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1 **A dual legume-rhizobium transcriptome of symbiotic nodule senescence reveals**
2 **coordinated plant and bacterial responses**

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18

19 **Running title:** Dual transcriptome of symbiotic nodule senescence

20 **Keywords:** RNA sequencing, nitrogen fixation, root nodule, senescence, symbiosis, NAC
21 transcription factor, S40, cytokinin.

22

23 **Abstract**

24 Senescence determines plant organ lifespan depending on aging and environmental
25 cues. During the endosymbiotic interaction with rhizobia, legume plants develop a specific
26 organ, the root nodule, which houses nitrogen (N)-fixing bacteria. Unlike earlier processes of
27 the legume–rhizobium interaction (nodule formation, N fixation), mechanisms controlling
28 nodule senescence remain poorly understood. To identify nodule senescence-associated
29 genes, we performed a dual plant-bacteria RNA sequencing approach on *Medicago*
30 *truncatula*-*Sinorhizobium meliloti* nodules having initiated senescence either naturally (aging)
31 or following an environmental trigger (nitrate treatment or salt stress). The resulting data
32 allowed the identification of hundreds of plant and bacterial genes differentially regulated
33 during nodule senescence, thus providing an unprecedented comprehensive resource of new
34 candidate genes associated with this process. Remarkably, several plant and bacterial genes
35 related to the cell cycle and stress responses were regulated in senescent nodules, including
36 the rhizobial RpoE2-dependent general stress response. Analysis of selected core nodule
37 senescence plant genes allowed showing that *MtNAC969* and *MtS40*, both homologous to leaf
38 senescence-associated genes, negatively regulate the transition between N fixation and
39 senescence. In contrast, overexpression of a gene involved in the biosynthesis of cytokinins,
40 well-known negative regulators of leaf senescence, may promote the transition from N
41 fixation to senescence in nodules.

42

43 **Introduction**

44 Nitrogen (N) deficiency is a major environmental constraint limiting plant growth.
45 Legume plants overcome this limitation through a symbiotic interaction with soil bacteria
46 named rhizobia, which results in the development of a new plant organ, the root nodule (Roy
47 et al., 2020). Rhizobia colonize nodules where they differentiate into bacteroids capable of
48 reducing atmospheric N for the benefit of the plant. *Medicago truncatula* nodules have an
49 apical meristem that remains active, giving them an elongated cylindrical shape, and leading
50 to a gradient of differentiated plant and bacterial cells. Cells originating from this apical
51 meristem are infected by rhizobia within the adjacent infection zone, where both bacteria and
52 plant cells start to differentiate through a process involving cell enlargement and genome
53 endoreduplication. Full differentiation is achieved in the N-fixation zone where differentiated
54 bacteria, called bacteroids, reduce atmospheric N (N₂) into ammonium thanks to the
55 nitrogenase enzymatic complex. In nodules, both expression and functioning of the
56 nitrogenase rely on the microaerobic environment of the N fixation zone, partly achieved
57 through oxygen scavenging by plant leghemoglobins specifically expressed in this zone and
58 responsible for the pink color of nodules (Ott et al., 2005).

59 Aging plant organs eventually enter a senescence program, characterized by the
60 gradual degradation of cell structures through the activation of catabolic enzymes, including
61 various proteases and the proteasome pathway, which are thus molecular markers of the onset
62 of senescence (Balazadeh, 2014). Degradation of cell components generates nutrients that are
63 recycled and exported towards non-senescent plant organs thanks to specific transporters and
64 the vasculature (Thomas and Ougham, 2014). Environmental stresses can also provoke
65 premature organ senescence, which in leaves reduces the photosynthesis capacity and leads to
66 a breakdown of carbon/N sink-to-source relationships (Lim et al., 2007). Similarly, a nodule
67 senescence process, leading to a N fixation decline, associated with an increased cellular
68 catabolism, is induced either by intrinsic physiological and developmental signals such as
69 aging, flowering and pod filling, or by environmental conditions such as abiotic stresses or N
70 provision (Puppo et al., 2005; Pérez Guerra et al., 2010; Cabeza et al., 2014). Thus, nodule
71 senescence can be induced by drought, salt stress, high nitrate levels, or dark treatment
72 (Matamoros et al., 1999; Aranjuelo et al., 2013; Marquez-Garcia et al., 2015). Nodule
73 senescence initiates in the root-proximal part of the N fixation zone, and is characterized by a
74 pink-to-green color shift resulting from leghemoglobin alteration, which is thus a macroscopic
75 marker of nodule senescence progression (Puppo et al., 2005; Pérez Guerra et al., 2010;

76 Navascués et al., 2012). At the microscopic level, degradation of cellular structures appears to
77 be a two-step process, bacteroids being degraded before plant cells (Van de Velde et al.,
78 2006).

79 Little is known about the molecular mechanisms controlling nodule senescence. On
80 the plant side, several transcriptomic studies conducted on aging *M. truncatula* nodules (Van
81 de Velde et al., 2006), or nodules from plants treated with a herbicide (Seabra et al., 2012) or
82 nitrate (Cabeza et al., 2014), identified plant genes differentially expressed during both
83 developmental and environmentally-induced nodule senescence, including several Cysteine
84 Proteases (CP)-encoding genes. This is consistent with knowledge gained in leaves, where
85 some Senescence Associated Genes (SAGs) encode CPs, such as *AtSAG12* in *Arabidopsis*
86 *thaliana* (Lohman et al., 1994). Three *M. truncatula* CP genes, *MtCP6*, *MtVPE* and *MtCP77*,
87 showing an expression pattern associated to the nodule senescence zone, were functionally
88 characterized. The use of CP gene mutants, RNA interference (RNAi), or overexpression
89 approaches revealed that these CPs promote nodule senescence (Pierre et al., 2014; Deng et
90 al., 2019).

91 Few transcription factors (TF) potentially involved in the N fixation-senescence switch
92 were identified in nodules. The MtNAC969 TF was proposed to inhibit nodule senescence as
93 nodules expressing a RNAi construct showed an upregulation of CP senescence markers (de
94 Zélicourt et al., 2012). Interestingly, MtNAC969 is closely related to AtNAP that is associated
95 to the regulation of *A. thaliana* leaf senescence (Podzimska-Sroka et al., 2015). More
96 recently, the MtbHLH2 TF was associated with nodule senescence in *M. truncatula* and
97 shown to directly bind and repress the activity of the *MtCP77* promoter (Deng et al., 2019).
98 Regarding the hormonal regulation of nodule senescence, previous transcriptomic analyses
99 revealed that several genes related to ethylene and jasmonic acid biosynthesis were induced in
100 senescent nodules, as well as genes encoding gibberellic acid (GA) degradation enzymes (Van
101 de Velde et al., 2006; Cabeza et al., 2014).

102 On the bacterial side, even less is known apart from the inhibition of N fixation.
103 Several bacterial functions were proposed to prevent nodule senescence, such as those
104 involved in buffering oxidative and nitrosative stresses (Redondo et al., 2009; Cam et al.,
105 2012; Yang et al., 2020). Rhizobia toxin-antitoxin (TA) systems were also shown to regulate
106 the N fixation-senescence transition, although the underlying mechanisms are unclear (Syska
107 et al., 2019). In a recent simultaneous analysis of both *M. truncatula* – *Sinorhizobium medicae*

108 nodule transcriptomes in response to a N satiety treatment, no massive reprogramming of
109 gene expression was detected in symbiotic bacteria (Lambert et al., 2020).

110 In this study, we used a dual plant-bacteria transcriptomic RNAseq approach on *M.*
111 *truncatula* nodules having initiated senescence either naturally upon aging, or following
112 exposure to environmental conditions detrimental for N fixation, namely nitrate treatment or
113 salt stress. The resulting dataset allowed identifying hundreds of plant and bacterial genes
114 differentially regulated during nodule senescence, thus providing an unprecedented
115 comprehensive resource for identifying new candidate genes associated to the regulation of
116 the nodule N fixation-senescence transition. Selected candidate regulatory pathways were
117 functionally analyzed and shown to regulate the N fixation-senescence transition.

118 **Results**

119 **Set-up of the conditions used for studying developmental and stress-induced nodule** 120 **senescence**

121 *M. truncatula* plants grown in aeroponic tanks in N-free medium were inoculated with
122 *S. meliloti*. Whereas 14 dpi nodules were pink-colored, indicating an active N fixation, 21-35
123 dpi nodules additionally displayed a green color in their proximal part, suggesting that the
124 developmental/aging nodule senescence had started between 14 and 21 dpi. This was
125 confirmed by an increased expression of *MtCP5* and *MtCP6* genes, used as markers of nodule
126 senescence, between 14 and 21 dpi (Fig. S1A; (Van de Velde et al., 2006; Pierre et al., 2014)).
127 *CP* gene expression was also found higher in the proximal part of the nodule at all time points
128 analyzed from 21 dpi (Fig. S1A), as expected since senescence initiates in this region. To
129 maximize chances of identifying genes differentially regulated at the onset of developmental
130 nodule senescence, we performed transcriptomic analyses on 14, 21, and 28 dpi whole
131 nodules, as well as on the distal and proximal halves of 21 dpi nodules.

132 Exogenous treatments known to induce nodule senescence were additionally used.
133 After a 24 hours salt or nitrate treatment on 13 dpi plants, the proximal parts of the nodules
134 were completely green, and *CP* gene expression was increased relative to untreated 14 dpi
135 nodules, while N fixation was reduced showing activated nodule senescence (Fig. S1B-C).
136 Thus, these samples were also included in the transcriptomic analysis to study
137 environmentally induced nodule senescence.

138 In order to obtain the bacterial and plant symbiont transcriptomes in parallel, RNA
139 samples were ribodepleted prior to RNA sequencing (see Materials and Methods). Reads were
140 mapped to the Sm2011 genome and to the latest release of the *M. truncatula* Jemalong A17
141 genome (v5; summary of libraries and raw sequencing data, Table S1; full absolute and
142 relative normalized expression data, Tables S2 and S3).

143

144 **Global analysis of transcriptome changes in the selected conditions highlight an early** 145 **establishment of the nodule senescence and coordinated plant and bacterial responses**

146 Hierarchical clustering analyses of whole normalized RNAseq data replicas showed
147 that time-course samples clustered apart from exogenously treated samples (Fig. S2A). Salt

148 and nitrate samples clustered separately from each other, as did the proximal and distal halves
149 of 21 dpi nodule samples. In contrast, 14, 21 and 28 dpi nodules did not separate clearly,
150 suggesting that sample data are similar, as confirmed subsequently by Differentially
151 Expressed Gene (DEG) analyses.

