

2 **Title of article:** Role of Cytosolic, Tyrosine-Insensitive Prephenate Dehydrogenase in
3 *Medicago truncatula*

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27 **ABSTRACT**

28 L-Tyrosine (Tyr) is an aromatic amino acid synthesized *de novo* in plants and microbes
29 downstream of the shikimate pathway. In plants, Tyr and a Tyr pathway intermediate,
30 4-hydroxyphenylpyruvate (HPP), are precursors to numerous specialized metabolites, which are
31 crucial for plant and human health. Tyr is synthesized in the plastids by a TyrA family enzyme,
32 arogenate dehydrogenase (ADH/TyrA_a), which is feedback inhibited by Tyr. In addition to ADH
33 enzymes, many legumes possess prephenate dehydrogenases (PDH/TyrA_p), which are insensitive
34 to Tyr and localized to the cytosol. Yet the role of PDH in legumes is currently unknown. This
35 study isolated and characterized *Tnt1*-transposon mutants of *MtPDH1* (*pdh1*) in *Medicago*
36 *truncatula* to investigate PDH function. The *pdh1* mutants lacked *PDH* transcript, PDH activity,
37 and displayed little aberrant morphological phenotypes under standard growth conditions
38 providing genetic evidence that *MtPDH1* is responsible for the PDH activity detected in *M.*
39 *truncatula*. Though plant PDH enzymes and activity have been specifically found in legumes,
40 nodule number and nitrogenase activity of *pdh1* mutants were not significantly reduced
41 compared to wild-type (Wt) during symbiosis with nitrogen-fixing bacteria. Although Tyr levels
42 were not significantly different between Wt and mutants under standard conditions, when carbon
43 flux was increased by shikimate precursor feeding, mutants accumulated significantly less Tyr
44 than Wt. These data suggest that *MtPDH1* is involved in Tyr biosynthesis when the shikimate
45 pathway is stimulated, and possibly linked to unidentified legume-specific specialized
46 metabolism.

47 INTRODUCTION

48 L-Tyrosine (Tyr) is an aromatic amino acid synthesized *de novo* in plants and microbes,
49 but not animals; thus, humans must acquire Tyr through their diet or by enzymatic conversion of
50 L-phenylalanine (Phe, Fitzpatrick, 1999). In addition to its involvement in protein synthesis, Tyr
51 and a Tyr-pathway intermediate 4-hydroxyphenylpyruvate (HPP) are the precursors to numerous
52 specialized metabolites crucial for plant and animal health (Schenck and Maeda, 2018).
53 Tyr-derived plant specialized metabolites have roles as photosynthetic electron carriers
54 (plastoquinone, Metz et al., 1989), pollinator attractors (betalain pigments, Strack et al., 2003)
55 and in defense (dhurrin and rosmarinic acid, Møller, 2010; Petersen, 2013). In grasses, Tyr also
56 serves as a precursor to lignin, the main structural polymer in plants (Higuchi et al., 1967; Rosler
57 et al., 1997; Barros et al., 2016). Humans have co-opted some of these natural products to serve
58 nutritional and medicinal roles such as some benzylisoquinoline alkaloids, which have
59 antitussive, analgesic, and antimicrobial activities (Barken et al., 2008; Beaudoin and Facchini,
60 2014; Kries and O'Connor, 2016), and the antioxidant properties of tocopherols (collectively
61 referred to as vitamin E, Bramley et al., 2000).

