

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

2 **DMI2**

3

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9 Author Contributions: D.W. and H.P. conceived the original research plans; H.P. performed the
10 experiments; D.W. and H.P. analyzed the data; and D.W. and H.P. wrote the manuscript.

11

12 Short title: Proteasome control of DMI2

13

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

16 **Abstract**

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

37 **Introduction**

38

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

46

47 Plants use receptors to discriminate between symbiotic and pathogenic microbes. In both AM
48 and nitrogen-fixing symbiosis, plant roots detect the existence of beneficial microbes by a
49 group of receptor-like kinases (RLKs). In nitrogen-fixing symbiosis, rhizobia secrete a group
50 of lipo-chitooligosaccharides, or Nod factors, to initiate nodule development and symbiosis.
51 In *Medicago truncatula*, Nod factors are perceived by two RLKs: NFP (Nod Factor
52 Perception) and LYK3 (LysM Domain-containing Receptor-like Kinase 3), which are named
53 NFR1 (Nod Factor Receptor 1) and NFR5 in *Lotus japonicus*, respectively. The perception of
54 Nod factors activates the common symbiosis signaling pathway, including root
55 hair-associated calcium spiking, early nodulation gene activation and cortical cell division
56 (Schauser et al., 1999). DMI2 (DOES NOT MAKE INFECTIONS 2, the name in *M.*
57 *truncatula*)/SYMRK (the name in *L. japonicus*)/NORK (Nodulation Receptor Kinase, the
58 name in *M. sativa*) is believed to interact with Nod factor receptors (Antolín-Llovera et al.,
59 2014). Mutations in *DMI2* lead to the abortion of rhizobial infection at a very early stage
60 (Endre et al., 2002; Stracke et al., 2002). *DMI2* is also indispensable for AM symbiosis and
61 plant-*Frankia* symbiosis, another independently-evolved nitrogen-fixing symbiosis between
62 certain plants and *Frankia* bacteria (Endre et al., 2002; Stracke et al., 2002; Gherbi et al.,
63 2008), indicating a conserved role of *DMI2* throughout the evolution of plant-microbe
64 symbioses.

65

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

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

74

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

87

88 During nitrogen-fixing symbiosis, the host is required to provide nutrients to the rhizobia
89 (Oldroyd et al., 2011). This burden on the plant has promoted the evolution of a sophisticated
90 regulatory network controlling the scale and timing of nodule development (Oldroyd et al.,
91 2011). Several reports show that overexpressing the full-length SYMRK/DMI2 or the
92 intracellular kinase domain of SYMRK/DMI2 leads to spontaneous nodule formation even in
93 the absence of rhizobia (Ried et al., 2014; Saha et al., 2014), suggesting the protein level of
94 DMI2 needs to be regulated precisely. SYMRK/DMI2 has also been reported to interact with
95 two E3 ligases: SINA4 (SEVEN IN ABSENTIA 4) and SIE3 (SYMRK-INTERACTING E3
96 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

112 **Results**

113

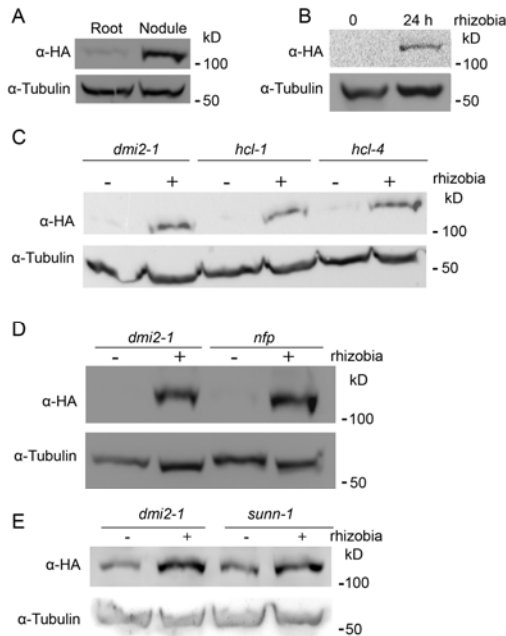
114 **Rhizobia induce DMI2 protein at the posttranscriptional level**