152 For identifying DEGs, 14 dpi nodules were taken as a reference to calculate mean gene
153 expression fold changes (FC) relative to all other conditions. Plant genes were arbitrarily
154 considered as DEGs for $0.5 > FC > 2$ (i.e. $-1 > \log_2FC > 1$) with a False Discovery Rate
155 (FDR) < 0.05 , whereas bacterial genes were considered as DEGs for $0.55 > FC > 1.8$ (i.e. $-$
156 $0.85 > \log_2FC > 0.85$) with a FDR < 0.05 . 59% (9280) of plant DEGs and 37% (938) of
157 bacterial DEGs were found regulated in two conditions or more. Hierarchical clustering
158 analysis of gene expression profiles revealed two metaclusters, in which plant and bacterial
159 DEGs were globally either up or down regulated, respectively, in comparison to the control
160 (Fig. S2B). Only ~4% of the DEGs displayed opposite regulations in the different conditions
161 selected, suggesting that related biological processes were affected, including senescence.

162 Hierarchical clustering analyses of experimental conditions, relative to the 14 dpi
163 nodule reference condition, showed that the mean plant and bacterial gene expression profiles
164 of 14 and 21 dpi nodules were the most similar (Fig. S2B), as only 95 *M. truncatula* and 7 *S.*
165 *meliloti* DEGs were identified (Tables S2 and S3), whereas the distal part of 21 dpi nodules
166 was also very similar to whole 14 dpi nodules, with only 150 *M. truncatula* and 4 *S. meliloti*
167 DEGs. These observations reveal that even if senescence had already started in 21 dpi
168 nodules, it did not yet strongly affect the whole nodule transcriptome. In contrast, the
169 proximal part of 21 dpi nodules was very different from 14 dpi nodules, with 5555 *M.*
170 *truncatula* and 472 *S. meliloti* DEGs. This suggests that the proximal nodule part, which
171 includes the senescence zone, has a minor contribution to the whole nodule transcriptome at
172 21 dpi, justifying the interest of isolating this region to enrich for senescence-related genes.
173 Expression profiles were also very different between 14 and 28 dpi whole nodules, with 3578
174 *M. truncatula* and 508 *S. meliloti* DEGs. Interestingly, 2239 *M. truncatula* and 139 *S. meliloti*
175 DEGs were shared between 28 dpi nodules and the proximal region of 21 dpi nodules. This
176 suggests that senescence has progressed between 21 and 28 dpi, to significantly contribute to
177 the whole nodule transcriptome at 28 dpi. Altogether, these data highlight that 28 dpi nodules
178 and the proximal region of 21 dpi nodules are the most appropriate conditions for identifying
179 DEGs related to developmental nodule senescence.

180 Nodules treated with NaCl or KNO₃ displayed expression profiles very different from
181 those of untreated nodules, with NaCl showing the highest number of DEGs. A total of 13673
182 *M. truncatula* and 2176 *S. meliloti* genes were differentially expressed in at least one of these
183 two conditions, including 53% (*M. truncatula*) and 29% (*S. meliloti*) shared DEGs between
184 NaCl and KNO₃ treatments.

185 To identify a core of senescence-associated genes (SAGs), intersections between the
186 following conditions were therefore considered (Fig. 1A): “old” (28 dpi) nodules; the
187 proximal region of 21 dpi nodules, which includes the senescence zone; and nodules treated
188 with salt or nitrate, both inducing nodule senescence. Strikingly, the proportions of plant and
189 bacterial genes upregulated in these conditions showed parallel trends (Fig. 1B, Pearson
190 correlation $R^2=0.67$; $p=0.0002$), suggesting that coordinated plant and bacterial responses
191 occur.

192

193 **Nodule senescence leads to a biased expression of plasmidic genes, an induction of a** 194 **general stress response, and a reinitiation of the cell cycle in bacteria**

195 The *S. meliloti* genome is organized in three replicons, one chromosome and two
196 megaplasmids (pSymA and pSymB). Interestingly, a bias in the representation of plasmidic
197 genes was observed among bacterial DEGs retrieved in at least three of the selected
198 conditions (Fig. 2A). Whereas the number of chromosomal and plasmidic DEGs
199 corresponded to the respective gene proportions within each replicon (χ^2 test, $p>0.05$),
200 pSymB genes were overrepresented in upregulated senescence-induced DEGs and
201 underrepresented among down-regulated genes, while pSymA genes displayed the opposite
202 profile (χ^2 test, $p<0.0001$). This bias may be linked to the functional specialization of each
203 megaplasmid relatively to the symbiotic interaction. Indeed, pSymA encodes a majority of
204 genes upregulated during the symbiosis, such as genes involved in N fixation, and the
205 downregulation of pSymA genes is thus consistent with senescence inducing a decline of the
206 symbiotic process. In contrast, pSymB encodes many genes responding to the environment
207 such as stress-related genes (Domínguez-Ferreras et al., 2006; Sauviac et al., 2007), whose
208 induction may be consistent with cellular responses activated in the nodule senescence zone.

209 Sixty-three *S. meliloti* genes were downregulated in more than three senescence-
210 inducing conditions (Fig. 1A; Table S2, sheet 5). Overrepresented pSymA genes (26 genes,
211 41%) include symbiotic genes known for their involvement in Nod factor biosynthesis (*nodH*,

212 *nodF*) and N fixation (*fixB*, *nifX*, *fdxB*, *nifN*) (Table 1). Among the three pSymB and 34
213 chromosomal down-regulated genes, two are of particular interest as they are known to play
214 essential roles in bacteroid differentiation: *bacA*, associated to the import of plant Nodule
215 Cysteine Rich (NCR) peptides (see below), and *acpXL* involved in the synthesis of very long
216 chain fatty acids (Table 1). Altogether, these observations are consistent with nodule
217 senescence being associated with an arrest of bacterial differentiation and/or symbiotic N
218 fixation.

219 Among the 169 *S. meliloti* genes upregulated in ≥ 3 conditions (Fig. 1A; Table S2,
220 sheet 4), 29 (17%) are under the control of the general stress regulator RpoE2 (Sauviac et al.,
221 2007), representing 19% of the known regulon (Table S4; Fig. 2B). 37 additional RpoE2-
222 dependent genes were upregulated in only one or two of the senescence inducing conditions
223 (Table S4; Fig. 2B). These observations suggest that during nodule senescence, bacteria are
224 exposed to stressful conditions.

225 Twenty-six (15%) of the *S. meliloti* genes induced in ≥ 3 conditions were also
226 upregulated during the *S. meliloti* cell cycle in the transcriptomic analysis performed by De
227 Nisco et al., 2014 (Table S5). In that study, the expression of these genes peaked between 140
228 and 160 min of the cell cycle, which roughly corresponds to the time of cell division.
229 Accordingly, these 26 genes include the *ftsQ*, *ftsA*, *ftsZ1* cell division genes that are down-
230 regulated during bacteroid differentiation (Roux et al., 2014). In addition, 103 other cell
231 cycle-regulated genes were upregulated in only one or two conditions. Altogether, these
232 observations suggest that some bacteria reinitiate their cell cycle during the senescence
233 process. As *S. meliloti* bacteroids are terminally differentiated (Mergaert et al., 2006), this cell
234 cycle reactivation likely concerns undifferentiated bacteria released from infection threads.

235

236 **The analysis of a core nodule senescence transcriptional program highlights candidate** 237 **plant regulators of the N fixation-senescence transition**

238 799 plant genes were upregulated in at least three of the senescence-inducing
239 conditions, including 201 (25%) in all four conditions (Fig. 1A), which were thus considered
240 as the core of nodule SAGs (Table S3, sheet 4). Because of the large number of plant DEGs,
241 we first evaluated the biological processes and responses differentially regulated under the

242 selected senescence-inducing conditions using the Mapman software (see Supplemental
243 Results). A selection of SAGs is presented in Table 2 and further described below.

244 Eleven up-regulated genes encode putative CPs, seven that were already previously
245 identified (*MtCPI-6* and *VPE*) and three new ones that were named *MtCP7-9*, as well as a
246 caspase ICE-like protease (Pierre et al., 2014; Deng et al., 2019). CPs being known as
247 markers of nodule senescence, their upregulation thus validates our experimental design. The
248 two most highly expressed *CP* genes in all conditions were *CP4* and *CP6*, representing 54 to
249 69 % of the total *CP* transcripts) (Fig. S3). Two phytohemoglobin genes (*MtPgb1.1* and *MtPgb1.2*)
250 were upregulated, which were already described as up-regulated in aging nodules (Berger et
251 al., 2020). The *MtTML2* gene, encoding a E3 ubiquitin ligase subunit that targets proteins for
252 degradation through the proteasome, and which is a known negative regulator of early
253 nodulation stages (Gautrat et al., 2019), was upregulated in senescent nodules. This suggests
254 that the TML2 inhibitory role is maintained in differentiated nodules. In addition, five
255 pathogenesis Related (PR) proteins, one PAMP-inducible peptide, four glucanases, and three
256 class III peroxidases were upregulated in senescence-inducing conditions, suggesting an
257 activation of plant defense, in agreement with previous studies establishing relationships
258 between plant senescence and defense responses in nodules (Bourcy et al., 2013; Berrabah et
259 al., 2014). Twelve annotated nutrient transporter-encoding genes were additionally retrieved,
260 including two zinc/iron permease (*MtZIP1* and *MtZIP7*), a sulfate transporter (*MtSULTR3.1*),
261 a phosphate transporter (*MtPT6*), two nitrate transporters (*MtNPF6.4*, *MtNPF7.7*), a
262 molybdate transporter (*MtMOT1.1*), four transporters of the Multidrug And Toxic Compound
263 Extrusion (MATE) family, and a sucrose transporter (*MtSWEET13*). These transporters are
264 likely associated to the senescence-associated remobilization of mineral nutrients for
265 recycling.

266 Concerning regulatory pathways, 17 genes encoding small secreted peptides of various
267 functions were upregulated (Table 2), whereas not a single NCR peptide involved in bacteroid
268 maturation was upregulated in senescing conditions (Guefrachi et al., 2014). Several
269 hormone-related genes were additionally upregulated: the Ethylene Insensitive 3 [EIN3]-
270 binding F box protein 1 (*MtEBF1*), in agreement with the previously proposed positive role of
271 ethylene in nodule senescence (Van de Velde et al., 2006), and the Gibberellin 2-oxygenase 1
272 (*MtGA2ox*; (Fonouni-Farde et al., 2017) associated to GA inactivation, accordingly to the
273 previously proposed negative role of GA on nodule senescence (Van de Velde et al., 2006;
274 Serova et al., 2019). A gene encoding one of the four histidine kinase cytokinin (CK)

275 receptors (MtCHK2; Boivin et al., 2016) was identified, suggesting a role for CK hormones in
276 nodule senescence, as previously observed in senescing leaves and in lupin nodules (Coba de
277 la Peña et al., 2008; Zwack and Rashotte, 2013).