62 The aromatic amino acids (AAAs; Tyr, Phe, and tryptophan [Trp]) are synthesized in the
63 plastids from chorismate, the final product of the shikimate pathway (Tzin and Galili, 2010;
64 Maeda and Dudareva, 2012). Chorismate mutase (CM, EC number 5.4.99.5) catalyzes the
65 committed step of Tyr and Phe synthesis — the isomerization of chorismate into prephenate
66 (Goers and Jensen, 1984; Kuroki and Conn, 1989; Eberhard et al., 1996; Mobley et al., 1999).
67 Prephenate is converted to Tyr via two reactions, oxidative decarboxylation catalyzed by a TyrA
68 dehydrogenase enzyme and transamination. These reactions can occur in either order, leading to
69 alternative Tyr pathways (**Fig. 1**, Schenck and Maeda, 2018). In most microbes, a
70 prephenate-specific TyrA dehydrogenase (PDH/TyrA_p EC 1.3.1.13, **Fig. 1**) first converts

71 prephenate into HPP followed by transamination to Tyr via Tyr-aminotransferase (Tyr-AT or
72 TyrB, EC2.6.1.5). In plants, these reactions occur in the reverse order with transamination of
73 prephenate to form aroenate by a plastidic prephenate aminotransferase (PPA-AT, EC 2.6.1.78),
74 followed by oxidative decarboxylation catalyzed by a plastidic aroenate-specific TyrA
75 dehydrogenase (ADH/TyrA_a EC 1.3.1.78) producing Tyr (**Fig. 1**). The ADH-mediated Tyr
76 pathway appears to be essential for normal growth and development in plants as indicated by the
77 severe phenotype of *Arabidopsis thaliana adh2/tyra2* knockout mutant, which was further
78 exacerbated by transient suppression of the other *ADH1/TyrA1* gene (de Oliveira et al., 2019).
79 PDH and ADH are the key enzymes in their respective pathways, as they compete for substrates
80 that are shared with Phe biosynthesis, and are generally feedback inhibited by Tyr (Rubin and
81 Jensen, 1979; Gaines et al., 1982; Connelly and Conn, 1986; Rippert and Matringe, 2002).

82 PDH activity, which is commonly found in microbes, has been detected in tissue extracts
83 of some plants, all restricted to the legume family (Gamborg and Keeley, 1966; Rubin and
84 Jensen, 1979; Siehl, 1999). In soybean, leaf tissue had the highest PDH activity of all analyzed
85 tissues (Schenck et al., 2015). Phylogenetic analyses of plant and microbial TyrAs showed that
86 *PDH* genes are uniquely present in legumes, suggesting that legume PDH enzymes are the result
87 of a recent legume-specific gene duplication event of a plant *ADH* rather than horizontal gene
88 transfer from a *PDH*-possessing rhizobia (Schenck et al., 2015). However, not all legumes
89 possess *PDH*, which suggests gene loss in some legumes (Schenck et al., 2015; Schenck et al.,
90 2017a). PDH recombinant enzymes from *Glycine max* (soybean; GmPDH1 and GmPDH2) and
91 *Medicago truncatula* (Medicago; MtPDH1) preferred prephenate over aroenate as their
92 substrates (Schenck et al., 2015; Schenck et al., 2017a) and, unlike plant ADH enzymes, were
93 insensitive to Tyr inhibition and localized to the cytosol (Schenck et al., 2015; Schenck et al.,
94 2017a). These recently diverged plant ADH and PDH enzymes were used to identify a single

95 amino acid residue (Asn222 of GmPDH1) of TyrA dehydrogenases that switches TyrA substrate
96 specificity and underlies the evolution of legume PDH enzymes (Schenck et al., 2017a; Schenck
97 et al., 2017b). While these biochemical and evolutionary studies established that some legumes
98 have an alternative Tyr-insensitive cytosolic PDH enzyme, its *in vivo* function is unknown.

