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3 **Running title:**

4 MtMOT1.2 delivers Mo to nodules

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34 **MtMOT1.2 is responsible for molybdate supply to *Medicago truncatula***
35 **nodules**

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48 **One Sentence summary:** MtMOT1.2 mediates molybdate transfer from the vasculature
49 to nitrogen-fixing nodules

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51 **List of author contributions:** P.G-D. carried out most of the experiments. Yeast
52 transport assays were performed by M.T-J. J. L-M. studied the complementation with
53 molybdate of the *mot1.2-1* mutant. J.W. and K.S.M performed *M. truncatula* mutant
54 screening and isolated the *mot1.2-1* allele. M.T-J, J.I., and M.G-G. designed the
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67 **ABSTRACT**

68 Symbiotic nitrogen fixation in legume root nodules requires a steady supply of
69 molybdenum for synthesis of the iron-molybdenum cofactor of nitrogenase. This nutrient
70 has to be provided by the host plant from the soil, crossing several symplastically
71 disconnected compartments through molybdate transporters, including members of the
72 MOT1 family. MtMOT1.2 is a *Medicago truncatula* MOT1 family member located in
73 the endodermal cells in roots and nodules. Immunolocalization of a tagged MtMOT1.2
74 indicates that it is associated to the plasma membrane and to intracellular membrane
75 systems, where it would be transporting molybdate towards the cytosol, as indicated in
76 yeast transport assays. A loss-of-function *mot1.2-1* mutant showed reduced growth
77 compared to wild-type plants when nitrogen fixation was required, but not when nitrogen
78 was provided as nitrate. While no effect on molybdenum-dependent nitrate reductase
79 activity was observed, nitrogenase activity was severely affected, explaining the observed
80 difference of growth depending on nitrogen source. This phenotype was the result of
81 molybdate not reaching the nitrogen-fixing nodules, since genetic complementation with
82 a wild-type *MtMOT1.2* gene or molybdate-fortification of the nutrient solution, both
83 restored wild-type levels of growth and nitrogenase activity. These results support a
84 model in which MtMOT1.2 would mediate molybdate delivery by the vasculature into
85 the nodules.

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101 INTRODUCTION

102 Symbiotic nitrogen fixation carried out by the legume-rhizobia partnership is one
103 of the main sources of assimilable nitrogen in natural ecosystems and in sustainable
104 agriculture (Downie, 2014). The symbiosis is established in differentiated root organs,
105 the nodules, developed after a complex chemical exchange between the symbionts
106 (Oldroyd, 2013). Nodule inner cells are infected by rhizobia in an endocytic-like process
107 that results in organelle-like structures, the symbiosomes (Vasse, 1990; Limpens et al.,
108 2009). There, surrounded by a plasmalemma-derived membrane, the symbiosome
109 membrane, rhizobia will differentiate into nitrogen-fixing bacteroids (Roth, 1989, Vasse,
110 1990). As a result, dedicated membrane transporters would be required to transfer the
111 fixed nitrogen to the host plant, while the bacteroid receives photosynthates and mineral
112 nutrients (Udvardi and Poole, 2013).

113 Transition metal nutrients, such as iron (Fe), copper (Cu), zinc (Zn), or
114 molybdenum (Mo), are required in relatively large amounts for symbiotic nitrogen
115 fixation (O'Hara, 2001; Brear et al., 2013; González-Guerrero et al., 2014). While Fe,
116 Cu, and Zn are employed as cofactors of multiple enzymes present in the nodule (Brear
117 et al., 2013; González-Guerrero et al., 2014), Mo is required by just two when nitrogen
118 fixation is active. One of them is a xanthine dehydrogenase that might be required for
119 nitrogen delivery out of the nodules (Kaiser et al., 2005); while the other is nitrogenase,
120 the protein complex directly responsible for converting N_2 into NH_4^+ by the bacteroids.
121 In this enzyme, Mo is a key element of its unique Fe-Mo cofactor (FeMoco) (Rubio and
122 Ludden, 2005). Consequently, Mo uptake and delivery to the nodules is an essential
123 process for legumes and for symbiotic nitrogen fixation.

124 Mo is present in soil as molybdate, a close structural analogue to sulfate (Stiefel,
125 2002). In fact, for many years it was believed that molybdate was mainly transported by
126 sulfate carriers, since, under the right conditions, they can also carry molybdate across
127 membranes (Stout et al., 1951; Mendel and Hansch, 2002; Kaiser et al., 2005). However,
128 more recently, molybdate-specific transporters have been identified (Tejada-Jiménez et
129 al., 2007; Tomatsu et al., 2007; Tejada-Jiménez et al., 2011). Among them, the better
130 known is the MOT1 family of transporters (Tejada-Jimenez et al., 2007; Tomatsu et al.,
131 2007; Baxter et al., 2008). These proteins are evolutionarily related to sulfate transporters
132 of the SULTR family (Tejada-Jimenez et al., 2007). However, the conserved STAS
133 domain common to the latter proteins is not present in the MOT1 family members, which
134 are characterized by the domains: P-PVQPMKXIXA-A and FG-MP-CHGAGGLA-QY-

135 FGGR-G (Tejada-Jimenez et al., 2007). In Arabidopsis, two *MOT1* genes have been
136 found: *AtMOT1*, involved in Mo uptake (Tomatsu et al., 2007; Baxter et al., 2008); and
137 *AtMOT2*, associated to the vacuole, and playing a role in inter-organ molybdate allocation
138 (Gasber et al., 2011).

139 On an average, legume genomes encode more copies of MOT1 gene family
140 members than other dicots. This could be the result of evolutionary pressures to expand
141 this family to account for the increased demand of Mo in symbiotic nitrogen fixation
142 (O'Hara et al., 2001). For instance, *Glycine max* has seven members; *Phaseolus vulgaris*,
143 four; and *Medicago truncatula* has five (MtMOT1.1 to MtMOT1.5). Two legume MOT1
144 proteins have been characterized to date (Gao et al., 2016; Duan et al., 2017; Tejada-
145 Jimenez et al., 2017). LjMOT1 would be responsible for molybdate uptake from soil and
146 its distribution to plant sink organs (Gao et al., 2016; Duan et al., 2017), in a role similar
147 to that played by *AtMOT1* (Tomatsu et al., 2007). This is indicated by the expression of
148 this transporter in the epidermis and in the root vasculature, as well as by the reduction
149 of Mo content in nodules and leaves in mutant plants (Duan et al., 2017). However,
150 LjMOT1 would not primarily be involved in molybdate delivery to the nodules, since
151 lack of this transporter has no significant effect on nitrogen fixation capabilities. In
152 contrast, mutation of the other characterized legume MOT1 protein, nodule-specific
153 MtMOT1.3, results in nearly a total loss of nitrogenase activity (Tejada-Jiménez et al.,
154 2017). Immunolocalization of this transporter in the plasma membrane and
155 characterization of its transport capabilities indicate that its main function would be
156 introducing molybdate into nodule cells. However, *mot1.3-1* mutant nodules still
157 accumulate more Mo than wild-type ones, suggesting that the delivery of this metal to the
158 nodules is not impaired by *MtMOT1.3* mutation (Tejada-Jiménez et al., 2017).
159 Consequently, we still need to identify the transporters responsible for molybdate release
160 from the vasculature into the nodules. This function would require two types of
161 transporters: a MOT1-like for uptake from the vessels, and a yet-to-be-defined molybdate
162 efflux protein (perhaps a sulfate transporter) to extrude Mo into the nodule apoplast.