115

116 To determine the dynamics of DMI2 protein levels during nitrogen-fixing symbiosis, a stable
117 transgenic *Medicago dmi2-1* line was used. This line expresses the *DMI2* genomic sequence
118 driven by its native promoter; it is also fused to a dual affinity tag containing three copies of
119 the hemagglutinin epitope (HA) and a single StrepII (ST); therefore, the resulting protein is
120 named DMI2-HAST. The *gDMI2:HAST* construct complements the phenotype of *dmi2*
121 mutants and the protein is easy to detect (Riely et al., 2013). After inoculating the transgenic
122 lines with *Sinorhizobium meliloti* strain ABS7, we compared the protein accumulation of
123 DMI2-HAST between nodules and untreated roots. The result showed that before rhizobia
124 treatment, the DMI2-HAST protein level in the roots was very low (Figure 1A), which is
125 consistent with previous reports that DMI2-HAST protein is almost undetectable (Riely et al.,
126 2013). In nodules, the protein level of DMI2-HAST was much higher compared with
127 rhizobia-free roots (Figure 1A). Furthermore, by analyzing the expression of *DMI2* in nodules
128 and un-inoculated roots in the *M. truncatula* gene expression atlas database (Benedito et al.,
129 2008), we found that the transcription of *DMI2* is not highly activated in whole nodules
130 (Supplemental Figure S1A and B), which is also consistent with a previous report using
131 Northern blot assays (Bersoult et al., 2005). These results suggest that the DMI2-HAST
132 protein accumulates in the nodules through posttranscriptional regulation.

133

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



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

156

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

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

171

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

179

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

190

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

192

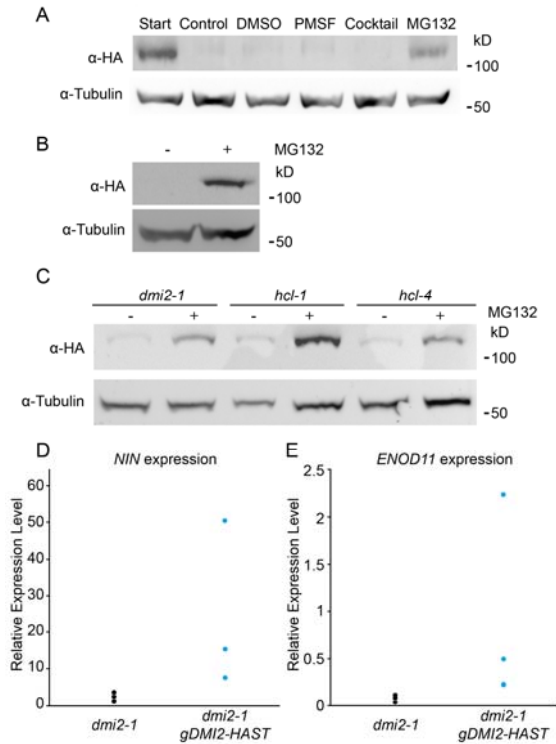
193 The accumulation of DMI2 protein by rhizobia is regulated at the posttranscriptional level,
194 indicating protein stability regulation may play a role. To test this hypothesis, we performed a
195 cell-free degradation assay using protein samples from *dmi2-1 gDMI2-HAST* roots grown in a
196 sterile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