278 Among the 65 genes encoding putative TFs (Table S3), several had previously been
279 associated to stress responses and/or nodule senescence, such as MtHB1 and MtZPT2-2, both
280 shown to regulate salt stress responses, and for the latter also N fixation (Frugier et al., 2000;
281 Merchan et al., 2007; Ariel et al., 2012), or MtATB2, associated to senescent nodules
282 (d'Haeseleer et al., 2010). The salt stress regulated MtNAC969 TF, previously proposed as a
283 negative regulator of nodule senescence (de Zélicourt et al., 2012), was also retrieved. As
284 initial data establishing the function of MtNAC969 were based on an RNAi approach, a plant
285 *Tnt1* insertional mutant was now identified (Fig. 3A). No significant difference in nodule
286 number and fresh weight was observed between the *nac969* mutant and the wild-type (WT)
287 (Fig. 3B-C), whereas the N fixation capacity significantly decreased (Fig. 3D) and the *MtCP5*
288 senescence marker expression increased (Fig. 3E). Histological analysis of *nac969* nodules
289 revealed a broader senescence zone than in WT nodules (Fig. 3F-H). Overall, these data
290 independently validate previous RNAi data indicating a negative role of NAC969 in the
291 nodule N fixation-senescence transition.

292 Another upregulated gene encodes a predicted nuclear protein similar to *AtS40*,
293 previously described as a positive regulator of leaf senescence in Arabidopsis (Fischer-
294 Kilbienski et al., 2010; Jehanzeb et al., 2017). To demonstrate that the core nodule senescence
295 dataset comprises novel regulators of the nodule N fixation-senescence transition, we used an
296 RNAi approach that efficiently downregulated the expression of the *MtS40* gene (Fig. 4A).
297 This revealed a decreased nodule N fixation capacity (Fig. 4B) associated to a decreased
298 expression of *Lb1* and *NCR001*, two N-fixation zone markers (Fig. 4C-D; Yang et al., 2020).
299 These results show that MtS40 is involved in the maintenance of the N fixation zone.
300 However, no upregulation of *MtCP6* expression, used as a senescence marker, was detected in
301 *MtS40* RNAi nodules (Fig. 4). MtS40 may thus promote the N fixation efficiency either
302 through the stimulation of the transition from the infection zone to the N fixation zone, and/or
303 through the inhibition of transition from the N fixation zone to the senescence zone, a role that
304 would be therefore opposite to that of *AtS40* in Arabidopsis leaf senescence.

305

306 **Plant genes downregulated in nodule senescence-inducing conditions highlight an**
307 **inhibition of all symbiosis stages, from infection to differentiation and N fixation**

308 2786 plant genes were downregulated in at least three senescence-inducing conditions,
309 including 1218 (44 %) in all four conditions (Table S3, sheet 5). A selection of these genes is
310 presented in the Table 3 and further described below. This large number of repressed genes
311 contrasts with the only 799 upregulated genes in the same conditions (see above).

312 Within these downregulated genes, many are related to nodule differentiation and N
313 fixation, including 18 nodule specific Glycine Rich Proteins-encoding genes (GRPs; Alunni et
314 al., 2007), 454 NCR peptide-encoding genes (out of ~700 annotated), *MtDNF1* which
315 encodes a signal peptidase required for NCR peptide activity (Van de Velde et al., 2010;
316 Wang et al., 2010), and the *MtDME* DNA demethylase regulating the expression of a subset
317 of *NCR* genes (Satgé et al., 2016). Five leghemoglobin genes were also downregulated
318 (*MtLb2*, *MtLb5*, *MtLb6*, *MtLb9*, *MtLb10*; Larrainzar et al., 2020), as well as two molybdate
319 transporter-encoding genes essential for N fixation (*MtMOT1.2*, *MtMOT1.3*; Gil-Díez et al.,
320 2019). These expression profiles are consistent with an arrest of bacterial differentiation and
321 an inhibition of N fixation in senescent nodules, in agreement with the bacterial
322 transcriptomic data. In addition to the numerous *NCR* and *GRP* genes, > 70 genes encoding
323 members of other families of small secreted peptides having various functions were
324 downregulated, including two *CLE* peptide-encoding genes participating in the systemic
325 negative autoregulation of nodulation (*MtCLE12*, *MtCLE13*; Mortier et al., 2010), suggesting
326 that beyond their well-known function at early nodulation stages, they may also inhibit
327 rhizobial infections in differentiated nodules.

328 Accordingly, several early nodulation genes were also downregulated (*MtNFP*,
329 *MtLYK3*, *MtLYK4*, *MtLYK10*, *MtRIP1*, *MtRPG*, *MtNSP2*, *MtERN1*, *MtNFY-A1*, *MtENOD11*,
330 *MtENOD12*, *MtENOD10*, *MtENOD16*, *MtENOD20*, *MtCCS52b*, *MtSYMREM1*, *MtNFH1*).
331 This highlights that a subset of genes actively expressed in mature nodules and linked to
332 symbiont recognition and infection are repressed upon senescence-inducing conditions.
333 Among these genes, *MtRPG* and *MtNFY-A1* are two direct targets of the MtNIN transcription
334 factor, a central regulatory hub of nodulation, recently shown to regulate not only early
335 nodulation stages but also the expression of genes associated with symbiosome development
336 and N fixation (Feng et al., 2021; Liu et al., 2021).

337 Thirteen downregulated genes encode cyclins that were recently proposed to act
338 during nodule initiation and development (Meng et al. 2020). Four downregulated genes are
339 related to root/nodule development (*MtLBD16*, *MtPLT1*, *MtPLT3*, *MtPLT4*), as well as the
340 *MtbHLH1* gene encoding a TF previously associated to nodule vascular patterning and
341 nutrient exchanges (Godiard et al., 2011). Three down-regulated genes relate to the
342 differentiation/maintenance of bacteroids in nodules: *MtEFD2* (Vernié et al., 2008), a
343 homolog of the *L. japonicus SEN1* gene (Hakoyama et al., 2012), and the *MtRSD* TF
344 (Sinharoy et al., 2013). These observations are consistent with an arrest of nodule
345 differentiation and functioning, as expected for senescent nodules. Finally, two genes linked
346 to the prevention of defense responses in nodules were retrieved, *MtSymCRK* (Berrabah et al.,
347 2014) and *MtNAD1* (Wang et al., 2016), in agreement with an activation of plant immune
348 responses.

349

350 **Several cytokinin-related genes are downregulated in senescence-inducing conditions,**
351 **and increasing the cytokinin pool in the senescence zone promotes the N fixation-**
352 **senescence transition**

353 Regarding hormone-related genes downregulated in senescence-inducing conditions,
354 five GA-related genes were identified: one linked to gibberellic acid (GA) inactivation, a
355 *GA2ox*, and four linked to GA biosynthesis, *MtCPS1*, one *GA3ox*, and two *GA20ox*
356 (Breakspear et al., 2014; Fonouni-Farde et al., 2017). Five genes were linked to auxin: *ASA2*
357 involved in auxin biosynthesis, the auxin carriers *MtLAX5* and *MtPIN10*, as well as the
358 *MtARF1/MtARF16a* and *MtPUCHI* TFs involved in auxin responses (Schnabel and Frugoli,
359 2004; Breakspear et al., 2014). Only one gene was linked to ethylene biosynthesis (*MtACS2*;
360 van Zeijl et al., 2015b), and one to strigolactones (*MtD27*, *DWARF27*; van Zeijl et al., 2015a).
361 In contrast, nine CK-related genes were identified: five CK biosynthesis genes, *MtIPT1*,
362 *MtIPT2*, *MtLOG1*, *MtLOG3* and a *CYP735A1-like* gene (Azarakhsh et al., 2015), three CK
363 signaling response genes, *MtRRA2*, *MtRRA3* and *MtRRA4* (Plet et al., 2011; Tan et al., 2019),
364 and one oxidase involved in CK degradation, *MtCKX3* (Fonouni-Farde et al., 2017). This
365 suggests that beyond the involvement of GA hormones highlighted by the biological process
366 enrichment analysis (see Supplemental Results), and reported as well by van de Velde et al.
367 (2006), other hormones likely regulate the transition from N fixation to senescence, including
368 CKs.

369 To specifically determine the role of CKs in the nodule N fixation-senescence
370 transition, knowing that these hormones affect previous stages of the symbiotic interaction
371 and the whole plant development, an *A. tumefaciens IPT* CK biosynthesis gene was expressed
372 from the *MtCP6* promoter that displays a senescence-specific expression pattern (Pierre et al.,
373 2014). No significant effect was observed on the number and weight of nodules formed (Fig.
374 S4), as expected when using such a late nodulin promoter. Interestingly, the N fixation
375 activity was reduced in *pCP6:IPT* nodules compared to *pCP6:GUS* control nodules, whether
376 normalized by plant or by nodule weight (Fig. 5A). qRT-PCR analyses demonstrated the
377 efficient ectopic expression of the *IPT* transgene in *pCP6:IPT* nodules, and identified a
378 reduced expression of the *Lb1* and *NCR001* markers of the N fixation zone (Fig. 5B), in
379 agreement with the decreased N fixation activity observed. This result thus suggests that,
380 unlike what was reported in leaves (Calderini et al., 2007), CKs do not delay, but rather
381 promote the transition from N fixation to senescence in nodules. However, as for the *MtS40*
382 RNAi nodules, we were not able to detect any significant change in the expression of the
383 *MtCP5* senescence marker in *pCP6:IPT* nodules (Fig. 5B).

384

385 Discussion

386 Using a dual plant/bacteria RNAseq approach, we determined global transcriptional
387 changes affecting both *M. truncatula* and *S. meliloti* during the nodule N fixation-senescence
388 transition. In order to identify the most robust “core” SAGs, we focused on DEGs shared
389 between aging nodules (28 dpi), the proximal nodule region (at 21 dpi), and nitrate and salt
390 treatments. Eleven *CPs* were found among the upregulated genes, but the *MtCP77* gene and
391 the associated *MtbHLH2* transcription factor, previously linked to nodule senescence (Deng et
392 al., 2019), were surprisingly not differentially expressed in any of the senescence-inducing
393 conditions tested. Focusing on this core of SAGs, it is striking that most plant DEGs
394 correspond to down-regulated genes, in contrast to bacterial DEGs. This suggests that the
395 inhibition of symbiosis, either at the level of rhizobial recognition and infection,
396 differentiation, or N fixation, correlates with a repression of numerous plant genes in nodules,
397 several of which were initially characterized as acting during early symbiotic stages. As these
398 downregulated rhizobial or plant genes demonstrate an arrest of symbiotic nodule infection,
399 bacteroid differentiation, and N fixation, this also validates the experimental design used to
400 study nodule senescence.