163 Among the four remaining MOT1 transporters in *M. truncatula*, MtMOT1.1 is the
164 most closely related to LjMOT1, and would likely play a similar role (Tejada-Jiménez et
165 al., 2017). *MtMOT1.4* and *MtMOT1.5* are expressed all over the plant, while *MtMOT1.2*
166 is the only one of these four genes that is expressed exclusively in roots and nodules, with
167 a maximum of expression in the latter (Tejada-Jimenez et al., 2017). Here, we
168 characterize the function of MtMOT1.2 as a likely candidate for molybdate uptake from

169 the vasculature by endodermal cells. MtMOT1.2 is located in the endodermis of nodule
170 and root vascular cylinders, in the plasma membrane and in an endomembrane
171 compartment. It shows molybdate uptake capabilities in yeast, and its mutation in *M.*
172 *truncatula* leads to a reduction in nitrogenase activity in nodules, likely the result of the
173 reduction in molybdate delivery to the nodules. Its function seems to be relevant for
174 symbiotic nitrogen fixation, with no major role being played under non-symbiotic
175 conditions, given how its mutation has no effect on plants grown on nitrate or on nitrate
176 reductase activity. This work represents a further step towards understanding how
177 molybdate in particular, and transition metals, in general, are delivered to legume nodules,
178 and represents the first case in which a metal transporter has been associated with root-
179 to-nodule vascular metal delivery.

180

181 **RESULTS**

182

183 **MtMOT1.2 is a molybdate transporter**

184 It has been reported that proteins belonging to MOT1 family are involved in the
185 transport of the oxyanion molybdate into the cytosol of cells (Tejada-Jimenez et al.,
186 2013). Members of MOT1 family showed a high sequence similarity to other molybdate
187 transporters, including the signature motives of MOT1 family (Tejada-Jimenez et al.,
188 2007). To confirm that MtMOT1.2 was able to transport molybdate, a yeast expression
189 system was used, since these *Saccharomyces cerevisiae* is among the rare organisms
190 lacking Mo-containing proteins and having no Mo transporters (Mendel and Bittner,
191 2006). When grown in the presence of molybdate, yeast expressing *MtMOT1.2* were able
192 to accumulate Mo following a Michaelian kinetics (Fig. 1A), with a V_{max} of 155 ± 12
193 pmol 10^{-6} cells h^{-1} and a $k_{1/2}$ of 488 ± 105 nM. This transport was not inhibited by the
194 structural analogue sulfate, even at concentrations up to 4,000 times higher (Fig. 1B).

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196 **MtMOT1.2 is located in the plasma membrane and intracellular compartments in** 197 **the endodermal/pericicle layer in roots and nodules**

198 According to Symbimics database (<https://iant.toulouse.inra.fr/>) (Roux et al.,
199 2014) and previously reported data (Tejada-Jiménez, 2017), *MtMOT1.2* is expressed in
200 nodules and roots of *M. truncatula*. In order to identify the tissues in which its expression
201 peaks, 1,446 bp upstream of the start codon of *MtMOT1.2* were fused to a β -

202 glucuronidase (*gus*) gene. *M. truncatula* plants were transformed with this genetic
203 construct and GUS activity was visualized at 28 days-post-inoculation (dpi). The results
204 confirmed that *MtMOT1.2* was expressed in nodules and roots (Fig. 2A). Root sections
205 showed that most of the GUS activity was confined to the endodermal layer around the
206 root vessels (Fig. 2B). Similarly, *MtMOT1.2* expression in the nodules was located
207 around the nodule vasculature (Fig. 2C), and no expression was observed in the inner
208 nodule regions, even when they were clarified with bleach (Supplemental Fig. S1).

209

210 Immunolocalization of MtMOT1.2 was performed by fusing the full genomic
211 region (comprising from 1,446 bp upstream of the start codon to the last codon before the
212 stop) to three hemagglutinin (HA) epitopes. Localization of the MtMOT1.2-HA protein
213 was carried out with a mouse anti-HA primary antibody and a secondary anti-mouse
214 Alexa594-conjugated antibody. The plants were inoculated with a *S. meliloti* strain
215 constitutively expressing green fluorescent protein (GFP), and DNA was stained blue
216 with 4'-6-diamino-phenylindole (DAPI). The result of this staining showed that
217 MtMOT1.2-HA was located in a cell layer around the root and nodule vessels (Fig. 3A
218 and B), thus validating the *gus*-reporter assays. The detection of autofluorescence bands
219 corresponding to the Casparian strip suggests that these cells form the endodermis of the
220 nodule vessels (Supplementary Fig. S2). The Alexa594 signal was observed in two
221 locations within a cell: in the periphery of the cells and in a perinuclear region (Fig. 3A).
222 In the root, MtMOT1.2-HA had a similar cellular distribution (Fig. 3B). This pattern of
223 detection was not the result of autofluorescence detected in the Alexa594 emission
224 channel, since negative controls with exactly the same conditions did not show any signal
225 in this emission range (Supplemental Fig. S3).

226 To obtain further detail on the subcellular distribution of MtMOT1.2, *Nicotiana*
227 *benthamiana* leaves were co-agroinfiltrated with a C-terminal GFP-tagged MtMOT1.2
228 and the plasma membrane-marker AtPIP2 labelled with cyan fluorescent protein (CFP).
229 Figure 3C shows that both signals co-localize, indicating that in *N. benthamiana*
230 MtMOT1.2 is located in the plasma membrane. Again, these signals were not due to
231 autofluorescence, since neither GFP nor CFP were detected in leaves expressing just
232 AtPIP2-CFP, or MtMOT1.2-GFP, respectively (Supplemental Fig. S4). MtMOT1.2-HA
233 localization was also determined with transmission electron microscopy and a gold-
234 conjugated secondary antibody (Fig. 3D). In these sections, the epitope was detected in
235 the plasma membrane and in an intracellular membrane compartment, likely the

236 endoplasmic reticulum. No gold particles were detected in control nodules (Supplemental
237 Fig. S5).