99 To address this issue, we hypothesized and tested four non-mutually exclusive *in planta*
100 functions of the PDH enzyme in legumes. **Hypothesis I: PDH functions as a part of a**
101 **redundant Tyr biosynthetic pathway in the cytosol (Fig. 1)**. Although AAA biosynthesis is
102 localized to the plastids (Bickel et al., 1978; Jung et al., 1986), some plants including legumes
103 identified cytosolic isoforms of CM and Tyr-AT, which catalyze immediately up- and
104 down-stream steps of PDH and complete the cytosolic Tyr biosynthetic pathway from chorismate
105 (**Fig. 1**) (D'Amato et al., 1984; Eberhard et al., 1996; Ding et al., 2007; Schenck et al., 2015;
106 Wang et al., 2016). Also, a cytosolic Phe pathway was recently identified in plants (Yoo et al.,
107 2013; Qian et al., 2019). **Hypothesis II: PDH is involved in the production of Tyr or**
108 **HPP-derived metabolite(s) (Fig. 1)**. Tyr is the precursor of many plant specialized metabolites
109 (Schenck and Maeda, 2018) and duplicated primary metabolic enzymes can be co-opted to
110 efficiently provide Tyr and HPP precursors to support their downstream specialized metabolism
111 (Weng et al., 2012; Moghe and Last, 2015; Maeda, 2019). **Hypothesis III: PDH is involved in**
112 **the Tyr catabolism pathway to the tricarboxylic acid (TCA) cycle**. Tyr catabolism proceeds
113 through HPP, the product of PDH, and feeds intermediates (e.g. fumarate) of the TCA cycle (**Fig.**
114 **1**). Thus, HPP produced from PDH may be directly incorporated into the Tyr degradation
115 pathway. **Hypothesis IV: PDH is involved in the legume-rhizobia symbiosis**. Many legumes
116 form a symbiotic relationship with rhizobia, and PDH activity is uniquely present in legumes.
117 Furthermore, *MtPDH1* expression is upregulated in the nodules after treatment with nitrate
118 (NO_3^- , Benedito et al., 2008) or phosphinothricin (PPT, Seabra et al., 2012), both of which

119 stimulate nodule senescence (Streeter and Wong, 1988; Matamoros et al., 1999; Pérez Guerra et
120 al., 2010). To systematically test the above four hypotheses, this study isolated and characterized
121 *pdh* mutants in the model plant *M. truncatula*, which conveniently has a single *PDH* gene
122 compared with some other legumes that have multiple copies (Schenck et al., 2015; Schenck et
123 al., 2017a) using genetics, biochemistry, metabolomics, histochemical staining, microscopy, and
124 gene expression analyses.

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126

127 RESULTS

128 *Isolation of pdh mutants in Medicago truncatula*

129 To investigate the *in vivo* function of PDH in legumes, two independent homozygous alleles of
130 *Tnt1*-transposon mutants of *M. truncatula* (Tadege et al., 2008) were identified for the *MtPDH1*
131 locus (Mt3g071980) (Cheng et al., 2014). The *pdh1-1* and *pdh1-2* mutants carried a transposon
132 insertion in the first and last exons of the *MtPDH1* gene, respectively (**Fig. 2a**). *MtPDH1* was
133 constitutively expressed across many tissues and under various conditions with the highest and
134 lowest expression being detected in aerial tissues and seeds, respectively (**Supplementary Fig.**
135 **1**). Both alleles did not produce any *PDH* transcript in the leaves (**Fig. 2b**). The *M. truncatula*
136 genome contains two *ADH* genes, *MtADH* (Mt4g115980) and *MtncADH* (Mt5g083530)
137 (Schenck et al., 2017a), but neither were enhanced in the *pdh1* mutants (**Fig. 2b**). Consistent with
138 their transcript levels, PDH activity was almost completely abolished in both *pdh1* mutants
139 without significant reduction in ADH activity (**Fig. 2c**). After six weeks of growth, *pdh1-1*
140 showed no phenotypic difference from wild-type (Wt; R108). The *pdh1-2* mutant, on the other
141 hand, had a slight bushy phenotype, which could be due to an unknown secondary transposon
142 insertion(s) (**Fig. 2d**); however, multiple attempts of backcrossing were unsuccessful. These data
143 provide genetic evidence that *MtPDH* is responsible for the PDH activity detected in *M.*
144 *truncatula*. The minor impacts of eliminating PDH activity on overall plant growth in two
145 independent *pdh1* mutants suggests that the PDH enzyme is not essential during standard growth
146 conditions in *M. truncatula*.