401

402 The rhizobial cell cycle is reactivated in senescent nodules

403 The upregulation of a subset of rhizobial genes suggests the reactivation of the
404 bacterial cell cycle, which likely concerns undifferentiated bacteria released from infection
405 threads. Such saprophytic bacteria were previously observed in the proximal region of
406 nodules having entered senescence following a stress (Van de Velde et al., 2006; Seabra et al.,
407 2012; Cabeza et al., 2014). However, during nodule aging, such proliferation of saprophytic
408 bacteria was already described only in the senescence zone of old (> 42 dpi) *M. sativa*
409 nodules (Timmers et al., 2000). Our data indicate that, at least in *M. truncatula*, this occurs
410 much earlier, i.e. already at the onset of the senescence process. This also suggests that the *S.*
411 *meliloti* transcriptome in senescent nodules includes a mixture of bacteroids and saprophytic
412 non-differentiated bacteria. It is therefore likely relevant to consider that the observed
413 apparent differential expression of rhizobial N fixation-related vs cell cycle-related genes may
414 at least partly reflect changing proportions of bacteroids and saprophytic bacteria in nodules.
415 On the plant side, the cell cycle regulation also appears as a major biological function affected
416 during the nodule N fixation-senescence transition (see Supplemental Results and Table S6).

417 Whereas cell divisions were promoted in the case of rhizobia, plant cell endocycles and cell
418 proliferation were however likely inhibited during senescence progression, as suggested by
419 the downregulation of numerous cyclin genes (Table 3).

420

421 **Both rhizobia and plant cell stress responses are activated in senescent nodules**

422 Rhizobial and plant genes upregulated in nodule senescence-inducing conditions
423 include several stress-related genes (Supplemental Results; Table S4; Table S6). In the
424 bacteria, targets of the general stress response regulator RpoE2 were notably identified (Fig.
425 2B). A related induction of the *S. medicae* general stress response was previously deduced
426 from a proteome analysis of *MtsymCRK* mutant nodules, which display a premature
427 senescence phenotype (Berrabah et al., 2018). Concordantly, the up-regulation of *S. meliloti*
428 RpoE2 target genes in the senescence zone was observed using transcriptional reporter
429 fusions on sections of old *M. sativa* nodules (Oke et al., 2001; Jamet et al., 2003). Note that
430 saprophytic bacteria (Timmers et al., 2000) and the activation of the bacterial general stress
431 response were both similarly observed in the senescence zone of old *M. sativa* nodules,
432 suggesting that the observed stress response likely concerns saprophytic bacteria rather than
433 bacteroids. Previous studies noticed that the pSymB plasmid is enriched in stress-responsive
434 genes, including RpoE2-dependent genes (Domínguez-Ferrerías et al., 2006; Sauviac et al.,
435 2007). The bias towards pSymB genes among up-regulated SAGs is another indication that
436 rhizobia are likely exposed to stress conditions in senescent nodules. Although oxidative and
437 nitrosative stresses were expected to be involved on the bacterial and plant sides, we did not
438 observe any massive response to these stress conditions in the core of SAGs, suggesting the
439 specific involvement of distinct redox associated genes in the different senescence induced
440 conditions.

441 In the case of environmentally-induced senescence, it is tempting to assume that
442 nitrate or salt are the inducing cues directly sensed by plant and bacterial cells within nodules.
443 For instance, the up-regulation of the NR1 and NR2 plant nitrate reductases by KNO_3
444 indicates that nitrate indeed affected nodules. Similarly, some genes involved in the *S. meliloti*
445 osmotic stress response were up-regulated in salt-treated nodules, such as those involved in
446 osmolyte transport, glutamate biosynthesis or potassium efflux, demonstrating that salt stress
447 reached nodule infected cells. Nevertheless, the bacterial transcriptome of salt-treated nodules
448 only partially overlaps the one of free-living bacteria exposed to an osmotic stress

449 (Domínguez-Ferreras et al., 2006) suggesting that additional cues are perceived by bacteria
450 during stress-induced nodule senescence.

451

452 **Several hormonal pathways are associated with nodule senescence, and cytokinins affect**
453 **the N fixation-senescence transition**

454 Several plant regulatory pathways are associated to the core SAGs. These include
455 components of the Autoregulation of Nodulation (AON) pathway that inhibits early
456 nodulation by affecting symbiotic root infections (Tsikou et al., 2018; Moreau et al., 2021),
457 namely the *CLE12* and *CLE13* genes encoding CLAVATA-like signalling peptides that are
458 downregulated under nodule senescence inducing conditions, and the *TML2* target repressed
459 by AON, which is conversely upregulated. This, together with the downregulation of
460 numerous early rhizobial recognition- and infection-related genes, strongly suggests that
461 nodule senescence establishment correlates with an inhibition of symbiotic infections. In
462 addition, the production of flavonoid secondary metabolites and of several phytohormones is
463 differentially regulated during the nodule N fixation-senescence transition. Among those, GA-
464 associated genes were significantly differentially regulated, in agreement with previous
465 reports (Van de Velde et al., 2006; Cabeza et al., 2014). In contrast to these previous studies,
466 no enrichment for ethylene pathways was highlighted, either in the Mapman global analysis,
467 or when focusing on ethylene biosynthesis, as only one *ACS* gene was retrieved among the
468 core senescence genes. CK-associated genes, not highlighted in the previous studies, were in
469 contrast the most represented among phytohormone-related SAGs. CKs were previously
470 identified as inhibiting leaf senescence, such as with the Arabidopsis *ore12* gain of function
471 mutant affecting the AHK3 cytokinin receptor that has a delayed leaf senescence (Kim et al.,
472 2006), or with the ectopic expression of an *IsoPentyl-Transferase (IPT)* CK biosynthetic gene
473 from a *SAG* promoter (*AtSAG12*, encoding a CP) that delays leaf senescence in different
474 species including *Medicago sativa* (“stay green” phenotype; Calderini et al., 2007).
475 Concerning nodule senescence, a previous study in *L. japonicus*, using stable IPT3 RNAi
476 transgenic plants revealed a reduced nitrogenase activity and premature nodule senescence
477 (Chen et al., 2014). Based on these observations, we anticipated a positive effect on N fixation
478 in nodules transformed with a *pCP6:IPT* construct, but in contrast, a reduced N fixation
479 activity was observed. This apparent contradiction could result from the different strategies
480 used, i.e. to express the *IPT* gene specifically within the N fixation-senescence transition zone

481 vs constitutively across the whole plant. In the latter case, *LjIPT3* RNAi stable transgenic
482 plants indeed also showed a premature leaf senescence phenotype, associated with decreased
483 chlorophyll contents, which is anticipated to negatively impact N fixation, and thus to
484 indirectly provoke early nodule senescence. Overall, our approach specifically affecting the
485 CK pool within nodules to avoid such pleiotropic effects now suggests that CKs promote the
486 nodule N fixation-senescence transition, unlike their reported function in leaves. Knowing
487 that CKs were proposed in *L. japonicus* to inhibit nodule formation not only locally but also
488 systemically from shoots (Sazaki et al. 2014), the local and/or systemic nature of the CK
489 regulation of the nitrogen fixation-senescence transition remains to be established.

490 The two other plant candidate genes that were selected to identify regulators of the
491 nodule N fixation-senescence transition, namely *NAC969* and *S40*, both inhibited the nodule
492 N fixation to senescence transition despite their expression was upregulated by senescence-
493 inducing conditions. It has to be noticed however that, in contrast to the *nac969* mutant, a
494 significant deregulation was detected only for N fixation zone markers, but not for *CP*
495 senescence marker genes. Even though previous studies mentioned that *CP* genes may not
496 always be appropriate markers to evaluate nodule senescence phenotypes (Yang et al., 2020),
497 it remains thus unclear whether the observed N fixation activity phenotypes may relate to a
498 role of the targeted genes in nodule senescence, or not. Speculating anyway on a putative role
499 of *S40* and cytokinins in nodule senescence, it is striking that their deduced role would be
500 opposite to the one reported in Arabidopsis leaves, as also observed for *NAC969*. This may
501 relate to the very opposite metabolic behavior of leaves and nodules, respectively fixing
502 carbon and being N sinks, or fixing N and being carbon sinks. In addition, the expression of
503 about half of the NAC TF family is regulated during Arabidopsis leaf senescence (Kim et al.,
504 2016), and several *NAC* up-regulated genes also have a negative function, such as the “NAC
505 troika” (Kim et al., 2018), as observed in *M. truncatula* nodules with MtNAC969. This
506 indicates that a complex and dynamic combination of positive and negative regulations
507 mediated by NAC TFs modulate leaf senescence progression. In nodule also, CKs and
508 transcriptional complexes involving NAC TFs and probably other TF families, may
509 dynamically fine-tune the progression of the N fixation-senescence transition, and the
510 respective induction of *S40* and *NAC969*, or the repression of *IPT* expression by senescence-
511 inducing conditions may similarly participate in negative feedback regulations.

512 **Material and Methods**

513 **Plant material and growth conditions**

514 *Medicago truncatula* Jemalong A17 seeds were surface sterilized, germinated on agar
515 plates and transferred to aeroponic caissons under controlled growth conditions (temperature
516 22°C; 75% hygrometry; light intensity 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 16h light/8h dark photoperiod). Plants
517 were grown for seven days in the “caisson growth medium” (Journet et al., 2001)
518 supplemented with 10mM NH_4NO_3 , before transfer into N-free medium for three days. The
519 *Sinorhizobium meliloti* 2011 strain (GMI 11495) (Sallet et al., 2013) was grown overnight in
520 TY liquid medium (Sauviac et al., 2007) supplemented with 6mM CaCl_2 , bacteria were
521 collected by centrifugation, resuspended in sterile water, and inoculated together with the
522 “caisson N-free medium” to a final Optical Density ($\text{OD}_{600\text{nm}}$) of 0.001.

523 To avoid harvesting immature symbiotic organs, nodules were collected on the first 40
524 cm of roots below the root collar at 14, 21, 28 and 35 days post inoculation (dpi). At each
525 time-point, the growth medium was replaced with a fresh one. For each sample, whole
526 nodules were harvested on ice from at least 10 plants, immediately frozen in liquid nitrogen,
527 and stored at -80°C before RNA extraction. For distal and proximal nodule samples, 21, 28
528 and 35 dpi nodules were transversally cut in two equivalent halves (14 dpi nodules were too
529 small to be hand-cut). For exogenous treatments, 10 mM KNO_3 or 150 mM NaCl was added
530 to the medium at 13 dpi and whole nodules were harvested after 24 hours. These
531 concentrations and treatment length were chosen based on previous studies (de Lorenzo et al.,
532 2007; Cabeza et al., 2014; Pierre et al., 2014). All experiments were performed in triplicates,
533 each biological repeat corresponding to an independent caisson.

534 The *M. truncatula* *Tnt1* insertional mutant *nac969* (R108 genotype) was obtained from
535 the Noble Foundation (USA; <https://medicago-mutant.dasnr.okstate.edu/mutant/index.php>;
536 line NF9286). *Tnt1* insertion sites and primers used for genotyping are detailed in Fig. 3A and
537 Table S7, respectively. *M. truncatula* seeds were sterilized and germinated as described
538 above. Seedlings were transferred to a growth chamber (22°C, 16h light 8h darkness, 160
539 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 70% hygrometry) in pots containing perlite/sand (3:1 v/v) and irrigated with a
540 PlantPro solution without N at $1\text{g}\cdot\text{L}^{-1}$ (Fertil, Ref. 211). After seven days, plants were
541 inoculated as described above. Nodules were collected seven weeks post-inoculation to
542 analyse gene expression and N fixation activity, from five to eight plants, grown within two
543 independent pots, and three biological repetitions were performed.

