAST* containing amino acid substitutions in MLD could not generate detectable DMI2-HAST protein, with or without rhizobia inoculation (Figure 3C). To rule out the possibility that the disappearance of MLD point mutated protein is regulated at the transcriptional level, we checked the transcripts of wild-type *gDMI2-HAST* and the MLD point mutation versions using RT-qPCR. We found that transgenic plants could generate comparable amounts of wild-type *gDMI2-HAST* and MLD point mutation transcripts (Supplemental Figure S11), albeit the expression level varied due to variations in hairy root transformation. These results show that the proper function of MLD is critical for the precise regulation of DMI2 protein abundance at the post-transcriptional stage.

To find out whether the degradation of MLD amino acid substitution versions of DMI2 protein is dependent on the proteasome apparatus, we treated *dmi2-1* plants expressing wild-type *gDMI2-HAST* and MLD point mutation versions with MG132 and checked DMI2 levels before and after MG132 treatment. As shown in Figure 3D, 4 hours after MG132 treatment, the protein level of wild-type full length DMI2-HAST was strongly induced, while MLD point mutations versions could not accumulate the protein. This result shows that the degradation of DMI2 containing point mutations in MLD could not be rescued by inhibiting proteasome activity with MG132. Considering that the human Malectin protein binds to carbohydrate and functions in ER quality control (Schallus et al., 2008; Qin et al., 2012), it is possible that the MLD is required for proper folding of DMI2 in the ER; blocking MLD

316 function may activate ER quality control signaling and result in the degradation of DMI2.

317

318 As MLD is indispensable for the function of DMI2 in *Medicago*, we further investigated the
319 origin of MLD and whether it is conserved in the plant kingdom. We constructed a
320 phylogenetic tree of DMI2 in dicots, monocots and basal angiosperm species and found that
321 MLD is not present in monocot homologs of DMI2, but the protein from *Amborella*
322 *trichopoda*, a basal angiosperm species, has a MLD (Supplemental Figure S12). On the other
323 hand, we examined the phylogenetic tree of the closest *DMI2* paralog in *M. truncatula*,
324 Medtr7g057170. To our surprise, the orthologs of this gene from every species had a MLD.
325 These results suggest that MLD is important for the function of DMI2 in dicots and basal
326 angiosperms; however in monocot plants, the MLD is missing, indicating that there may be
327 other proteins functioning together with DMI2 to perform the function of MLD
328 (Supplemental Figure S12).

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## Discussion

### 1. Rhizobia inoculation can block the protein degradation of DMI2.

Localized to the plasma membrane, RLKs are able to detect environmental changes through extracellular domains and transduce the signals into cells, through their intracellular catalytic domains (Lemmon and Schlessinger, 2010). To ensure the proper activation of RLKs during development and stress response processes, the protein level of RLKs must be kept in check by plants to avoid run-away intracellular responses. Here we report that the protein level of *M. truncatula* symbiosis receptor DMI2 is regulated at the posttranslational level to modulate proper response to rhizobia inoculation.

DMI2 plays a key role regulating plant-microbe symbiosis, as it is required for the symbiosis between plants and AM fungi, plants–actinomycete nitrogen-fixing symbiosis and legume plant–rhizobia nitrogen-fixing symbiosis (Gherbi et al., 2008). Furthermore, DMI2 displays protein level increases coinciding with rhizobia inoculation. When plants are grown in the absence of rhizobia, the DMI2 protein is kept at a very low level (Figure 1A, 1B); during rhizobia infection, through a currently unknown mechanism, rhizobia block the degradation of DMI2, resulting in increased protein levels. As a consequence, accumulated DMI2 induces plant roots to start the process of nodule development. This is consistent with previous reports that, when the DMI2/SYMRK kinase domain or full length protein is overexpressed in legume roots, plants will generate spontaneous nodules without rhizobia infection (Ried et al., 2014; Saha et al., 2014).