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239 **MtMOT1.2 is required for molybdate delivery to nitrogen fixing root nodules**

240 A *M. truncatula* Transposable Element from *N. tabacum* (*Tnt1*) insertion lines
241 (Tadege et al., 2008) were used for a reverse genetics screening (Cheng et al., 2011;
242 Cheng et al., 2014) to identify a mutant line (NF9961, *mot1.2-1*) carrying *Tnt1* in
243 *MtMOT1.2* to determine the physiological role of MtMOT1.2. *Tnt-1* insertion in the
244 mutant *mot1.2-1* is located in the second exon of the gene, 1,576 bp downstream of the
245 start codon (Fig 4A). No *MtMOT1.2* transcript was detected by RT-PCR in either roots
246 or nodules of *mot1.2-1* (Fig. 4B). Since *MtMOT1.2* was expressed in roots of plants
247 inoculated and non-inoculated with *S. meliloti*, *mot1.2-1* phenotype was assessed under
248 both conditions.

249 When the plants received an assimilable form of nitrogen (KNO₃) in their nutrient
250 solution, and no *S. meliloti* inoculum was added, no significant differences in growth (Fig.
251 4C) or in biomass production (Fig. 4D) were observed between wild-type and mutant
252 plants. Since nitrate was the sole nitrogen source for these plants, they would require the
253 activity of the Mo-containing enzyme nitrate reductase to grow (Bernard and Habash,
254 2009), and any defect on Mo uptake or source-to-sink delivery in these plants would lead
255 to a reduction of Mo-dependent enzymatic activities. However, nitrate reductase activity
256 in *mot1.2-1* plants watered with KNO₃ was equivalent to that of wild-type plants (Fig.
257 4E). No significant change was observed even when no molybdate was added to the
258 nutrient solution (Supplemental Fig. S6).

259 In contrast, under symbiotic conditions, when the plant depends on symbiotic
260 nitrogen fixation as the sole source of nitrogen, *mot1.2-1* plants showed reduced growth
261 when compared to the controls (Fig. 5A), with smaller nodules (Fig. 5B) and a biomass
262 reduction of 54% and 38% in shoot and root, respectively (Fig. 5C). The reduction of
263 growth and nodule size did not seem to be the consequence of alterations on nodule
264 development, or defects in nodulation kinetics (neither the number of nodules per plant,
265 nor the rate of nodulation were affected; Supplemental Fig. S7). Growth reduction in
266 *mot1.2-1* plants is the likely result of a reduction of nitrogenase activity in those nodules,
267 which was only 12% of that in wild-type plants (Fig. 5D). Mutant nodules exhibited a
268 significant reduction in Mo content, while no significant changes were observed in roots
269 (Fig. 5E). This phenotype was the result of the mutation of *MtMOT1.2*, since transforming

270 *mot1.2-1* with *MtMOT1.2-HA* improved growth and restored wild-type levels of
271 nitrogenase activity (Fig. 5). Similarly, increasing molybdate content in the nutrient
272 solution restored wild-type growth and nitrogenase activity in *mot1.2-1* nodules
273 (Supplemental Figure S8).

274

275 **DISCUSSION**

276 Mo is an essential oligonutrient for plants. As part of the Mo cofactor (Moco), it
277 is used by five different proteins: i) Nitrate reductase (NR), for the reduction of nitrate to
278 nitrite, a key step in the inorganic nitrogen assimilation process; ii) sulfite oxidase (SO),
279 which oxidizes sulfite to sulfate producing hydrogen peroxide and thus has a role in ROS
280 production; iii) aldehyde oxidase (AO), which is related with the production of abscisic
281 acid and auxin; iv) xanthine dehydrogenase (XDH), which catalyses hydroxylation of
282 aldehydes and aromatic heterocycles in the purine degradation metabolic pathway; and
283 v) the amidoxime reducing component (ARC), which catalyse the reduction of N-
284 hydroxylated products (Mendel and Bittner, 2006; Hille et al., 2001). Consequently,
285 plants need a regular supply of this nutrient from soil to sink organs. Mo requirements by
286 legumes are substantially higher than those of other dicots (Clark, 1984; Tisdale et al.,
287 1985), as a result of the synthesis of large quantities of nitrogenase by rhizobia in their
288 root nodules (Miller et al., 1993). These bacteria use Mo to synthesize FeMoco required
289 for nitrogenase activity (Rubio and Ludden, 2008). While much is known on how Moco
290 and FeMoco are synthesized (Rubio and Ludden, 2008; Mendel, 2013) much less is
291 known on how Mo is ferried to the enzymes synthesizing each cofactor. A major
292 breakthrough has been the identification of molybdate-specific transporters (Tejada-
293 Jimenez et al., 2013), such as those of the MOT1 family (Tejada-Jimenez et al., 2007;
294 Tomatsu et al., 2007) and the description of the role of these proteins in molybdate uptake
295 from soil in *Arabidopsis* and in *L. japonicus* (Tomatsu et al., 2007; Baxter et al., 2008;
296 Duan et al., 2017). More recently, a MOT1 protein has been reported as responsible for
297 molybdate uptake by sink organ cells in *M. truncatula* nodules, participating in symbiotic
298 nitrogen fixation (Tejada-Jimenez et al., 2017). However, how Mo reaches this sink organ
299 still remains obscure.

300 *MtMOT1.2* is a *M. truncatula* MOT1 family member that is expressed in roots and
301 nodules (Tejada-Jimenez et al., 2017). All MOT1 members identified so far have shown
302 Mo transport activity (Tejada-Jiménez et al., 2007; Tomatsu et al., 2007; Baxter et al.,
303 2008; Gasber et al., 2011; Duan et al., 2017; Tejada-Jiménez et al., 2017). Yeast transport

304 assays confirm that MtMOT1.2 is able to transport molybdate, showing kinetic
305 parameters comparable to those of previously characterized MtMOT1.3 and LjMOT1
306 transporters (Duan et al., 2017; Tejada-Jimenez et al., 2017), with lower affinity and
307 higher speed than MOT1 proteins from *Chlamydomonas reinhardtii* and *A. thaliana*
308 (Tejada-Jimenez et al., 2007; Tomatsu et al., 2007). This difference could be due to a
309 higher local concentration of molybdate in nodules corresponding to the increased Mo
310 demand of these organs, for which a low-affinity system would be enough, but that would
311 need to work at higher rates. In spite of its relatively low molybdate affinity, MtMOT1.2
312 is a transporter specific for this anion, since the addition of up to a 4,000-fold excess of
313 the structurally similar anion sulfate did not inhibit Mo transport.