544

545 **RNA extraction and Illumina sequencing**

546 RNAs were prepared as described (Sallet et al., 2013). Briefly, total plant and bacterial
547 RNAs were extracted using the MirVana™ miRNA isolation kit (Ambion), treated with the
548 Turbo DNase (Ambion) and purified with the SurePrep™ RNA cleanup and concentration
549 kit (Fisher BioReagents™). A ribosomal RNA depletion was performed, using an
550 oligocapture strategy based on the Plant Ribominus kit (Invitrogen), with an oligonucleotide
551 set specifically targeting *M. truncatula* and *S. meliloti* rRNAs and the highly abundant *S.*
552 *meliloti* tRNA-Alanine. Samples were then once more treated with the Turbo DNase
553 (Ambion) and purified on Zymo Research RNA Clean & Concentrator TM-5 columns
554 (Proteogene). RNA sample quality was assessed using the RNA 6000 Nano kit and a
555 Bioanalyser (Agilent).

556 RNA-seq libraries were prepared according to Illumina's protocols and the Illumina
557 TruSeq Stranded mRNA sample prep kit. Oriented paired-ended RNA sequencing (2 x 150
558 bp) was performed on Illumina HiSeq3000.

559

560 **RNA-seq reads mapping and transcriptome analysis**

561 Reads were mapped on the merged *M. truncatula* Jemalong A17 (Genbank_accession:
562 PSQE00000000.1) and *S. meliloti* 2011 (Genbank accessions: CP004138.1, CP004139.1,
563 CP004140.1) genomes using the Glint software ([http://lipm-
564 bioinfo.toulouse.inrae.fr/download/glint/](http://lipm-bioinfo.toulouse.inrae.fr/download/glint/)) with parameters set as follows: matches ≥ 75
565 nucleotides, ≤ 5 mismatches, no gap allowed. Only the best unambiguous hits were kept and
566 pair counts were performed at the gene level. The Legoo knowledge base ([https://lipm-
567 browsers.toulouse.inra.fr/k/legoo/](https://lipm-browsers.toulouse.inra.fr/k/legoo/)) was used to identify genes of interest (Carrère et al., 2020),
568 as well as the Mapman software (<https://mapman.gabipd.org/>) for the analysis of gene
569 functions (Thimm et al., 2004). The data have been deposited with links to BioProject
570 accession number PRJNA813411 in the NCBI BioProject database
571 (<https://www.ncbi.nlm.nih.gov/bioproject/>).

572

573 **Real-time PCR analyses**

574 For real time RT-PCR, total RNAs were isolated from *M. truncatula* nodules using the
575 Plant RNeasy mini kit (Invitrogen), and subjected to a DNase I treatment (Fermentas). The
576 first-strand cDNA was synthesized from 1µg of total RNA using the SuperScript II First
577 Strand Synthesis kit according to the manufacturer's instructions (Invitrogen). Primers were
578 designed using the Primer 3 software (<http://frodo.wi.mit.edu/primer3/>). Primer combinations
579 displaying a minimum amplification efficiency of 85% were used in real-time RT-PCR
580 experiments (Table S7), and the specificity was checked using a dissociation curve. RT-PCR
581 reactions were performed on a Roche Light Cycler 480 using the Light Cycler Fast Start DNA
582 Master SYBR Green I kit according to manufacturer's instructions (Roche). Cycling
583 conditions were as follows: 95°C for 10 min, 50 cycles at 95°C for 5 sec, 60°C for 5 sec, and
584 72°C for 15 sec.

585 Expression levels were calculated from one or two technical replicates (based on two
586 independent cDNA syntheses derived from the same RNA sample) for each of the three
587 independent biological experiments. All expression values were normalized with the
588 expression of two reference genes, *MtACT* and *MtRBP1*, or *Mtc27* and *A38*, or *Mt0085* and
589 *Mt0089* (primers listed in Table S7). The experimental control condition was used as a
590 calibrator to determine gene expression fold changes.

591

592 **Cloning procedures**

593 The RNAi construct was obtained by amplification of the *MtS40* cDNA with the
594 RNAi-MtS40-F and RNAi-MtS40-R primers (Table S7), to generate a 160 bp PCR product
595 which was inserted into the pDONR207 vector (Invitrogen), and then recombined into the
596 pK7GWIWG6D(II),0 plasmid using Gateway Technology (Pierre et al., 2014). Efficiencies of
597 RNAi and absence of off-targets were predicted for both constructs using the RNAiScan tool
598 (Xu et al., 2006)(<https://www.zhaolab.org/pssRNAi/>). A control experiment was conducted
599 with the pK7GWIWG2(II) vector expressing a GFP reporter gene (Marino et al., 2011).

600 For the *pCP6:IPT* cloning, the *pCP6* promoter region was isolated from a *pCP6:GUS*
601 construct (Pierre et al., 2014) as a 1.7 kb *KpnI* - *AatII* fragment, and inserted into a pFRN
602 binary vector upstream of the *Agrobacterium tumefaciens IPT* transgene (kindly provided by
603 Thomas Schmülling (Faiss et al., 1997); Mathias Brault, IPS2, unpublished).

604

605 **Microscopy analyses**

606 Nodules (15 from five plants per genotype) were embedded in 5% agarose (Eurobio).
607 Longitudinal sections (100 μm) were cut with a vibratome (Leica VT1200S), mounted in
608 water, and imaged using an optical microscope (Olympus BX53). For starch detection, nodule
609 sections were stained using a 5% lugol solution (Sigma 62650) for 5-10 min, then rinsed, and
610 mounted in water.

611 For semi-thin sections, nodules were immersed in a fixative mixture (3%
612 glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate pH 6.8) for three hours,
613 and washed with 0.1 M cacodylate buffer, pH 6.8. Nodules were then dehydrated through
614 graded concentration of ethanol (10-20-30-50-70-90-100%-absolute ethanol-acetone),
615 infiltrated with epoxy resin (low viscosity Premix Kit medium, Agar), and polymerized 16h at
616 60°C. Semi-thin sections (200 nm) were cut with an ultramicrotome EM UC6 (Leica
617 Microsystems), collected on a glass slide, stained with 1% toluidine blue during one minute,
618 and observed on a Macroscope (Nikon AZ 100).

619

620 **Acetylene Reduction Assays**

621 N fixation activity was determined by acetylene reduction assays (ARA), as previously
622 described (Boivin et al., 2016). Briefly, nodules were detached from plant roots and put inside
623 sealed vials. In each vial, part of the atmosphere was substituted with acetylene. After 1-2
624 hours of incubation, gas samples were analyzed using a gas chromatographer (Agilent).

625

626 **Statistical analyses**

627 For RNAseq datasets, the EdgeR Bioconductor package version 3.30.3 (Robinson and
628 Smyth, 2008) was used to detect DEGs. Genes showing no count in any library were
629 removed. After normalization (trimmed mean of Mvalues method, Robinson and Oshlack,
630 2010), principal component analysis (Ade4 version 1.7-15 package, Dray and Dufour, 2007)
631 and Spearman correlation were used to assess the quality and reproducibility of biological
632 repeats. Heatmaps (package pheatmap version 1.0.12 (<https://CRAN.Rproject.org/package/pheatmap>))
633 were generated to illustrate sample-to-sample Euclidean distances. Fitted
634 generalized linear models (GLM) with a matrix multiple factor (biological repeats and factors

635 of interest) were designed for further analyses with the Cox-Reid profile-adjusted likelihood
636 method to estimate dispersions. DEGs were called using the GLM likelihood ratio test using
637 an FDR (Benjamini and Yekutieli, 2001) adjusted P value < 0.05 . Clustering on filtered DEGs
638 (P value < 0.05 in at least one biological condition) was generated with the heatmap.2
639 function available in the gplots Bioconductor package version 3.0.1.
640 (<https://CRAN.Rproject.org/package=gplots>) using a Ward's minimum variance clustering
641 method on Euclidean distances (Murtagh and Legendre, 2014).

642 For other experiments, dedicated statistical tests were performed using either the
643 XLSTAT or PRISM GraphPad softwares, as detailed in figure legends.

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655

656 **Author contributions**

657 CB, FF, PF defined the project; AR, CM, EB, LS, EH, MD, SV, TK performed
658 experiments; ACC, AR, BG, BR, CB, CM, EB, EH, EM, FF, LD, LL, LS, MFJ, PF, TK, VG
659 analyzed the data; CB and FF drafted the manuscript, with help from BG, EB, EM, LD, LS,
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661

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926 **Figure legends**

927 **Figure 1. Venn and upset plot analyses of plant and bacterial DEGs.**

928 A. Venn diagram analysis of the *M. truncatula* and *S. meliloti* genes differentially expressed
929 in at least one of the four senescence-inducing conditions (KNO₃, NaCl, 28 dpi whole
930 nodules, and the proximal part of 21 dpi nodules), generated using
931 <https://bioinformatics.psb.ugent.be/webtools/Venn/>. Genes differentially expressed in at least
932 three of the four senescence-inducing conditions analyzed are surrounded with a blue line. B.
933 Upset plot analysis of *M. truncatula* (grey bars) and *S. meliloti* (blue bars) genes upregulated
934 in at least one of the four senescence inducing conditions (KNO₃, NaCl, 28 dpi whole
935 nodules, and the proximal part of 21 dpi nodules), indicated by dots below the graph.

936

937 **Figure 2. Nodule senescence leads to a biased expression of plasmidic genes and an**

938 **induction of the general stress response.** A. Biased distribution of DEGs on the different *S.*
939 *meliloti* replicons. *S. meliloti* genes differentially regulated in at least three of the four
940 senescence-inducing conditions (KNO₃, NaCl, 28 dpi whole nodules, and the proximal part of
941 21 dpi nodules) were used for this analysis. Proportions of up- (blue) and down- (red)
942 regulated genes belonging to the chromosome, the pSymA, or the pSymB megaplasmid,
943 expressed in %, are compared to their proportions of occurrence in these different replicons
944 (grey). Total numbers of genes in the different replicons are 1858 (pSymA), 2025 (pSymB),
945 and 5113 (chromosome). B. Induction of the general stress response. Venn diagram showing
946 the proportion (in %) of up-regulated *S. meliloti* genes known to be transcriptionally
947 controlled by the sigma factor RpoE2, the regulator of the general stress response (see also
948 Table S4), in each of the tested conditions.