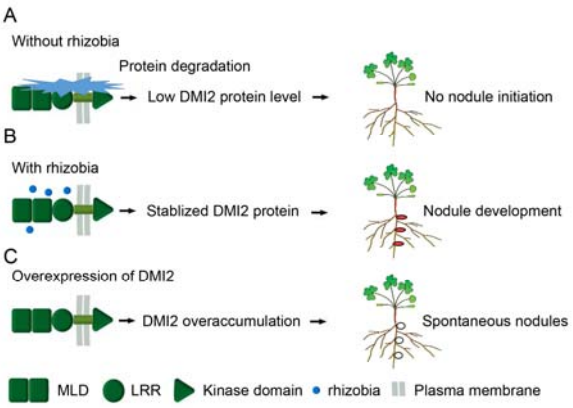
We also found that MG132 treatment caused accumulation of the protein level of DMI2 in un-inoculated *Medicago* roots (Figure 2A and B), indicating that the turnover of DMI2 in the absence of rhizobia infection is through proteasome-mediated protein degradation. More importantly, MG132 treatment mimicked the effect of rhizobia inoculation in the respect of activating *NIN* and *ENOD11* (Figure 2D and 2E). In inoculated roots, MG132 treatment did not increase the protein level of DMI2 (Supplemental Figure S6), suggesting that after

rhizobia inoculation, DMI2 protein is already protected from proteasome degradation. The robust induction of DMI2 protein levels by MG132 also suggests that MG132 treatment can be used for further characterization of the DMI2 protein. For example, it may be used to find DMI2 interacting partners, especially the substrates of the DMI2 kinase domain.

Although the mechanisms coupling ligand recognition in the extracellular parts and the activation of intracellular catalytic domains are surprisingly diverse (Lemmon and Schlessinger, 2010), there are reports that ligand binding induces the protein level of RLKs. For instance, it is widely known that in human cells insulin treatment can increase the intracellular protein abundance of insulin receptors (Lemmon and Schlessinger, 2010). In *Arabidopsis thaliana*, FLS2 (FLAGELLIN SENSING 2) is a LRR RLK, which functions as a receptor for bacterial flagellin protein or flg22, a 22 amino acid active peptide derivative (Gómez-Gómez and Boller, 2000). FLS2 can be ubiquitinated by two related U-box E3 ligases, PUB12 and PUB13 without the presence of pathogen (Lu et al., 2011). The ubiquitination and subsequent degradation of FLS2 protects plant cells from the harm of excessive activation of defense response. These findings indicate that the induction of RLK protein levels by their ligands may be a widespread phenomenon.

## 2. DMI2 protein level is at the center of regulating nodule development and rhizobial initiation

It is broadly known that when growing in environments with abundant nitrogen, legume plants do not make nodules despite the presence of rhizobia. Similarly, when nodulating plants are provided with bio-available nitrogen, the plant will shut down the nodules immediately in order to save energy and resources. Hyper-nodulation mutants, which generate many more nodules than the wild type, are reported to have severe growth defects, suggesting that excessive nodules have negative effects on the fitness of plants (Elise et al., 2005). Understanding how plants maintain the ability to make the right number of nodules at the right time is a great interest of the research community. Our results show that the DMI2 protein accumulates during rhizobial inoculation. Taken together with reports that




overexpressing the kinase domain or whole length DMI2/SYMRK results in spontaneous nodulation or a hyper-nodulation phenotype in legumes (Ried et al., 2014; Saha et al., 2014), we conclude that the protein level of DMI2/SYMRK is a master determinate signal of nodule development (Figure 4).

Our results show that the induction of DMI2 protein is independent of Nod factor reception (Figure. 1C), pointing to a possibility that there is another hidden signaling pathway used by rhizobia to regulate DMI2 protein levels, likely through blocking protein degradation. It has been reported that DMI2/SYMRK interacts with several E3 ligases in *L. japonicus*, like SINA4 and SIE3 (Den Herder et al., 2012; Yuan et al., 2012). As these reports have shown that SINA4 and SIE3 could ubiquitinate DMI2/SYMRK, our results provide the necessary evidence that the protein level of DMI2/SYMRK is altered during rhizobia infection in a proteasome dependent manner *in vivo*. To truly confirm SINA4 and SIE3 are responsible for DMI2/SYMRK protein turnover, expressing *DMI2/SYMRK* in *sina4* and *sie3* mutants to investigate DMI2/SYMRK protein dynamics is necessary.