314 Within roots and nodules *MtMOT1.2* expression was detected around the vessels,
315 as indicated by promoter::*gus* fusions and immunolocalization of a HA-tagged version of
316 the protein. More specifically, the tagged protein could be detected in endodermal cells
317 in both nodule and root vessels. As it was the case for *A. thaliana* MOT1 (Tomatsu et al.,
318 2007), MtMOT1.2-HA was observed in the plasma membrane and in an endodermal
319 compartment resembling the endoplasmic reticulum. This could indicate a role in
320 introducing molybdate into the cytosol, either from the cell exterior or from intracellular
321 reserves. Alternatively, the endoplasmic reticulum subpopulation of MtMOT1.2 could
322 also correspond to newly synthesized protein being ferried towards the plasma
323 membrane. Surprisingly for a vascular transporter, no polar localization in the cell was
324 observed. Molybdate could be introduced into the cell from the apoplast or from the
325 vessels. As a result, in the absence of another driving force, it would result in a futile
326 cycle in which no net transfer of molybdate from sink to source would occur. Since this
327 is not what happens, molybdate is being delivered from root to nodules, it might be
328 speculated that molybdate delivery could be driven by a net mass-effect in which the
329 molybdate pulled from the nitrogen-fixing cells, with their high molybdate uptake
330 capability for FeMoco synthesis, would prevent a backward flux of Mo. The net transport
331 into rhizobia-infected cells would have to be driven by transforming molybdate into
332 different chemical species, rather than a substrate MOT1 proteins. Such a system would
333 also ensure that should Mo not be used and accumulated in a given compartment, it would
334 be rapidly recycled back for use elsewhere.

335 The localization of MtMOT1.2 in the vasculature and its function in molybdate
336 uptake into the cell is suggestive of a role in the sink-to-source transport of this
337 oligonutrient. Its position in the root endodermis indicates that it would be facilitating the

338 transfer of apoplastic molybdate to the vasculature, so that molybdate would then be
339 transferred to leaves or nodules. However, our data indicate that MtMOT1.2 does not play
340 an essential role in molybdate transport to the leaves, since mutant plants in this gene did
341 not have any significant growth alteration compared to wild-type plant, and, more
342 importantly, Mo-dependent nitrate reductase activity was not affected in *mot1.2-1* when
343 nitrate was the sole nitrogen source to these plants. In contrast, when plants relied on
344 symbiotic nitrogen fixation for assimilable nitrogen, *mot1.2-1* plants showed a severe
345 growth defect. This difference in growth between the two different nutritional situations
346 could be the result of two non-incompatible possibilities: i) MtMOT1.2 is functionally
347 substituted by another molybdate transporter in roots when mutated, and ii) MtMOT1.2
348 is only essential for molybdate release to the nodule. The endodermal localization in
349 nodule vessels and the predicted direction of transport is indicative of a role in introducing
350 the molybdate delivered by the vessels into endodermal cells. This would be the first step
351 towards transferring molybdate to the nodule apoplast for uptake by MtMOT1.3. The
352 plant growth defect observed in nitrogen-fixing conditions arises from the reduction of
353 nitrogenase activity in these plants, consequence of insufficient molybdate reaching the
354 nitrogen-fixing cells, as indicated by the lower levels of Mo in the *mot1.2-1* nodules and
355 the restoration of the wild-type phenotype when more Mo was added to the nutrient
356 solution. The pattern of Mo accumulation in *mot1.2-1* plants compared to their controls,
357 with no significant changes in roots and a decrease in nodules, indicates that the defect in
358 molybdate delivery for nitrogen fixation is occurring at the level of nodule vessels and
359 not in loading the root vasculature with Mo. Otherwise, an accumulation of Mo in *mot1.2*
360 roots would be expected as well as a decrease in shoots, and none was detected in either
361 (in this case, even slightly higher levels were detected).

362 In summary, MtMOT1.2 would position itself between molybdate root uptake
363 transporter, likely MtMOT1.1 as the closest LjMOT1 orthologue, and the nodule apoplast
364 molybdate uptake protein MtMOT1.3 (Fig. 6). MtMOT1.2 would facilitate the transfer
365 of this oligonutrient into endodermal cells mediating the sink-to-source molybdate
366 trafficking, which would be controlled by mass-effects to ensure that it reaches its
367 destination. However, a critical point remains to be solved, which is the identity of the
368 proteins mediating molybdate efflux from the cytosol to the symbiosome. Whether these
369 are sulfate transporters, or whether a novel family of Mo transporters with a direction of
370 transport opposite to MOT1 proteins, remains to be unveiled.

371

372 **METHODS**

373 **Biological material and growth conditions**

374 *M. truncatula* R108 seeds were scarified by incubating with concentrated sulfuric
375 acid for 7 min. After several washes with cold water, the seed surfaces were sterilized in
376 50 % (v/v) bleach for 90 s, and left in sterile water in the dark overnight, followed by a
377 48 h incubation at 4 °C. Seed germination was done in water-agar plates 0.8 % (w/v).
378 Seedlings were planted in sterile perlite pots, and inoculated with *Sinorhizobium meliloti*
379 2011 or the same bacterial strain transformed with pHc60 (Cheng and Walker, 1998).
380 Plants were grown in a greenhouse with 16 h of light and 22 °C, and watered every two
381 days with Jenner's solution or water alternatively (Brito et al., 1994). Nodule collection
382 was carried out at 28 dpi. Non-nodulated plants were supplemented every two weeks with
383 2 mM KNO₃, instead of being inoculated with *S. meliloti* 2011. For hairy root
384 transformation of *M. truncatula* seedlings, *Agrobacterium rhizogenes* strain ARqual
385 having the appropriate vector was used (Boisson-Dernier et al., 2001). Agroinfiltration
386 experiments for transitory expression were done in *N. benthamiana* leaves using *A.*
387 *tumefaciens* C58C1 as a vector for the corresponding genetic construct. *N. benthamiana*
388 plants were grown in the greenhouse under the same conditions as *M. truncatula*.

389 In heterologous expression assays the yeast *S. cerevisiae* strain 31019b (MATa
390 *ura3 mep1Δ mep2Δ::LEU2 mep3Δ::KanMX2*) was used (Marini et al., 1997). Yeasts
391 were grown in synthetic dextrose (SD) or yeast peptone dextrose (YPD) medium
392 supplemented with 2 % glucose (Sherman et al., 1986).

393

394 **Molybdate uptake**

395 *S. cerevisiae* cells grown in SD medium were transferred to 10 mM MES-Ca(OH)₂
396 buffer (pH 5.8) containing 0.1 mM MgCl₂, 2 mM CaCl₂ and 0.5 % glucose. Molybdate
397 uptake was measured after 30 min incubation at 28 °C in the presence of 500 nM
398 Na₂MoO₄. For molybdate transport kinetics yeast cells were transferred to the same MES
399 buffer supplemented with 100, 200, 500, 1000 and 2000 nM Na₂MoO₄ and incubated for
400 30 min at 28 °C. Molybdenum determination was carried out in 10 mL cell-free MES
401 buffer using the method previously described (Cardenas and Mortenson, 1975)

402

403 **GUS Staining**

404 A transcriptional fusion between *MtMOT1.2* promoter region and the *gus* gene
405 was obtained by amplifying 1.4 kb upstream of the *MtMOT1.2* start codon using the

406 primers 5MtMOT1.2-1446GW and 3MtMOT1.2pGW (Supplemental Table S1). This
407 amplicon was inserted into pGWB3 (Nakagawa et al., 2007) using the Gateway cloning
408 technology (Invitrogen). Roots were transformed as indicated above. Visualization of
409 GUS activity was done in 28 dpi plants as described (Vernoud et al., 1999). Clearing of
410 nodule sections was carried out with a 50 % bleach treatment for 30 min.