949

950 **Figure 3. Nodule N fixation and senescence phenotypes of the *nac969* mutant.**

951 A. Localization of the *Tnt1* insertion in the *NAC969* gene. The localization of the *Tnt1*
952 insertion in the *NAC969* gene of the NF9286 mutant line is shown, as well as the LTR4-
953 >LTR6 orientation. Red boxes, NAC domain; dotted line, exon; and full lines, exon outside
954 the NAC domain. B. Total nodule number per plant. C. Mean nodule fresh weight (in g). D.
955 Specific N fixation activity of Wild-Type (WT) or *nac969* nodules evaluated using an ARA
956 relative to nodule weight (nmol ethylene.h⁻¹.mg⁻¹). In (B-D), a Mann and Whitney test was
957 performed ($\alpha < 0.05$, n=50 from two independent experiments), and statistical differences are

958 indicated with asterisks. E. Expression of the *MtCP5* senescence marker in WT or *nac969*
959 nodules analyzed by qRT-PCR. Means of three biological replicates are shown, each replicate
960 containing a pool of nodules from five plants, and error bars indicate the standard deviation. A
961 Mann and Whitney test was performed ($\alpha < 0.05$), and statistical differences are indicated with
962 asterisks. F, G. Histological structure of nodule sections in optical microscopy. F, Thick
963 vibratome sections (100 μm). From left to right, WT nodules, in bright field or in dark field
964 after a lugol staining of amyloplasts (brown color); and *nac969* mutant nodules, in the same
965 conditions. The senescence zone is indicated with a black line in bright field pictures.
966 Representative samples from two independent experiments are shown. Scale bars = 500 μm .
967 G, Semi-thin ultramicrotome sections (200nm). From left to right, WT and *nac969* mutant
968 nodules, in bright field after a toluidine blue staining of cells. Representative samples from
969 two independent experiments are shown. Scale bars = 500 μm . H. Relative length of the
970 senescence zone in WT or *nac969* mutant nodules. Pools of $n=50$ nodules from two
971 independent experiments were used ($n > 12$ plants). Error bar represents confidence interval
972 ($\alpha=5\%$). The asterisk represents a statistical difference based on a Student's t test ($P < 0.05$).

973

974 **Figure 4. *MtS40* is a negative regulator of nodule senescence.**

975 A. Expression of *MtS40* in control or *S40-RNAi* nodules. B. N_2 fixation activity of control or
976 *S40-RNAi* nodules evaluated using an ARA, normalized by the nodule fresh weight (FW).
977 Error bars indicate the standard deviation. C. Expression of *Leghemoglobin Lb1* (left graph)
978 and *NCR001* (middle graph), used as markers of the N fixation region, and of *CP6* (right
979 graph), used as a senescence marker, in control or *S40-RNAi* nodules, analyzed by qRT-PCR
980 and normalized relative to the control, as indicated by the dotted line. Means of three
981 biological replicates are shown, and error bars indicate the standard deviation. A Mann and
982 Whitney test ($\alpha < 0.05$, $n = 3$ biological replicates with 8 plants) was performed, and
983 statistical differences are indicated with letters.

984

985 **Figure 5. A *pCP6:IPT* construct decreases nitrogen fixation.**

986 A. N fixation activity of *pCP6:GUS* (control) or *pCP6:IPT* nodules evaluated using an ARA,
987 normalized by plant ($\text{nmol ethylene.h}^{-1}$, left graph) or by the nodule fresh weight (FW, nmol
988 $\text{ethylene.h}^{-1}.\text{mg}^{-1}$, right graph). A Mann and Whitney test was performed ($\alpha < 0.05$, $n > 10$
989 plants/condition), and statistical differences are indicated with letters. B. Expression of the *A.*
990 *tumefaciens IPT* transgene (upper left graph), of a *Leghemoglobin (Lb1)* gene (upper right

991 graph) and of *NCR001* (lower left graph), used as markers of the N fixation region, and of
992 *CP5* (lower right graph), used as a senescence marker, in WT or *pCP6:IPT* nodules, analyzed
993 by qRT-PCR and normalized relative to the *pCP6:GUS* control, as indicated by the dotted
994 line. Means of three biological replicates are shown, and error bars indicate the standard
995 deviation. A Mann and Whitney test was performed ($\alpha < 0.05$, $n = 3$ biological replicates with
996 12 plants), and statistical differences are indicated with letters.

997

998 **List of Tables**

999 **Table 1.** Selected *S. meliloti* genes downregulated in at least three nodule senescence-
1000 inducing conditions.

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1002 inducing conditions.

1003 **Table 3.** Selected *M. truncatula* genes downregulated in at least three nodule senescence-
1004 inducing conditions.

1005

1006 **Supplementary Material**

1007 **Table S1.** Summary of libraries and sequencing.

1008 **Table S2.** RNAseq expression data of *S. meliloti* genes.

1009 **Table S3.** RNAseq expression data of *M. truncatula* genes.

1010 **Table S4.** *S. meliloti* RpoE2-dependent genes upregulated in senescence-inducing conditions.

1011 **Table S5.** *S. meliloti* cell cycle genes upregulated in senescence-inducing conditions.

1012 **Table S6.** Enrichment for Mapman bins in senescence-inducing conditions.

1013 **Table S7.** Primers used in this study.

1014

1015 **Figure S1. Selection of the senescence-inducing conditions to be used in the RNAseq**
1016 **analysis.**

1017 A, B. qRT-PCR analysis of *CP* gene expression. *MtCP5* (upper panels) and *MtCP6* (lower
1018 panels) genes were selected, following the nomenclature of Pierre et al., 2014. (A) For
1019 developmental senescence (aging), experiments were carried out on RNA prepared from
1020 whole nodules at 14, 21, 28 or 35 days post rhizobium inoculation (dpi). In addition, isolated
1021 proximal (P) and distal (D) parts of the same 21-35 dpi nodules were analysed. (B) For
1022 environmentally triggered senescence, RNA were extracted from whole 13 dpi nodules before
1023 (Control, Ctrl) or 24 hours after a treatment with 10 mM KNO₃ or 150 mM NaCl. In (A) and
1024 (B), results were normalized to the mean expression value of two reference genes (Mt0085
1025 and Mt0089; Satgé et al., 2016) and expressed relative to 14 dpi nodules (A), or to control
1026 nodules (B), taken as references. The means and standard errors of the means of three
1027 independent biological experiments, each carried out on nodules collected from 8-28 plants,
1028 are shown. Statistical analyses were performed on normalized expression values using a one
1029 way ANOVA, followed by a Dunnett multiple comparison test (*, P<0.05). C. N fixation
1030 efficiency measured by ARA on isolated nodules collected at 13 dpi (Ctrl) or 24 hours after a
1031 treatment with 10 mM KNO₃ or 150 mM NaCl, as indicated below the graphs. Statistical
1032 analyses were performed using a t-test (*, P<0.05). The results shown are means of three to
1033 four independent biological experiments, each carried out using 10-40 mg of nodules.

1034

1035 **Figure S2. Clustering analyses of transcriptomic data.**

1036 A. Sample-to-sample Euclidean distances using the three replicates (.1, .2, .3) of whole (*M.*
1037 *truncatula* and *S. meliloti*) normalized RNAseq data, for each experimental condition; 14, 21,
1038 28: whole nodules collected at 14, 21 or 28 dpi, respectively; 21D, 21P: distal or proximal
1039 regions of 21 dpi nodules, respectively; KNO₃ or NaCl: whole nodules collected 24h after
1040 addition of 10 mM KNO₃ or 150 mM NaCl at 13 dpi, respectively. B. Hierarchical clustering
1041 of mean normalized gene expression data and experimental conditions, using the 15731 *M.*
1042 *truncatula* (left) and 2532 *S. meliloti* (right) DEGs retrieved in at least one of the selected
1043 conditions, relative to the control (14 dpi nodules).

1044

1045 **Figure S3. Absolute expression levels of cysteine protease genes**

1046 Absolute mean expression data (normalized counts retrieved from Table S3, sheet 2) of the
1047 eleven cysteine protease genes up-regulated in at least three senescence-inducing conditions
1048 are represented in a stacked bar chart, showing their relative transcript abundance in all tested
1049 conditions.

1050

1051 **Figure S4. The *pCP6:IPT* construct does not affect nodule number or nodule weight.**

1052 Number (left graph) and weight (in mg, right graph) of nodules formed on composite plants
1053 expressing a *pCP6:GUS* or a *pCP6:IPT* construct, corresponding to the ones for which N
1054 fixation was measured in the Fig. 5. A Mann and Whitney test was performed ($\alpha < 0.05$, $n > 10$
1055 plants/condition), and statistical differences are indicated with letters.

1056 **Table 1. Selected *S. meliloti* genes downregulated in at least three nodule senescence-inducing**
 1057 **conditions**

Id ^a	Gene name	Description	Log ₂ fold-change relative to 14 dpi ^b			
			21 dpi prox	28 dpi	NaCl	KNO ₃
Nod factor biosynthesis						
SMa0852	<i>nodF</i>	NodF acyl carrier protein	-1.1	-	-1.7	-1.9
SMa0851	<i>nodH</i>	NodH sulfotransferase	-	-1.1	-1.4	-0.9
Nitrogen fixation						
SMa0834	<i>fdxB</i>	FdxB ferredoxin III	-1.1	-	-2.0	-1.5
SMa0819	<i>fixB</i>	FixB electron transfer flavoprotein alpha chain	-1.2	-	-1.8	-1.3
SMa0873	<i>nifN</i>	NifN Nitrogenase Fe-Mo cofactor biosynthesis protein	-1.1	-	-2.0	-1.2
SMa0831	<i>nifX</i>	NifX iron-molybdenum cluster-binding protein	-1.2	-	-1.9	-1.3
Bacteroid differentiation						
SMc04278	<i>acpXL</i>	Acyl carrier protein,involved in the transfer of long hydroxylated fatty acids to lipid A	-1.3	-	-1.3	-1.8
SMb20999	<i>bacA</i>	Putative transport protein,essential for bacteroid development	-1.9	-	-2.6	-2.3

1058 ^aA selection of genes of known function is shown, grouped in functional classes

1059 ^bValues are indicated when ≤ -0.85 with FDR < 0.05, otherwise noted (-)

Table 2. Selected *M. truncatula* genes upregulated in at least three nodule senescence-inducing conditions