Since DMI2 is also required for the symbiosis between plants and arbuscular mycorrhizal fungi and *Frankia* bacteria, it will be interesting to find out whether the protein level of DMI2 is induced by AM treatment. We would predict that this is the case because we found that the induction of DMI2 protein by rhizobia is independent of Nod factor perception (Figure 1C). Furthermore, any differences in DMI2 protein dynamics between rhizobia and AM infection might be related to how plants distinguish these two different symbionts, even though they

413 utilize a shared symbiosis signaling pathway.

414

415 **3. MLD is required for proper folding of DMI2 protein.**

416

417 The extracellular part of DMI2 contains a MLD domain and a LRR. Considering the MLD
418 reside at the extracellular domain of RLKs in plants, it is speculated that it may directly
419 recognize some microbial signals or signals generated from plant-microbe interaction, and
420 subsequently control the proper activation of the kinase domain. We found that the MLD is at
421 least required for the proper folding of DMI2 protein, as after we introduced amino acid
422 substitutions into the MLD, plants could not generate full-length DMI2 protein even in the
423 presence of proteasome inhibitor MG132 or rhizobia inoculation (Figure 3C and D). Human
424 Malectin protein is reported to function in the ER (Schallus et al., 2008). Through its affinity
425 to carbohydrate molecules, human Malectin protein could bind to misfolded proteins and
426 activate ER quality control signaling (Qin et al., 2012). From our results, we speculate that
427 DMI2-MLD may also function in the ER quality control process to guide proper folding and
428 successful secretion of the protein of which MLD itself is a part. The exact ligand for
429 DMI2-MLD has yet to be found, but if this hypothesis is true, the likely scenario is that
430 DMI2-MLD could bind to some carbohydrate ligands similar to di-glucosylated
431 Glc2Man9GlcNAc2 during protein folding in the ER lumen (Schallus et al., 2008). A
432 carbohydrate microarray is required to find the exact ligand of DMI2-MLD.

433

434 By showing the protein level dynamics of DMI2 during rhizobia inoculation and the altered
435 protein behavior of DMI2 with amino acid substitutions in the MLD, we show the probable
436 function of MLD and present a previously unidentified signaling pathway of how rhizobia
437 affect DMI2 protein levels.

438

439

## Methods

### Plant materials and growth conditions

*Medicago truncatula* ecotype A17 was used for all the experiments in this study. Plants were grown under 16 hours light, 8 hours dark at 22℃. *Agrobacterium rhizogenes* strain Arqua1 was used for plant hairy root transformation, and the procedure was conducted as previously described (Boisson-Dernier et al., 2001). Transgenic roots were selected based on antibiotic resistance against kanamycin at the concentration of 15 μg/mL in Fahraeus medium for 10 days.

*Sinorhizobium meliloti* strain ABS7 *hemA:LacZ* was used for rhizobia inoculation. Fresh overnight rhizobia culture was centrifuged and suspended in 1/2 BNM liquid medium to a concentration of $OD_{600}$= 0.05. Five mL liquid rhizobia culture was used per *Medicago* plant. Nodulation and infection thread phenotypes were checked 14 days and 7 days post inoculation respectively; for protein sample collection, tissues were collected at different time points as indicated in the figure legends.

### Evaluation of symbiotic phenotypes

To analyze nodule development phenotype, 14 days after ABS7 *HemA:: LacZ* inoculation, plants were harvested and total and pink nodules were counted. For infection thread analysis, 7 days post ABS7 *hemA:LacZ* inoculation, *Medicago* roots were stained for β-galactosidase activity as previously described (Pan et al., 2016). Root samples were filled with staining buffer (0.5 M sodium phosphate buffer, pH 7.2, 10% Triton X-100, 100 mM potassium ferrocyannide, 100 mM potassium ferricyanide) containing 2 mM X-Gluc, then incubated at 37℃ overnight. Infection threads were checked and the pictures were taken using a Nikon E200 microscope. Number of infection threads was counted on at least 10 individual transformed plants for each construct.