411

412 **Immunohistochemistry and confocal microscopy**

413 Plasmid pGWB13 (Nakagawa et al., 2007) was used to clone a DNA fragment
414 containing *MtMOT1.2* full gene and 1,446 kb upstream of its start codon using Gateway
415 cloning technology (Invitrogen), adding three C-terminal hematoagglutinin (HA)
416 epitopes in frame to MtMOT1.2. *M. truncatula* root transformation was carried out as
417 indicated above. Transformed plants were inoculated with *S. meliloti* 2011 containing
418 plasmid pHC60 that constitutively expresses GFP. Nodules and roots, collected at 28 dpi,
419 were fixed in 4 % (w/v) paraformaldehyde and 2.5 % (w/v) sucrose in phosphate-buffered
420 saline (PBS) at 4 °C and left overnight. Fixed plant material was sectioned with a
421 Vibratome 1000 Plus (Vibratome), in 100 µm sections. Dehydration of the sections was
422 performed by incubation with methanol dilution series (30 %, 50 %, 70 % and 100 % in
423 PBS) for 5 min and then rehydrated following the same methanol series in reverse order.
424 Cell wall permeabilization was done by incubating with 2 % (w/v) cellulase in PBS for 1
425 h, and 0.1 % (v/v) Tween 20 in PBS for 15 min. Bovine serum albumin 5% (w/v) was
426 used to block the sections. As primary antibody, a 1:50 dilution in PBS of anti-HA mouse
427 monoclonal antibody (Sigma) was used. This dilution was incubated with the sections for
428 2 h at room temperature and then washed away three times with PBS for 10 min.
429 Secondary antibody used was 1:40 Alexa594-conjugated anti-mouse rabbit monoclonal
430 antibody (Sigma) in PBS. The incubation was performed at room temperature for 1 h and
431 then sections were washed three times with PBS for 10 min. DNA was stained using
432 DAPI. Images were obtained with a confocal laser-scanning microscope (Leica SP8).

433

434 **Gold-immunohistochemistry and electron microscopy**

435 Plants were transformed with plasmid pGWB13 containing *MtMOT1.2* full gene
436 and 1,446 bp upstream of its start codon. Transformed plants were inoculated with *S.*
437 *meliloti* 2011. Nodules were collected at 28 dpi and were fixed in 1 % formaldehyde and
438 0.5 % glutaraldehyde in 50 mM potassium phosphate (pH 7.4) for 2 h. After that the
439 fixation solution was renewed for 1.5 h. Samples were washed in 0.05 M potassium

440 phosphate (pH 7.4) 3 times during 30 min and 3 times for 10 min. Nodules were
441 dehydrated by incubation with ethanol dilution series of 30 %, 50 %, 70 %, 90 % during
442 10 min, 96 % for 30 min and 100 % during 1 h. Samples were included in a series of
443 ethanol and LR–white resin (London Resin Company Ltd, UK) dilutions: 1:3 during 3 h,
444 1:1 were left overnight and 3:1 during 3 h. Nodules were included in resin during 48 h.
445 All the process was performed at 4 °C. Nodules were placed in gelatine capsules and filled
446 with resin and polymerized at 60 °C for 24 h. One-micron thin sections were cut at Centro
447 Nacional de Microscopia Electrónica (Spain) with Reichert Ultracut S-ultramicrotome
448 fitted with a diamond knife. Thin sections were blocked in 2 % bovine serum albumin in
449 phosphate buffer saline (PBS) for 30 min. As primary antibody, a 1:20 dilution in PBS of
450 anti-HA rabbit monoclonal antibody (Sigma) was used. Samples were washed 10 times
451 in PBS for 2 min. Secondary antibody used was 1:150 anti-rabbit goat conjugated to a 15
452 nm gold particle (BBI solutions) diluted in PBS. Incubation was performed for 1 h, after
453 that samples were washed 10 times in PBS for 2 min and 15 times in water for 2 min.
454 Sections were stained with 2 % uranyl acetate and visualised in a JEM 1400 electron
455 microscope at 80 kV.

456

457 **Transient expression in *Nicotiana benthamiana* leaves**

458 Experiment was performed as is described by Wood et al (2009). GFP was fused
459 to the C terminus of *MtMOT1.2* coding sequence by cloning it into pGWB5 (Nakagawa
460 et al., 2007) by Gateway cloning technology (Invitrogen). Four-week-old *N. benthamiana*
461 leaves were injected with *A. tumefaciens* C58C1 (Deblaere et al., 1985) cells
462 independently transformed with MtMOT1.2-GFP, with the plasma membrane marker
463 pm-CFP pBIN (Nelson et al., 2007) or with the silencing suppressor p19 of *Tomato bushy*
464 *stunt virus* (Wood et al., 2009). Expression in the leaves was analyzed after 3 d by
465 confocal laser-scanning microscopy (Leica SP8).

466

467 **Nitrogenase activity**

468 Nitrogenase activity was measured by the acetylene reduction assay (Hardy et al.,
469 1968). 28 dpi wild-type and mutant plants were introduced in 30 ml tubes and sealed with
470 rubber stoppers. Each tube contained at least four independently transformed plants. 10
471 % of the gas phase from each bottle was replaced by the same volume of acetylene. Tubes
472 were incubated for 30 min at room temperature. Ethylene production was measured by

473 analyzing 0.5 ml gas samples with a Shimadzu GC-8A gas chromatograph using a
474 Porapak N column (Shimadzu, Kyoto, Japan).

475

476 **Metal content determination**

477 Metal content was determined by inductively coupled plasma optical emission
478 spectrometry in three sets of 28 dpi roots, shoots, and nodules, each set originating from
479 a pool of five plants. The experiment was carried out at the Unit of Metal Analysis in the
480 Scientific and Technology Centers of the Universidad de Barcelona (Spain). These
481 samples were digested with HNO₃, H₂O₂ and HF in a Teflon reactor at 90 °C. The sample
482 was diluted with deionized water. Final volume was calculated by weight and weight:
483 volume ratios. The samples were digested with three blanks in parallel. Metal
484 determination was carried out in an Agilent 7500cw instrument under standard
485 conditions. Calibration was carried out with five solutions prepared from certified NIST
486 standards.

487

488 **Nitrate reductase activity**

489 Nitrate reductase activity was analyzed as described by Tejada-Jimenez *et al*
490 (2017). Briefly, a crude extract was obtained from approximately 100 mg of fresh
491 material in 100 mM potassium phosphate, pH 7.5, 5 mM magnesium acetate, 10%
492 glycerol (v/v), 10 % polyvinylpyrrolidone (w/v), 0.1% Triton X-100, 1 mM EDTA,
493 0.05 % β -mercaptoethanol and 1 mM PMSF. Plant material was homogenized with liquid
494 nitrogen and 1:6 extraction buffer (v/v), and centrifuged at 14,000 xg at 4 °C for 15 min.
495 The reaction was started adding 50 µl of crude extract to 0.5 ml of reaction buffer and
496 incubated at 30 °C for 20 min. The reaction buffer contained 50 mM potassium phosphate,
497 pH 7.5, 10mM KNO₃, 5 mM EDTA and 0.5 mM NADH. The reduction reaction was
498 stopped by adding 1 volume of 1 % sulfanilamide in 2.4 M HCl, and 1 volume of 0.02 %
499 N-1-naphtyl-ethylenediamine. After centrifugation, the supernatant was collected and its
500 absorbance at 540 nm measured in a UV/visible spectrophotometer (Ultrospect 3300 pro;
501 Amersham Bioscience).