Complete id ^a	Acronym	Description	Log ₂ fold-change relative to 14 dpi ^b			
			21 dpi prox	28 dpi	NaCl	KNO ₃
Protein degradation^c						
MtrunA17Chr4g0040881	MtCP1	cystein protease 1	1.6	-	2.8	3.8
MtrunA17Chr4g0041061	MtCP2	cystein protease 2	2.4	-	3.3	3.4
MtrunA17Chr4g0024411	MtCP3	cystein protease 3; AtSAG12-like	1.6	-	1.2	2.0
MtrunA17Chr5g0405751	MtCP4	cystein protease 4	1.9	-	2.5	3.3
MtrunA17Chr4g0060251	MtCP5	cystein protease 5	2.4	2.0	3.6	4.5
MtrunA17Chr4g0041081	MtCP6	cystein protease 6	1.6	-	2.7	3.5
MtrunA17Chr2g0315491	MtCP7	cystein protease 7	1.9	2.0	2.1	3.9
MtrunA17Chr4g0041481	MtCP8	cystein protease 8	2.1	2.6	3.9	5.1
MtrunA17Chr4g0041521	MtCP9	cystein protease 9	2.4	1.7	1.7	3.4
MtrunA17Chr1g0151801	MtVPE	Chitinase. vacuole processing enzyme	2.0	-	1.7	2.0
MtrunA17Chr2g0279261	MtCaspase	ICE-like protease (caspase) p20 domain protein	2.1	-	2.8	1.7
MtrunA17Chr6g0459491	MtTML2	TOO MUCH LOVE 2 F-box protein (AON) ^d	1.1	-	1.3	1.1
Polysaccharide degradation						
MtrunA17Chr2g0294601	MtBG15	beta-1,3-Glucanase 15	-	1.2	2.9	3.2
MtrunA17Chr2g0294591	MtBG16	beta-1,3-Glucanase 16	-	2.0	3.4	2.4
MtrunA17Chr4g0076581	MtBG18	beta-1,3-Glucanase 18	2.3	1.5	5.7	4.0
MtrunA17Chr6g0461601	MtBG32	beta-1,3-Glucanase 32	1.4	-	1.8	1.3
Hormone related functions						
MtrunA17Chr1g0149351	MtEBF1	EIN3-binding F box protein 1 (ethylene signaling)	1.4	1.6	1.1	-
MtrunA17Chr2g0313111	MtGA2ox1	Gibberellin GA2-oxidase 1 (GA inactivation)	1.1	1.9	2.4	-
MtrunA17Chr8g0377481	MtCHK2	CHASE-domain containing histidine kinase 2 (cytokinin receptor)	1.3	-	1.3	1.4
Transport						
MtrunA17Chr2g0310141	MtZIP1	Zinc-Iron Permease 1	1.4	-	1.7	2.3
MtrunA17Chr3g0102071	MtZIP7	Zinc-Iron Permease 7	2.2	1.1	-	1.7
MtrunA17Chr3g0120761	MtPT6	phosphate transporter	1.3	-	1.3	1.6
MtrunA17Chr4g0063591	MtNPF6.4	NRT1/ PTR FAMILY; nitrate / peptide transporter	3.4	3.1	3.4	2.9
MtrunA17Chr7g0217461	MtNPF7.7	NRT1/ PTR FAMILY; nitrate / peptide transporter	-	3.7	4.7	4.1
MtrunA17Chr1g0148451	MtMOT1.1	Molybdate transporter	2.0	1.6	2.7	3.3
MtrunA17Chr5g0412541	MtMATE15	multidrug and toxic compound extrusion family	1.8	1.1	2.8	1.7
MtrunA17Chr3g0132531	MtMATE16	multidrug and toxic compound extrusion family	-	2.4	5.8	4.1
MtrunA17Chr2g0317171	MtMATE43	multidrug and toxic compound extrusion family	1.3	-	3.1	2.5
MtrunA17Chr2g0317161	MtMATE44	multidrug and toxic compound extrusion family	1.3	-	1.6	1.3
MtrunA17Chr1g0185231	MtSULTR3.1	sulfate transporter	2.1	1.1	2.3	-
MtrunA17Chr3g0131951	MtSWEET13	probable Sucrose transporter	3.0	3.0	2.4	-
Phytoglobins						
MtrunA17Chr4g0034301	MtPhytogb1.1	non-symbiotic hemoglobin (ns-Hb)	1.4	-	1.4	2.1
MtrunA17Chr4g0034311	MtPhytogb1.2	non-symbiotic hemoglobin (ns-Hb)	1.1	-	2.4	1.8
Transcription regulation						
MtrunA17Chr8g0346251	MtHB1	HD-ZIP I transcription factor	1.8	-	3.4	3.0
MtrunA17Chr1g0207451	MtZpt2-2	Krüppel-like zinc finger protein	1.6	-	2.9	2.7
MtrunA17Chr3g0130731	MtNACsa	NAC domain protein	1.3	-	2.0	2.3
MtrunA17Chr4g0035421	MtATB2	S class bZIP transcription factor	1.1	-	1.1	1.0
MtrunA17Chr4g0042561	MtNAC969	NAC domain containing protein 969	2.1	2.4	5.8	4.7
MtrunA17Chr2g0285141	MtS40	Putative senescence regulator S40	1.8	2.9	5.6	4.6
MtrunA17Chr3g0122351	MtCAMTA3	Calmodulin-binding transcription activator 3	1.0	-	1.7	1.2
MtrunA17Chr5g0446491	MtNF-YB17	nuclear factor Y subunit B17	-	3.2	4.8	5.9
Small secreted peptides						
MtrunA17Chr1g0200531	MtGASA4	Gibberellic Acid-Stimulated in Arabidopsis	2.2	-	3.1	1.7
MtrunA17Chr3g0083271	MtIDA34	Inflorescence Deficient in Abscission	1.6	-	4.6	2.5
MtrunA17Chr6g0476961	MtKunitz36	Kunitz-P Trypsin Inhibitor	-	1.6	4.4	3.5
MtrunA17Chr6g0476951	MtKunitz37	Kunitz-P Trypsin Inhibitor	-	3.0	6.6	6.1
MtrunA17Chr6g0476921	MtKunitz38	Kunitz-P Trypsin Inhibitor	-	3.2	5.2	3.4
MtrunA17Chr6g0477281	MtKunitz44	Kunitz-P Trypsin Inhibitor	-	3.9	6.4	4.2
MtrunA17Chr5g0438441	MtNodGRP39	Nodule-specific Glycine-rich protein	1.4	1.8	3.9	3.1
MtrunA17Chr2g0304411	MtPRP669_10	Proline-rich Protein Group 669	1.9	-	2.4	1.8
MtrunA17Chr2g0304441	MtPRP669_8	Proline-rich Protein Group 669	2.2	1.7	1.2	1.4
MtrunA17Chr6g0460651	MtPSY8	Plant Peptide Containing Sulfated Tyrosine	2.3	-	3.2	3.5
MtrunA17Chr4g0053491	MtPIP5	PAMP-induced Secreted Peptide	2.7	3.0	2.1	-
MtrunA17Chr5g0406221	MtRTFL/DVL_10	Rotundifolia/Devil	2.2	-	4.3	2.5

MtrunA17Chr5g0442291	MtSubIn1	Subtilisin inhibitor	-	2.2	6.5	4.6
MtrunA17Chr1g0187161	MtSubIn3	Subtilisin inhibitor	-	2.3	4.3	2.8
MtrunA17Chr4g0014461	MtT2SPI2	Potato type II proteinase inhibitor	-	1.5	4.2	2.9
MtrunA17Chr2g0283451	MtTAX2	Taximin 2	1.1	1.4	4.0	2.5
MtrunA17Chr4g0040491	MtTAX3	Taximin 3	1.7	1.2	2.4	3.2
Plant defense and redox						
MtrunA17Chr2g0295371	AtPR-1-like; MtCAPE12	pathogenesis-related protein 1 (SA-inducible in Mt); CAP-derived peptide 12	-	3.3	4.1	4.0
MtrunA17Chr2g0295001	MtPR10-1	Pathogenesis-related protein 10-1 (Y08641.1)	-	1.7	1.3	1.5
MtrunA17Chr2g0295051	MtPR10-3	Pathogenesis-related protein 10 (TC76511)	-	1.2	3.3	2.3
MtrunA17Chr2g0295141	MtPR10-4	Pathogenesis-related protein 10 (TC122461)	-	2.7	4.7	2.8
MtrunA17Chr3g0145981	MtPR3	Pathogenesis related protein 3 (defense response-activated chitinase)	-	2.2	4.1	2.5
MtrunA17Chr4g0053871	MtPrx38	peroxidase	-	1.3	1.7	1.2
MtrunA17Chr5g0402571	MtPrx52	peroxidase	-	2.1	3.9	4.3
MtrunA17Chr1g0181431	MtPrx56	peroxidase	2.2	-	6.3	2.7

1062 ^aA selection of genes of known function is shown, grouped in functional classes

1063 ^bValues are indicated when ≥ 1 with FDR < 0.05, otherwise noted (-)

1064 ^cMtCP1 to MtCP6 were renamed according to Pierre et al., 2014; MtCP7 to MtCP9 nomenclature is proposed in the present study

1065 ^dAON, Autoregulation Of Nodulation

Table 3. Selected *M. truncatula* genes downregulated in at least three nodule senescence-inducing conditions