147 148 *Tyr and Tyr-derived compounds are unaltered in pdh mutants during standard growth* 149 *condition*

150 To test the hypothesis that the PDH pathway serves as a redundant Tyr biosynthetic route

151 **(hypothesis I)**, Tyr and Tyr/HPP-derived compounds were analyzed in Wt and mutants.
152 Metabolites were extracted from root and leaf tissue of 6-week-old plants grown under standard
153 conditions. Surprisingly, the levels of Tyr were not significantly different between Wt and
154 mutants in either leaf or root tissue (**Fig. 3a**). Additionally, the levels of Phe and Trp in the leaves
155 and roots were not significantly reduced in mutants as compared to Wt, though a slightly higher
156 Trp level was observed in *pdh1-2* (**Fig. 3a**). Potential effects on tocopherols, HPP-derived
157 metabolites, were also tested (**hypothesis II**), but their levels were not significantly different
158 between Wt and mutants in both leaf and root tissue (**Fig. 3b**). We also performed non-targeted
159 analysis using GC-MS for both polar and non-polar metabolites; however, no consistent
160 differences were observed between Wt and mutants (**Supplementary Table 1**). Together, these
161 data suggest that under standard growth conditions, the lack of the PDH enzyme had no
162 substantial effects on the overall accumulation of aromatic amino acids (AAAs) or
163 Tyr/HPP-derived metabolites analyzed.

164 In grasses, upwards of 50% of the total lignin is derived from Tyr, via a Tyr
165 ammonia-lyase (TAL) enzyme (Barros et al., 2016). Since TAL activity has also been detected in
166 some non-grass species, including legumes (Giebel, 1973; Beaudoin-Eagan and Thorpe, 1985;
167 Khan et al., 2003), the legume PDH enzyme may synthesize Tyr that is directly incorporated into
168 the phenylpropanoid pathway for downstream products such as lignin (**hypothesis II**). To test
169 this possibility, stem cross-sections from Wt and mutants were stained using two different
170 techniques, Mäule and phloroglucinol, which can detect potential differences in composition or
171 linkages of lignin (Mitra and Loqué, 2014, Pomar et al., 2002). Neither staining method showed
172 any obvious differences in stem lignification between Wt and mutants (**Supplementary Fig.**
173 **2a,b**). When phenylpropanoid intermediates, *p*-coumarate and ferulate, involved in lignin
174 biosynthesis were analyzed by GCMS, they were somewhat reduced in *pdh1-2* but not

175 consistently in *pdh1-1* (**Supplementary Fig. 2c**). Thus, the lack of PDH does not have
176 substantial impacts on lignin biosynthesis in *M. truncatula*.

177

178 ***Less tocopherols are accumulated in *pdh1* than Wt under high light treatment.***

179 Under various biotic and abiotic stresses, the shikimate pathway is induced, which often leads to
180 accumulation of AAAs (Dyer et al., 1989; Gilbert et al., 1998; Zhao et al., 1998; Betz et al.,
181 2009). To test the potential role of PDH in Tyr biosynthesis under stress (**hypothesis I**), Wt and
182 *pdh1-1* were subjected to 48-hour high light treatment (Gonzali et al., 2009), which is known to
183 induce production of AAA-derived antioxidants (Collakova and DellaPenna, 2003). The levels of
184 Tyr were not altered, except at 24 hours when Tyr increased slightly in *pdh1-1* compared with Wt
185 (**Supplementary Fig. 3a**). As expected, high light treatment enhanced tocopherol accumulation,
186 but to a significantly lesser extent in *pdh1-1* compared with Wt at both 24 and 48 hours
187 (**Supplementary Fig. 3b**). The levels of anthocyanins, which are also induced under various
188 stresses including high light stress (Collakova and DellaPenna, 2003), were increased after 24
189 and 48 hours of high light treatment but was not significantly different between Wt and *pdh1-1*
190 (**Supplementary Fig. 3c**). These results show that the lack of PDH negatively impacts the
191 accumulation of HPP-derived tocopherols when their production is induced under high light
192 conditions.