203

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

216

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



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

227

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

229

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

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

237

238 **MLD is vital for DMI2 function**

239

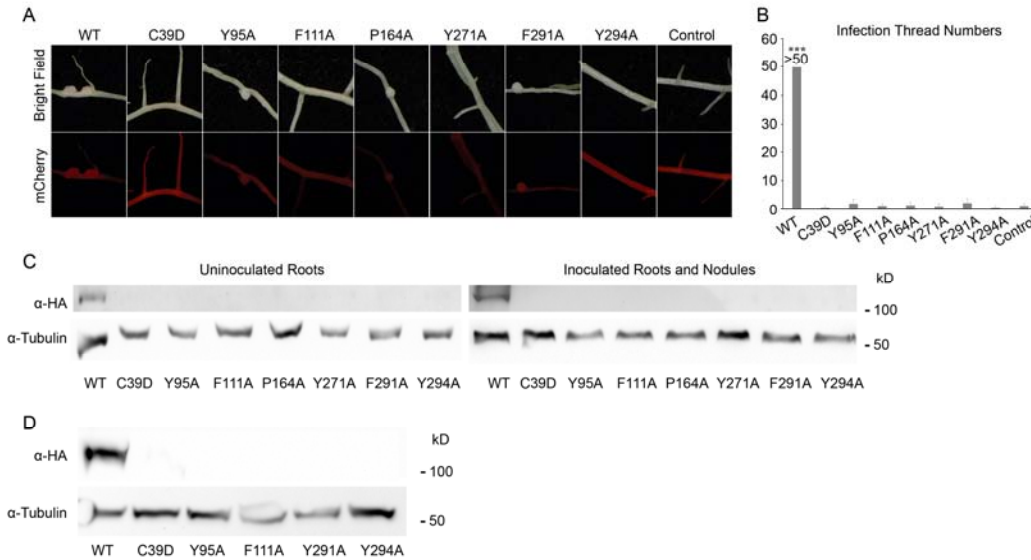
240 In the extracellular region, DMI2 has a LRR and a MLD. To gain more insights about the
241 MLD, we aligned the MLD with human Malectin protein using a homology modeling method
242 (<http://swissmodel.expasy.org/>) (Biasini et al., 2014). While originally DMI2-MLD was
243 described as a Malectin-like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

248

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

261

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



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

271

272 To enter legume roots, rhizobia normally have to penetrate plant cells through a plant-derived
 273 tubular structure at infected root hair cells, known as the infection thread (Oldroyd et al.,
 274 2011). It has been shown that, in *dmi2/symrk* mutants, the development of the infection thread
 275 is blocked (Endre et al., 2002; Stracke et al., 2002). To find out whether blocking the function
 276 of MLD could affect the role of *DMI2* in infection thread formation, we checked the infection
 277 thread phenotype of *dmi2-1* mutants transformed with wild type and the 7 MLD point
 278 mutation versions of *gDMI2-HAST*, respectively. Three days after rhizobia inoculation,
 279 infection threads could be seen under the microscope in *dmi2-1* mutants transformed with
 280 wild-type *gDMI2-HAST*. In contrast, there were few infection thread-like structures in the
 281 plants transformed with *gDMI2-HAST* containing point mutations in MLD (Supplemental
 282 Figure S10). Counting the numbers of infection threads, we found that *dmi2-1* roots
 283 transformed with wild-type *gDMI2-HAST* could produce large numbers of infection threads,
 284 but the roots transformed with *gDMI2-HAST* with point mutations in MLD had very few
 285 infection threads (Figure 3B). These results show that MLD is necessary for *DMI2* protein to

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

289

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

291

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

305

306 To find out whether the degradation of MLD amino acid substitution versions of DMI2
307 protein is dependent on the proteasome apparatus, we treated *dmi2-1* plants expressing
308 wild-type *gDMI2-HAST* and MLD point mutation versions with MG132 and checked DMI2
309 levels before and after MG132 treatment. As shown in Figure 3D, 4 hours after MG132
310 treatment, the protein level of wild-type full length DMI2-HAST was strongly induced, while
311 MLD point mutations versions could not accumulate the protein. This result shows that the
312 degradation of DMI2 containing point mutations in MLD could not be rescued by inhibiting
313 proteasome activity with MG132. Considering that the human Malectin protein binds to
314 carbohydrate and functions in ER quality control (Schallus et al., 2008; Qin et al., 2012), it is
315 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).