502

503 **Statistical analysis**

504 Student's unpaired *t*-test was used to calculate statistical significance of observed
505 differences. Test results with p-values less than 0.05 were considered as statistically
506 significant.

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541 **FIGURE LEGENDS**

542 **Figure 1.** *Medicago truncatula* Molybdate Transporter 1.2 (MtMOT1.2) introduces
543 molybdate towards the cytosol. (A) Molybdate uptake by *Saccharomyces cerevisiae*
544 strain 31019b transformed with PDR196 vector containing *MtMOT1.2* coding sequence
545 and grown at 28°C. Values for the pDR196 empty vector were subtracted from the data.
546 Data were fitted using Michaelis constant $k_{1/2} = 488 \pm 105$ nM and maximum speed $v_{max} =$
547 155 ± 12 pmol 10^6 cells $^{-1}$ h $^{-1}$. Data are the mean \pm SD. (B) Effect of sulfate on molybdate
548 uptake by *S. cerevisiae* strain 31019b transformed with PDR196 containing the
549 *MtMOT1.2* coding sequence. Data sets consist of *S. cerevisiae* incubated with 500 nM
550 Na₂MoO₄ and 0, 0.2, or 2 mM Na₂SO₄. Data are the mean \pm SD.

551
552 **Figure 2.** *Medicago truncatula* Molybdate Transporter 1.2 (*MtMOT1.2*) gene is
553 expressed in the around the vessels in roots and nodules. (A) β -glucuronidase (GUS)
554 staining of *M. truncatula* roots and nodules from 28 days-post-inoculation (dpi) plants
555 transiently expressing the *gus* gene under the control of *MtMOT1.2* promoter region.
556 Scale bar = 0.5 mm. (B) GUS activity localization in a cross section of a 28 dpi root from
557 *M. truncatula* plants transiently expressing the *gus* gene under the control of *MtMOT1.2*
558 promoter region. Scale bar = 0.025 mm. (C) GUS activity localization in a cross section
559 of a 28 dpi nodule from *M. truncatula* plants transiently expressing the *gus* gene under
560 the control of *MtMOT1.2* promoter region. Scale bar = 25 μ m.

561
562 **Figure 3.** *Medicago truncatula* Molybdate Transporter 1.2 (MtMOT1.2) is located in the
563 plasma membrane and an endomembrane compartment in endodermal cells. (A) Cross-
564 section of a 28 days-post-inoculation (dpi) *M. truncatula* nodule expressing *MtMOT1.2-*
565 *HA* and inoculated with a *Sinorhizobium meliloti* strain constitutively expressing the
566 green fluorescent protein (GFP) (green). MtMOT1.2-HA was detected using an
567 Alexa594-conjugated antibody (red). DNA was stained with DAPI (blue). Left panel,
568 localization of MtMOT1.2-HA; central panel, overlay with the green (*S. meliloti*)
569 channel; right panel, overlay of the central panel with the DAPI-stained DNA. Scale bars
570 = 10 μ m. (B) *M. truncatula* root expressing *MtMOT1.2-HA*. MtMOT1.2-HA was detected
571 using an Alexa594-conjugated antibody (red). DNA was stained with DAPI (blue). Left
572 panel, localization of MtMOT1.2-HA; central panel, overlay with the DAPI-stained DNA
573 and the xylem autofluorescence; right panel overlay with the transillumination image.

574 Scale bars = 50 μ m. (C) Transient co-expression of MtMOT1.3-GFP (green) and AtPIP2-
575 CFP (cyan) in *Nicotiana benthamiana* leaves. Left panel, AtPIP2-CFP signal, middle
576 panel MtMOT1.2-GFP signal; right panel overlay of the two channels with the
577 transillumination image. Scale bars = 25 μ m. (D) Subcellular localization of MtMOT1.2-
578 HA in nodule vessels using a gold-conjugated anti-HA antibody. Arrowheads indicate
579 the position of the gold particles. Scale bars = 250 nm and 500 nm.

580

581 **Figure 4.** *Medicago truncatula* Molybdate Transporter 1.2 (MtMOT1.2) is not required
582 for growth under non-symbiotic conditions. (A) Position of the *Transposable element*
583 *from Nicotiana tabacum1 (Tnt1)* insertion within the *MtMOT1.2* gene. (B) RT-PCR
584 amplification of *MtMOT1.2* coding sequence in 28 days-post-inoculation roots and
585 nodules of *M. truncatula* wild type (WT) or mutant (*mot1.2-1*) plants. *Ubiquitin carboxyl-*
586 *terminal hydrolase1 (MtUbl)* was used as a control. (C) Growth of representative WT
587 and *mot1.2-1* plants watered with KNO₃. Scale bar = 1 cm. (D) Dry weight of shoots and
588 roots. Data are the mean \pm SD of at least 6 independently transformed plants. (E) Nitrate
589 reductase activity. Nitrate reduction was measured in duplicate. Data are the mean \pm SD.

590

591 **Figure 5.** *Medicago truncatula* Molybdate Transporter 1.2 (MtMOT1.2) is required for
592 symbiotic nitrogen fixation. (A) Growth of representative WT, *mot1.2-1*, and *mot1.2-1*
593 transformed with *MtMOT1.2-HA* plants 28 days-post-inoculation (dpi). Scale bar = 1 cm.
594 (B) Detail of representative 28 dpi nodules from WT, *mot1.2-1*, and *mot1.2-1* transformed
595 with *MtMOT1.2-HA* plants. Scale bars = 50 mm. (C) Dry weight of shoots, and roots from
596 28 dpi WT, *mot1.2-1*, and *mot1.2-1* transformed with *MtMOT1.2-HA* plants. Data are the
597 mean \pm SD of at least 2 sets of 6 pooled independently transformed plants. (D)
598 Nitrogenase activity of 28 dpi WT, *mot1.2-1*, and *mot1.2-1* transformed with *MtMOT1.2-*
599 *HA* plants. Data are the mean \pm SD of 2 sets of 6 pooled plants. 100 % = 0.161 nmol
600 acetylene \cdot h⁻¹ plant⁻¹. * indicates a statistically significant difference (p<0.05). (E) Mo
601 content in shoots, roots, and nodules of WT, *mot1.2-1*, and *mot1.2-1* transformed with
602 *MtMOT1.2-HA* plants. Data are the mean \pm SD of at least 2 sets of 6 pooled independently
603 transformed plants. * indicates a statistically significant difference (p<0.05).