**Plasmids and vectors**

*pKGW-RR::gDMI2-HAST* was used to express wild type *gDMI2-HAST* in plants stably and transiently (Riely et al., 2013). To make point mutations in the MLD, an overlapping PCR method was used to generate desired amino acid substitutions in *pENTER::gDMI2-HAST* (Heckman and Pease, 2007). After sequencing to confirm the sequences were correct, the *gDMI2-HAST* sequences containing various point mutations in the MLD were introduced into the *pKGW-RR* vector using a LR recombination kit (Invitrogen). Then the plasmids containing the point mutations were transformed into *Agrobacterium rhizogenes* strain Arqua1 by electro-transformation.

**Protein extraction and immunoblot**

To extract proteins from un-inoculated roots, inoculated roots and nodules of *Medicago* plants, plant tissues were collected at the time points indicated in the figure legends and put into liquid nitrogen immediately. After grinding the tissues into a fine powder, proteins were extracted using Native Extraction Buffer 1 (50 mM Tris-MES, pH 8.0; 0.5 M sucrose; 1 mM $MgCl_2$; 10 mM EDTA; 5 mM DTT.) with or without the protease inhibitor cocktail (Sigma-Aldrich) (Liu et al., 2010). Extracted proteins were boiled for 5 minutes with 6 × SDS sampling buffer and stored at -20℃.

For immunoblots, various protein samples were loaded into a 12% acrylamide SDS running gel and the protein samples were transferred from the gel to nitrocellulose membrane (Adventec) by electro-blotting. After blocking the membrane with milk, the membrane was subjected to incubation with 1:500 diluted anti-HA antibody (New England Biolabs) and 1:4000 diluted anti-alpha tubulin antibody (Life Sciences). The secondary antibody was anti-rat and anti-mouse respectively (Life Sciences). As the secondary antibodies were conjugated with horseradish peroxidase, the membrane was treated with the detection reagent from Thermo Scientific (products #1859707 and #1859678). The bands on the membrane were visualized via a G-box machine (New England Biogroup).

**Cell free degradation assay and MG132 treatment**

Cell free degradation assays were performed following previous reports (Spoel et al., 2009). Extracted protein samples were split into individual centrifuge tubes using equal amounts, different reagents were added into the tubes as indicated (MG132 concentration, 40 μM/mL), and the tubes were shaken slowly at room temperature for 2 hours. Individual samples were boiled with 6 × SDS sampling buffer and subjected to immunoblot with anti-HA and anti-tubulin antibodies.

For MG132 *in vivo* treatments, 2-week old *Medicago* plants were washed clean and put into distilled water containing 100 μM/mL MG132 at 22℃. Root samples were collected at different time points during MG132 treatment as indicated.

**RNA extraction and RT-qPCR**

Total RNA extraction of rhizobia-inoculated or MG132-treated *Medicago* roots was carried out using TRIzol (Invitrogen) following the manufacturer's instructions. To eliminate possible DNA contamination, extracted RNA was treated with the Turbo DNA-free kit (Life Science Technologies, USA) using the manufacturer's instructions. iScript cDNA synthesis kit (Bio-Red) was used for second chain synthesis following the manufacturer's instructions step by step.

RT-qPCR was performed on the Eppendorf Mastercycler ep Realplex system. The PCR reaction was conducted using ExTaq polymerase (Takara) with SYBR Green dye (Thermo Fisher Scientific), in 20 μL total volume: dd$H_2O$, 9.9 μL; 10×Extaq buffer, 2 μL; 2.5 mM dNTP mix, 2 μL; 10 μM primer mix, 2 μL; SYBR Green dye, 2 μL; template cDNA, 2 μL; ExTaq polymerase, 0.1 μL. For the real-time PCR reaction, annealing temperature was set at 60℃ and elongation time was 30 seconds. When the PCR was finished, the melting curve was analyzed to rule out possible non-specific amplification, and

the result represented was the mean from threshold cycle value ($C_T$) of three technical replicates. Three biological replicates were used for each sample. *PROTODERMAL FACTOR 2* (*PDF2*), locus ID Medtr6g084690, was used as the internal control.