329

330

331 **Discussion**

332

333 **1. Rhizobia inoculation can block the protein degradation of DMI2.**

334

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

342

343 DMI2 plays a key role regulating plant-microbe symbiosis, as it is required for the symbiosis
344 between plants and AM fungi, plants-actinomycete nitrogen-fixing symbiosis and legume
345 plant-rhizobia nitrogen-fixing symbiosis (Gherbi et al., 2008). Furthermore, DMI2 displays
346 protein level increases coinciding with rhizobia inoculation. When plants are grown in the
347 absence of rhizobia, the DMI2 protein is kept at a very low level (Figure 1A, 1B); during
348 rhizobia infection, through a currently unknown mechanism, rhizobia block the degradation
349 of DMI2, resulting in increased protein levels. As a consequence, accumulated DMI2 induces
350 plant roots to start the process of nodule development. This is consistent with previous reports
351 that, when the DMI2/SYMRK kinase domain or full length protein is overexpressed in
352 legume roots, plants will generate spontaneous nodules without rhizobia infection (Ried et al.,
353 2014; Saha et al., 2014).

354

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

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

365

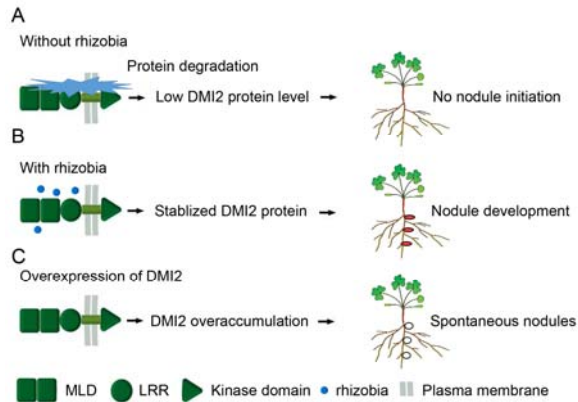
366 Although the mechanisms coupling ligand recognition in the extracellular parts and the
367 activation of intracellular catalytic domains are surprisingly diverse (Lemmon and
368 Schlessinger, 2010), there are reports that ligand binding induces the protein level of RLKs.
369 For instance, it is widely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

378

379 **2. DMI2 protein level is at the center of regulating nodule development and rhizobial** 380 **initiation**

381

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



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

395

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

406

407 Since DMI2 is also required for the symbiosis between plants and arbuscular mycorrhizal
 408 fungi and *Frankia* bacteria, it will be interesting to find out whether the protein level of DMI2
 409 is induced by AM treatment. We would predict that this is the case because we found that the
 410 induction of DMI2 protein by rhizobia is independent of Nod factor perception (Figure 1C).
 411 Furthermore, any differences in DMI2 protein dynamics between rhizobia and AM infection
 412 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

440 **Methods**

441

442 **Plant materials and growth conditions**

443

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

450

451 *Sinorhizobium meliloti* strain ABS7 *hemA:LacZ* was used for rhizobia inoculation. Fresh
452 overnight rhizobia culture was centrifuged and suspended in 1/2 BNM liquid medium to a
453 concentration of OD₆₀₀= 0.05. Five mL liquid rhizobia culture was used per *Medicago* plant.
454 Nodulation and infection thread phenotypes were checked 14 days and 7 days post
455 inoculation respectively; for protein sample collection, tissues were collected at different time
456 points as indicated in the figure legends.

457

458 **Evaluation of symbiotic phenotypes**

459

460 To analyze nodule development phenotype, 14 days after ABS7 *HemA::LacZ* inoculation,
461 plants were harvested and total and pink nodules were counted. For infection thread analysis,
462 7 days post ABS7 *hemA:LacZ* inoculation, *Medicago* roots were stained for β-galactosidase
463 activity as previously described (Pan et al., 2016). Root samples were filled with staining
464 buffer (0.5 M sodium phosphate buffer, pH 7.2, 10% Triton X-100, 100 mM potassium
465 ferrocyanide, 100 mM potassium ferricyanide) containing 2 mM X-Gluc, then incubated at
466 37°C overnight. Infection threads were checked and the pictures were taken using a Nikon
467 E200 microscope. Number of infection threads was counted on at least 10 individual
468 transformed plants for each construct.