Complete id ^a	Acronym	Description	Log ₂ fold-change relative to 14 dpi ^b			
			21 dpi prox	28dpi	NaCl	KNO ₃
Early nodulation (Nod factor perception and rhizobium infection)						
MtrunA17Chr5g0403371	MtNFP	Nod factor perception	-3.1	-1.7	-1.8	-
MtrunA17Chr5g0439631	MtLYK3	LysM domain-containing receptor-like kinase 3	-3.9	-1.5	-1.8	-
MtrunA17Chr5g0413071	MtLYK10	LysM domain-containing receptor-like kinase 10 (EPR3 / exopolysaccharide receptor)	-3.1	-1.8	-1.5	-2.3
MtrunA17Chr5g0439621	MtLYK4	LysM domain-containing receptor-like kinase 4	-4.3	-1.3	-1.6	-
MtrunA17Chr5g0432401	MtPrx01; MtRIP1	Rhizobium-induced peroxidase 1; peroxidase 29	-4.9	-2.8	-3.3	-3.4
MtrunA17Chr3g0114841	MtNSP2	Nodulation Signaling Pathway 2	-4.2	-1.4	-3.0	-
MtrunA17Chr7g0253421	MtERN1	ERF required for nodulation-1	-5.2	-2.4	-1.2	-
MtrunA17Chr1g0197491	MtRPG	Rhizobium-directed polar growth	-1.0	-1.1	-1.7	-2.4
MtrunA17Chr1g0177091	MtNF-YA1	Nuclear factor Y subunit A1	-1.7	-1.3	-2.1	-1.6
MtrunA17Chr3g0082991	MtENOD11	Early nodulin 11	-5.2	-1.9	-6.4	-3.0
MtrunA17Chr3g0082981	MtENOD12	Early nodulin 12	-4.0	-1.6	-4.3	-1.7
MtrunA17Chr8g0386521	MtSYMREM 1; MtREM2.2	Symbiotic remorin 1; remorin 2.2	-1.3	-	-2.2	-1.2
MtrunA17Chr4g0065501	MtNFB1	Nod factor hydrolase 1 (MtCHIT5)	-1.7	-	-1.9	-1.4
MtrunA17Chr3g0082921	MtENOD10	Early nodulin 10	-1.7	-	-2.3	-2.6
MtrunA17Chr4g0073901	MtENOD16; MtENODL27	Early nodulin 16; ENOD-like 27; Plantacyanin/Chemocyanin 70	-1.8	-1.1	-2.4	-1.6
MtrunA17Chr4g0073921	MtENOD20; MtENODL28; MtPCY72	Early nodulin 20; ENOD-like 28; Plantacyanin/Chemocyanin 72	-1.9	-	-2.5	-2.3
MtrunA17Chr3g0111961	MtCCS52b	cell cycle switch gene 52 b (anaphase-promoting complex activator)	-3.8	-	-2.8	-1.8
MtrunA17Chr5g0414651	MtMMPL1; MtN9	Matrix metalloproteinase-like 1; Nodulin9	-4.6	-1.8	-3.7	-2.9
Nodule development						
MtrunA17Chr7g0260971	MtLBD16	LOB Domain protein 16	-2.7	-2.2	-2.2	-
MtrunA17Chr2g0328971	MtPLT1	Plethora 1 ERF	-3.4	-1.6	-2.6	-
MtrunA17Chr5g0412151	MtPLT3	Plethora 3 ERF	-2.5	-1.4	-1.7	-
MtrunA17Chr7g0249621	MtPLT4	Plethora 4 ERF. BBM (BABYBOOM)-like	-2.0	-1.5	-2.0	-
MtrunA17Chr3g0092801	MtN11	Mtnodulin 11	-2.3	-	-2.3	-2.7
MtrunA17Chr4g0067921	MtN13	Mtnodulin 13	-4.2	-1.3	-1.4	-1.8
MtrunA17Chr4g0064831	MtN15; MtPDF2	Mtnodulin 15; Plant Defensin 2	-5.6	-2.1	-1.8	-
MtrunA17Chr6g0488881	MtN18	Nodulin 18	-3.6	-1.3	-2.8	-1.6
MtrunA17Chr5g0443191	MtN2	Nodulin 2	-4.1	-1.7	-4.0	-3.0
MtrunA17Chr3g0081511	MtN21	Nodulin 21	-	-1.1	-2.8	-1.3
MtrunA17Chr6g0450781	MtN24	Nodulin n 24	-1.3	-1.3	-1.9	-1.3
MtrunA17Chr7g0273961	MtN26	Nodulin 26	-1.4	-1.5	-2.5	-1.6
MtrunA17Chr5g0445131	MtN5	Nodulin 5	-1.8	-1.4	-	-2.4
MtrunA17Chr1g0180241	MtN6	Nodulin 6	-4.4	-1.2	-1.5	-2.0
MtrunA17Chr3g0111421	MtN7	Nodulin 7	-2.8	-1.1	-1.8	-1.5
MtrunA17Chr8g0379901	MtNIP1	NOD26-like intrinsic protein 1 (aquaporin)	-	-1.2	-1.7	-1.8
MtrunA17Chr8g0379911	MtNIP2	NOD26-like intrinsic protein 2 (aquaporin)	-1.1	-1.1	-1.4	-1.0
MtrunA17Chr3g0132471	MtHLH1	basic helix-loop-helix transcription factor 1	-1.3	-1.6	-1.6	-2.1
Bacteroid differentiation/maintenance						
MtrunA17Chr3g0137171	MtEFD2	AP2/ERF required for Nodule Differentiation 2	-2.8	-3.0	-1.5	-3.4
MtrunA17Chr4g0050851	MtSEN1	Stationary Endosymbiont Nodule 1 (Vacuolar iron transporter-like protein. symbiosome differentiation)	-1.8	-1.0	-2.5	-1.8
MtrunA17Chr7g0239441	MtRSD	regulator of symbiosome differentiation	-1.7	-1.1	-1.1	-
MtrunA17Chr3g0087791	MtDNF1	Does not fix nitrogen 1 (Signal peptidase complex 22-kD subunit, SPC22)	-1.7	-1.3	-1.6	-
MtrunA17Chr1g0148521	MtMOT1.2	Molybdate transporter	-	-1.7	-2.2	-3.5
MtrunA17Chr3g0105081	MtMOT1.3	Molybdate transporter	-	-2.0	-7.4	-6.9
MtrunA17Chr1g0198151	MtDME	DEMETER (DNA demethylase)	-1.7	-	-2.0	-1.5
MtrunA17Chr5g0436541	MtDAS25	DNF1-associated SPC25 (signal peptidase complex subunit)	-2.1	-1.7	-2.0	-1.3
Cyclins						
MtrunA17Chr3g0125051	MtCycA1;1	Cyclin A1;1	-3.9	-1.9	-3.4	-2.1
MtrunA17Chr2g0332171	MtCycA2;1	Cyclin A2;1	-3.0	-	-2.9	-2.1
MtrunA17Chr2g0332181	MtCycA2;2	Cyclin A2;2	-2.9	-1.1	-2.7	-1.6

MtrunA17Chr3g0138431	MtCycA2;4	Cyclin A2;4	-3.9	-	-3.8	-2.3
MtrunA17Chr3g0134401	MtCycA3;2	Cyclin A3;2	-1.8	-	-2.7	-3.5
MtrunA17Chr3g0134411	MtCycA3;3	Cyclin A3;3	-3.5	-1.7	-2.7	-2.5
MtrunA17Chr3g0134421	MtCycA3;4	Cyclin A3;4	-3.6	-2.7	-3.8	-3.9
MtrunA17Chr3g0134441	MtCycA3;6	Cyclin A3;6	-5.3	-3.0	-4.7	-4.0
MtrunA17Chr7g0255771	MtCycB1;1	Cyclin B1;1	-3.7	-1.0	-3.8	-1.7
MtrunA17Chr5g0441371	MtCycB1;2	Cyclin B1;2	-3.5	-1.2	-4.2	-1.4
MtrunA17Chr5g0406741	MtCycB2;1	Cyclin B2;1	-4.0	-1.4	-3.9	-1.6
MtrunA17Chr8g0370671	MtCycB2;2	Cyclin B2;2	-4.3	-1.3	-3.9	-2.0
MtrunA17Chr1g0208231	MtCycD3;1	Cyclin D3;1	-2.0	-1.0	-1.6	-1.3
MtrunA17Chr3g0134261	MtCycD3;2	Cyclin D3;2	-2.3	-1.4	-2.5	-
MtrunA17Chr4g0012811	MtCycU2;2	Cyclin U2;2	-1.2	-1.6	-1.9	-1.9
Phytoglobins						
MtrunA17Chr1g0170851	MtLb10	leghemoglobin10	-1.5	-	-3.2	-1.4
MtrunA17Chr5g0427351	MtLb2	leghemoglobin2 (previously Lb1) ^c	-1.7	-1.4	-3.5	-2.1
MtrunA17Chr5g0417631	MtLb5	leghemoglobin5	-1.3	-	-3.3	-1.4
MtrunA17Chr5g0435621	MtLb6	leghemoglobin6	-1.1	-	-3.1	-1.7
MtrunA17Chr5g0435991	MtLb9	leghemoglobin9	-1.6	-1.4	-3.6	-1.9
Hormone-related functions						
MtrunA17Chr7g0264181	MtLOG1	cytokinin biosynthesis. Lonely Guy 1	-3.9	-1.8	-3.1	-3.3
MtrunA17Chr1g0177391	MtLOG3	cytokinin biosynthesis. Lonely Guy 3	-3.0	-1.3	-2.5	-2.1
MtrunA17Chr1g0209731	MtIPT1	cytokinin biosynthesis. Isopentenyl transferase 1	-2.8	-	-2.1	-1.5
MtrunA17Chr4g0065731	MtIPT2	cytokinin biosynthesis. Isopentenyl transferase 2	-4.8	-1.6	-3.4	-1.9
MtrunA17Chr4g0037361	MtLAX5	Like Aux1 (auxin influx carrier)-5	-2.0	-1.4	-2.9	-
MtrunA17Chr6g0474391	MtGA20ox1	gibberellin biosynthesis. Gibberellin 20 oxidase	-1.3	-1.0	-2.4	-2.0
MtrunA17Chr3g0125421	MtGA20ox4	gibberellin biosynthesis. Gibberellin 20 oxidase	-5.1	-2.5	-5.1	-2.0
MtrunA17Chr4g0037611	MtGA20ox10	GA inactivation. Gibberellin GA2-oxidase 10	-3.7	-2.0	-4.5	-3.2
MtrunA17Chr2g0332191	MtGA3OX1	gibberellin biosynthesis. Gibberellin 3-oxidase 1; Gibberellin 3-beta-dioxygenase	-2.9	-1.6	-2.0	-
MtrunA17Chr1g0199681	MtARF1; MtARF16a	auxin response factor 1 / 16a	-1.6	-1.8	-2.3	-1.8
MtrunA17Chr3g0113261	ASA-2	auxin biosynthesis. anthranilate synthase	-2.2	-	-2.8	-2.6
MtrunA17Chr6g0457421	AtCYP735A1-like	cytokinin biosynthesis. CK hydroxylase	-5.3	-2.1	-1.6	-3.0
MtrunA17Chr7g0255941	MtPIN10	pin-formed auxin efflux carrier component	-1.1	-	-1.4	-1.1
MtrunA17Chr4g0066931	MtPUCHI	Auxin-responsive. Putative transcription factor AP2-EREBP family	-4.2	-2.3	-3.0	-3.6
MtrunA17Chr1g0184911	MtD27	strigolactone biosynthesis. DWARF27	-4.3	-1.8	-6.3	-2.2
MtrunA17Chr4g0054371	MtACS2	1-aminocyclopropane-1-carboxylate synthase (ethylene synthase)	-1.8	-2.8	-2.5	-4.7
MtrunA17Chr3g0118211	MtRRA2	cytokinin signaling. Type-A response regulator	-	-1.2	-1.7	-1.3
MtrunA17Chr3g0124861	MtRRA3	cytokinin signaling. Type-A response regulator	-4.3	-1.7	-2.4	-1.7
MtrunA17Chr5g0414931	MtRRA4;MtRR4	cytokinin signaling. Type-A response regulator	-2.9	-1.0	-1.1	-
MtrunA17Chr7g0218001	MtCPS1	gibberellin biosynthesis. Ent-copalyl diphosphate synthetase 1	-3.2	-	-3.1	-1.9
MtrunA17Chr2g0299371	MtCKX3	Cytokinin inactivation. Cytokinin oxidase 3	-2.0	-2.2	-3.4	-3.2
MtrunA17Chr4g0040971	MtCLE12	CLAVATA3/Endosperm Surrounding Region-related (CLE) family 12 (AON) ^d	-3.9	-2.4	-1.9	-
MtrunA17Chr4g0040951	MtCLE13	CLAVATA3/Endosperm Surrounding Region-related (CLE) family 13 (AON) ^d	-1.8	-1.3	-1.3	-
Prevention of plant defense responses						
MtrunA17Chr7g0222521	MtNAD1	Nodules with Activated Defense 1	-1.0	-1.7	-2.7	-1.7
MtrunA17Chr3g0119041	MtSymCRK	Symbiotic cys-rich receptor-like kinase	-	-1.1	-2.1	-1.2

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^aA selection of genes of known function is shown, grouped in functional classes

^bValues are indicated when ≤ -1 with FDR < 0.05, otherwise noted (-)

^cLarrainzar et al., 2020

^dAON, Autoregulation Of Nodulation