**Protein domain analysis and sequence alignments**

The gene expression analysis using existing databases was divided into two groups: for microarray analysis, the data were obtained from *Medicago truncatula* Gene Expression Atlas (MtGEA) (http://mtgea.noble.org/v3/) (Benedito et al., 2008); for RNA-seq analysis, *Medicago Truncatula* Genome Project v4.0 (Mt 4.0) (http://medicago.jcvi.org/medicago/index.php) and the symbimics website (https://iant.toulouse.inra.fr/symbimics/) were used (Young et al., 2011; Roux et al., 2014).

For protein structure prediction and conserved residue analysis, the SWISS-MODEL database (http://swissmodel.expasy.org/) was used, and the DMI2-MLD domain structure was predicted based on the structure of the human MALA (Malectin A) domain. The positions of conserved amino acids were mapped to the predicted structure in the same program (Biasini et al., 2014).

The phylogenic tree was built using the MEGA 7.0 program under the developer's instruction; all the sequences used are summarized in the Supplemental file.

**Accession numbers:** Sequence data from this article can be found in Supplemental Table 1. The GenBank accession numbers for the genes used to build phylogenetic tree are presented.

**Supplemental Data:**

**Supplemental Figure S1.** Expression of *DMI2* in nodules compared with roots.

**Supplemental Figure S2.** The induction of DMI2 by rhizobia.

**Supplemental Figure S3.** *DMI2* expression with rhizobia or Nod factor treatment.

**Supplemental Figure S4.** DMI2 level induction by rhizobia strains with defects in Nod factor synthesis.

**Supplemental Figure S5.** *DMI2-HAST* expression with MG132 treatment.

**Supplemental Figure S6.** DMI2 levels in rhizobia-inoculated *Medicago* roots treated with MG132.

**Supplemental Figure S7.** Predicted protein structure of two human MALA (PDB code: 2kr2.1.A) matches in DMI2-MLD.

**Supplemental Figure S8.** Alignment of DMI2-MLD with its closest plant homologs.

**Supplemental Figure S9.** Positions of amino acid substitutions in the DMI2-MLD protein sequence.

**Supplemental Figure S10.** Representative pictures showing infection thread development in wild-type and mutated MLD plants.

**Supplemental Figure S11.** *DMI2-HAST* expression in wild-type and mutated MLD plants.

**Supplemental Figure S12.** MLD presence or absence in *DMI2* orthologs across species.

**Supplemental Table 1.** Accession numbers.

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**Table 1**

| | WT | C39D | Y95A | F111A | P164A | Y271A | F291A | Y294A | Control |
|---|---|---|---|---|---|---|---|---|---|
| Plants in total | 13 | 14 | 11 | 12 | 13 | 14 | 12 | 11 | 14 |
| Transformed Plants | 13 | 13 | 11 | 12 | 13 | 14 | 12 | 11 | 14 |
| Plants with nodules | 13 | 0 | 9 | 4 | 9 | 1 | 5 | 3 | 0 |
| Nodule numbers | >300 | 0 | 29 | 4 | 17 | 1 | 19 | 4 | 0 |
| Pink nodules | >300 | 0 | 5 | 0 | 0 | 1 | 2 | 0 | 0 |

**Table 1.** Summary of the complementation assay using wild type and MLD point mutation versions of *gDMI2-HAST*. While *dmi2-1* plants transformed with wild type *gDMI2-HAST* could generate a large number of pink nodules, roots expressing *gDMI2-HAST* versions with amino acid substitutions in MLD were impaired in nodule development and generated very few pink nodules. Control, plants transformed with empty vector. Transformed roots were selected based on mCherry fluorescence marker of the *gDMI2-HAST* construct. Experiments were repeated more than three times with similar results.