469

470 **Plasmids and vectors**

471

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

480

481 **Protein extraction and immunoblot**

482

483 To extract proteins from un-inoculated roots, inoculated roots and nodules of *Medicago* plants,
484 plant tissues were collected at the time points indicated in the figure legends and put into
485 liquid nitrogen immediately. After grinding the tissues into a fine powder, proteins were
486 extracted using Native Extraction Buffer 1 (50 mM Tris-MES, pH 8.0; 0.5 M sucrose; 1 mM
487 MgCl₂; 10 mM EDTA; 5 mM DTT.) with or without the protease inhibitor cocktail
488 (Sigma-Aldrich) (Liu et al., 2010). Extracted proteins were boiled for 5 minutes with 6 × SDS
489 sampling buffer and stored at -20°C.

490

491 For immunoblots, various protein samples were loaded into a 12% acrylamide SDS running
492 gel and the protein samples were transferred from the gel to nitrocellulose membrane
493 (Adventec) by electro-blotting. After blocking the membrane with milk, the membrane was
494 subjected to incubation with 1:500 diluted anti-HA antibody (New England Biolabs) and
495 1:4000 diluted anti-alpha tubulin antibody (Life Sciences). The secondary antibody was
496 anti-rat and anti-mouse respectively (Life Sciences). As the secondary antibodies were
497 conjugated with horseradish peroxidase, the membrane was treated with the detection reagent
498 from Thermo Scientific (products #1859707 and #1859678). The bands on the membrane
499 were visualized via a G-box machine (New England Biogroup).

500

501 **Cell free degradation assay and MG132 treatment**

502

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

509

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

513

514 **RNA extraction and RT-qPCR**

515

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

522

523 RT-qPCR was performed on the Eppendorf Mastercycler ep Realplex system. The PCR
524 reaction was conducted using ExTaq polymerase (Takara) with SYBR Green dye (Thermo
525 Fisher Scientific), in 20 μ L total volume: ddH₂O, 9.9 μ L; 10 \times Extaq buffer, 2 μ L; 2.5
526 mM dNTP mix, 2 μ L; 10 μ M primer mix, 2 μ L; SYBR Green dye, 2 μ L; template
527 cDNA, 2 μ L; ExTaq polymerase, 0.1 μ L. For the real-time PCR reaction, annealing
528 temperature was set at 60°C and elongation time was 30 seconds. When the PCR was
529 finished, the melting curve was analyzed to rule out possible non-specific amplification, and

530 the result represented was the mean from threshold cycle value (C_T) of three technical
531 replicates. Three biological replicates were used for each sample. *PROTODERMAL FACTOR*
532 2 (*PDF2*), locus ID Medtr6g084690, was used as the internal control.

533

534 **Protein domain analysis and sequence alignments**

535

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

542

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

548

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

551

552 **Accession numbers:** Sequence data from this article can be found in Supplemental Table 1.

553 The GenBank accession numbers for the genes used to build phylogenetic tree are presented.

554

555 **Supplemental Data:**

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

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

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

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

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

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

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

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

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

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

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

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

575 **Supplemental Table 1.** Accession numbers.

576

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578

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586

587

588

589 **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 |

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

597

598 **Figure Legends**

599 **Figure 1.** Rhizobia treatment could induce the protein level of DMI2 in a Nod factor receptor
600 independent manner. A, DMI2-HAST protein accumulated in the nodules comparing with
601 un-inoculated root. Protein samples were taken from 14-day old nodules and 4-week old
602 un-inoculated roots. B, DMI2-HAST protein was induced to much higher level 24 hours after
603 rhizobia treatment. 4-week old Medicago roots were treated with *S. meliloti* strain ABS7
604 *hemA:LacZ* at the concentration of OD₆₀₀=0.05 for 24 hours. C and D, DMI2-HAST protein was
605 induced to a similar level by rhizobia treatment in wild type and Nod factor receptor mutant
606 backgrounds. *gDMI2-HAST* construct was transiently expressed in *dmi2-1*, *hcl-1*, *hcl-4*,
607 transformed roots were treated with or without *S. meliloti* strain ABS7 *hemA:LacZ*. E,
608 DMI2-HAST protein could be induced to a similar level by rhizobia in *dmi2-1* and *sun-1* plants
609 stably transformed with *gDMI2-HAST*. α -HA was used to detect the DMI2-HAST protein level,
610 Tubulin was used as the loading control. The experiments were repeated 5 times with similar
611 results.

612

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

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

627

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

644

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

654

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