604

605 **Figure 6** Model of molybdate transport in *Medicago truncatula*. Upper panel, molybdate
606 uptake from soil would be mediated by epidermal root transporters similar to LjMOT1,

607 perhaps MtMOT1.1. Symplastically and apoplastically-delivered molybdate will reach
608 the endodermis were MtMOT1.2 will introduce it from the apoplast. This role is not
609 carried out only by this protein, another molybdate transporter would also participate
610 delivering molybdate to the shoots. Molybdate efflux from the endodermis into the xylem
611 is mediated by a yet-to-be determined transporter. Lower panel, once molybdate reaches
612 the nodule, it is recovered from the vasculature by MtMOT1.2 that would introduce it
613 into the endodermal cells. Through an unknown protein, this molybdate is released into
614 the apoplast, where MtMOT1.3 will introduce it into nodule cells. SST1 and bacterial
615 ModABC would then direct the cytosolic molybdate to nitrogen-fixing bacteroids.

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Figure 1

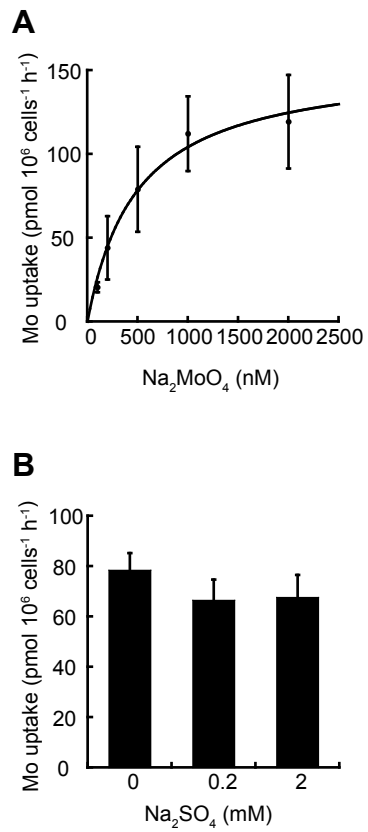


Figure 2

A



B



C

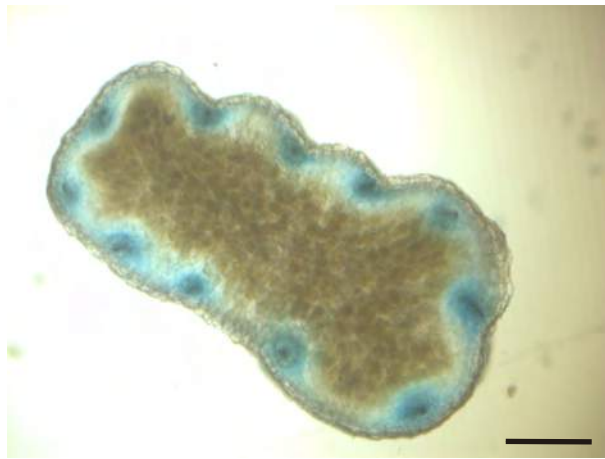


Figure 3

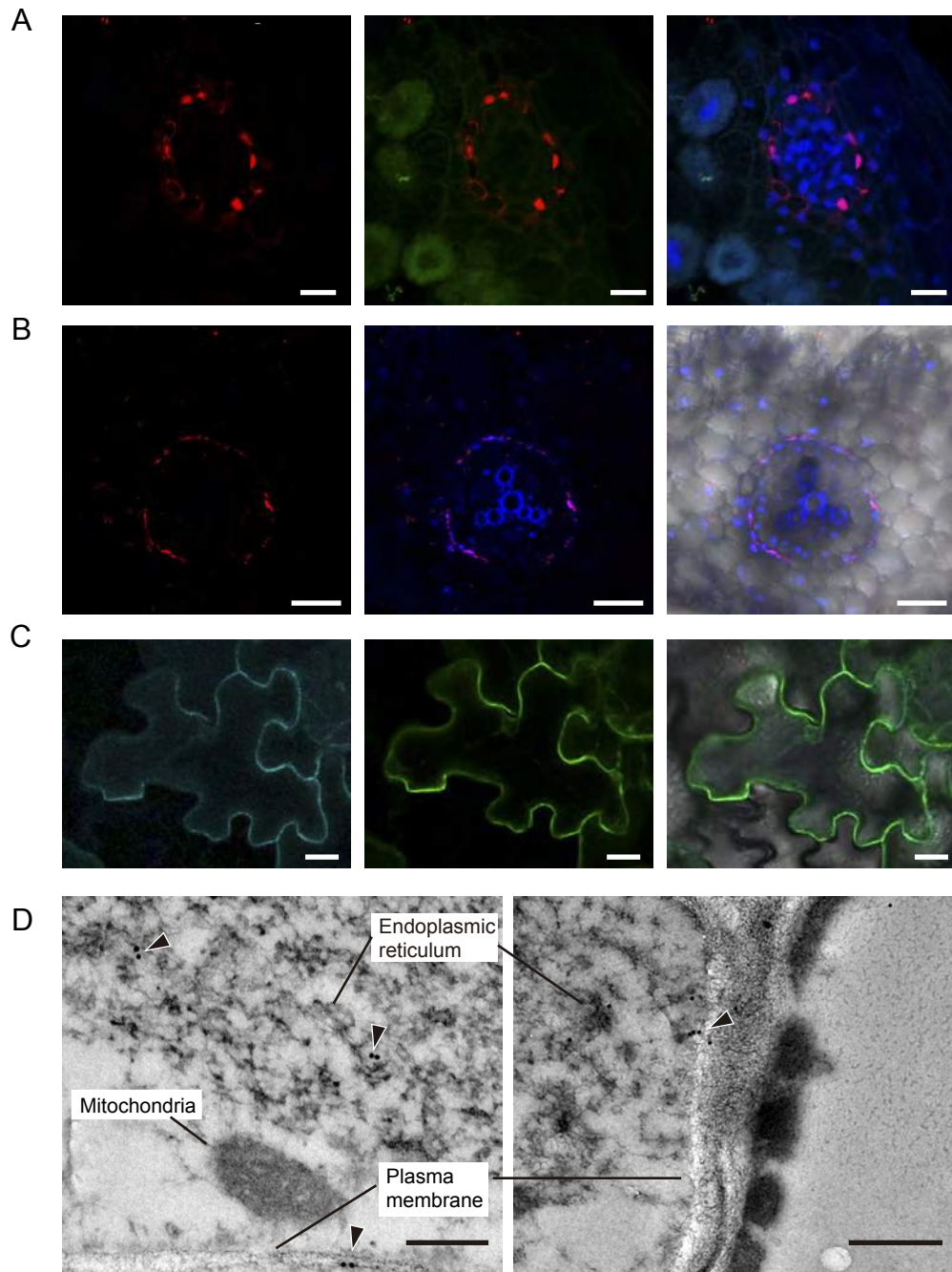


Figure 4

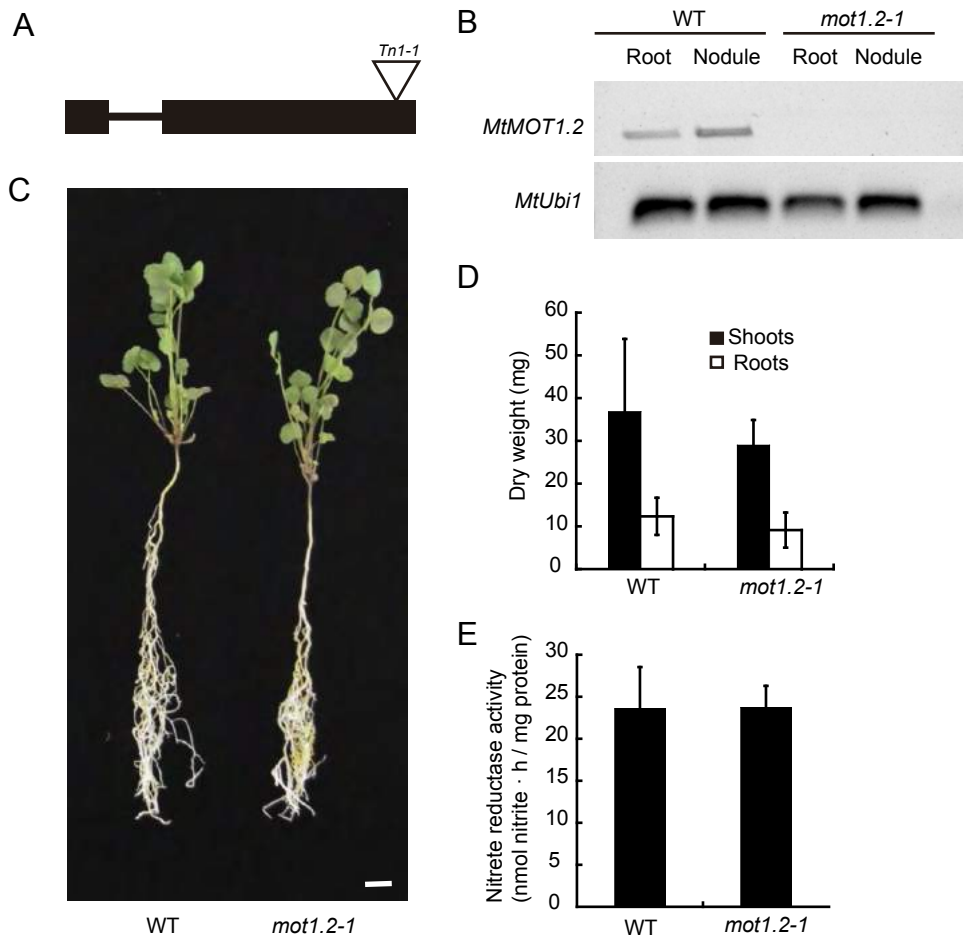


Figure 5

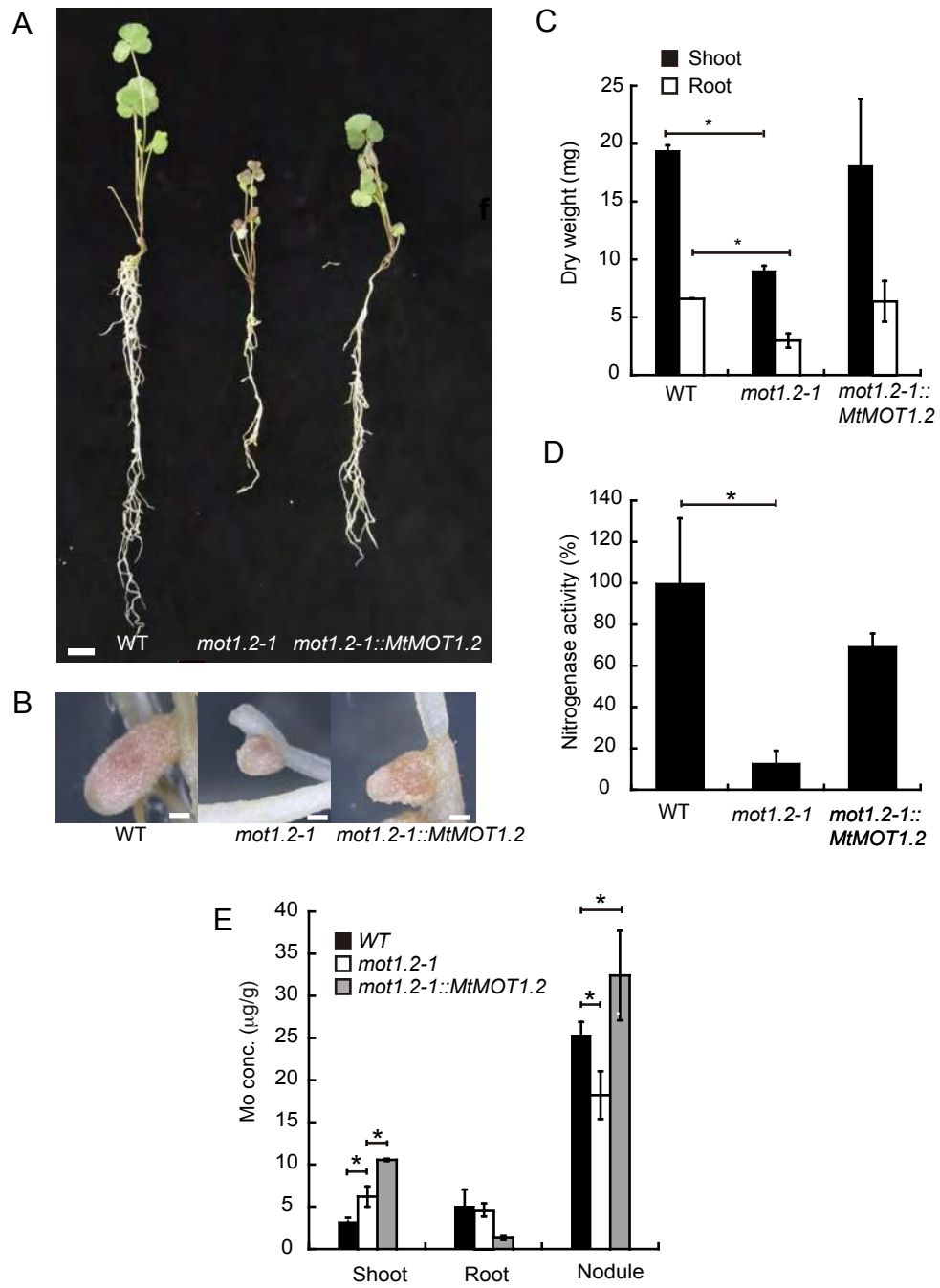


Figure 6