## Figure Legends

**Figure 1.** Rhizobia treatment could induce the protein level of DMI2 in a Nod factor receptor independent manner. A, DMI2-HAST protein accumulated in the nodules comparing with un-inoculated root. Protein samples were taken from 14-day old nodules and 4-week old un-inoculated roots. B, DMI2-HAST protein was induced to much higher level 24 hours after rhizobia treatment. 4-week old Medicago roots were treated with *S. meliloti* strain ABS7 *hemA:LacZ* at the concentration of $OD_{600}$=0.05 for 24 hours. C and D, DMI2-HAST protein was induced to a similar level by rhizobia treatment in wild type and Nod factor receptor mutant backgrounds. *gDMI2-HAST* construct was transiently expressed in *dmi2-1*, *hcl-1*, *hcl-4*, transformed roots were treated with or without *S. meliloti* strain ABS7 *hemA:LacZ.* E, DMI2-HAST protein could be induced to a similar level by rhizobia in *dmi2-1* and *sunn-1* plants stably transformed with g*DMI2-HAST*. α-HA was used to detect the DMI2-HAST protein level, Tubulin was used as the loading control. The experiments were repeated 5 times with similar results.

**Figure 2.** MG132 can block the degradation of DMI2 protein in un-inoculated Medicago roots. A, MG132 could partially rescue DMI2-HAST protein level in cell free degradation assay. Cell

freeDMI2-HAST protein samples were treated with different reagents for 2 hours respectively and subjected to Western blot using α-HA. Tubulin was used to show equal loading. “Start”, freshly extracted protein sample; “Cocktail” means plant-specific protease inhibitors mixture. B, MG132 treatment could induce the protein level of DMI2-HAST *in vivo*. Un-inoculated Medicago roots were treated with 100 μM MG132 and the level of DMI2-HAST protein was detected. Experiments were repeated at least 5 times with similar results. C, MG132 could block the degradation of DMI2 independent of Nod factor reception. Samples were taken from *dmi2-1*, *hcl-1*, *hcl-4* plants expressing *gDMI2-HAST* were treated with or without 100 μM MG132 for 4 hours. D and E, After MG132 treatment, the expression of *NIN* and *ENOD11* are modestly induced in 2-week old *dmi2-1 gDMI2-HAST* Medicago roots. Each dot represents a biological replicate. α-HA was used to detect the DMI2-HAST protein level, Tubulin was used as the internal control. Experiments were repeated two times with similar results.

**Figure 3.** MLD is required for DMI2 protein to function properly in early nodule development process and to stabilize full-length DMI2 proteins in plants. A, Pictures showing representative nodules in *dmi2-1* plants transforming wild type and MLD point mutation versions of *gDMI2-HAST*. Although some point mutation versions could generate nodules, most of them were white and small. mCherry fluorescence was used to select the transformed roots. B, *dmi2-1* plants expressing *gDMI2-HAST* containing point mutations in MLD had few infection threads in quantitative assay. The number of infection thread in *dmi2-1* plants expressing wild type *gDMI2-HAST* was estimated to be more than 50. More than 10 transformed plants were used for each line. Error bar indicates means and standard deviation. “***” shows significant difference ($P<0.001$, Student’s *t*-test). C, DMI2 protein containing amino acid substitutions in MLD is unstable in Medicago plants, with or without rhizobia inoculation. In *dmi2-1 gDMI2-HAST* plants DMI2-HAST protein could be seen, while the MLD amino acid substitution version proteins were totally undetectable. D, MG132 treatment could not rescue the constitutive degradation of DMI2 protein containing amino acids substitutions. *dmi2-1* plants expressing wild type *gDMI2-HAST* and MLD point mutation versions were treated with 100 μM MG132 and the protein level was tested. Experiments were repeated three times with similar results.

**Figure 4.** Plants control nodule development through fine regulation of DMI2 protein level. A, Without the presence of rhizobia, DMI2 protein is constitutively degraded by the proteasome apparatus and the nodule development signaling pathway is suppressed. B, Rhizobia inoculation could block the degradation of DMI2 protein, subsequently stabilized DMI2 could initiate nodule development signaling, and thus plants could develop functional nodules. C, When whole length DMI2 protein or the kinase domain is overexpressed, over-accumulated DMI2 protein can activate nodule development signaling pathway without the presence of rhizobia, leads to spontaneous nodulation phenotype. Red ellipsoid in (B) indicates functional nodules, empty ellipsoid in (C) represents spontaneous nodules respectively.

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