193

194 ***MtPDH1 is co-expressed with senescence-related genes but *pdh1* deficiency has no major
195 impacts on dark-induced senescence.***

196 To identify potential processes and pathways that are coordinately regulated with *PDH*, a gene
197 co-expression analysis with *MtPDH1* was performed using the Medicago gene expression atlas
198 (He et al., 2009). *MtPDH1* was co-expressed with genes mainly involved in senescence-related

199 processes (e.g., nucleases, proteases, and lipases **Supplementary Fig. 1**) and the gene encoding
200 HPP dioxygenase (HPPD, (Siehl et al., 2014), a senescence-activated enzyme involved in Tyr
201 catabolism (**Supplementary Fig. 5a**)(Wang et al., 2019). To experimentally test the potential
202 involvement of PDH in senescence, *PDH* gene expression and enzymatic activity were
203 monitored at different developmental stages during natural leaf senescence (**Supplementary Fig.**
204 **4a**). Expression of a senescence marker gene (*MtVPE*, Pérez Guerra et al., 2010) was monitored,
205 together with loss of chlorophyll in leaves collected at various developmental times from a single
206 plant, to define stages of leaf senescence (**Supplementary Fig. 4b**). *MtVPE* was basally
207 expressed in fully green leaves (defined here as the S1 stage), and gradually induced upon
208 senescence (defined here as S2, S3, and S4; **Supplementary Fig. 4b**), mirroring the loss of
209 chlorophyll. Fully senescent leaves (S5) were collected but did not yield high-quality RNA and
210 proteins and were unable to be further analyzed. The highest *MtPDHI* expression was detected in
211 green (S1) leaves, and PDH enzymatic activity was not induced upon senescence
212 (**Supplementary Fig. 4c,d**). Thus, *MtPDHI* is not upregulated during natural senescence in the
213 leaves. Similar to *PDH*, expression of the two *ADH* genes in *M. truncatula* did not follow the
214 developmental pattern of leaf senescence (**Supplementary Fig. 4c**). Unlike PDH activity,
215 however, ADH enzymatic activity was gradually induced upon senescence (**Supplementary Fig.**
216 **4d**).

217 Since *MtPDHI* was co-expressed with *MtHPPD* (**Supplementary Fig. 1**), and PDH
218 together with HPPD provides a direct route for catabolism of Tyr to homogentisate, eventually
219 leading to fumarate (**Fig. 1, Supplementary Fig. 5a**), we investigated potential impacts of *pdh1*
220 deficiency in Tyr catabolism (**hypothesis III**). The expression of genes encoding all enzymes of
221 the Tyr catabolic pathway were measured in the mutants and Wt under standard growth
222 conditions (**Supplementary Fig. 5a**, Dixon and Edwards, 2006). The expression of genes

223 encoding the first two steps of the pathway, *HPPD* and homogentisate dioxygenase (*HGO*), were
224 not significantly altered in the mutants compared with Wt. The subsequent step,
225 maleylacetoacetate isomerase (*MAAI*), was induced by 2- and 2.5-fold in *pdh1-1* and *pdh1-2*,
226 respectively (**Supplementary Fig. 5b**), though the final step in the pathway, fumarylacetoacetate
227 hydrolase (*FAH*), showed opposite expression patterns in the two mutants (**Supplementary Fig.**
228 **5b**). Thus, no consistent changes in the expression of the Tyr degradation pathway genes, beyond
229 *MAAI* were observed in *pdh1* mutants.

230 To further examine Tyr catabolism during leaf senescence under artificial, but
231 controlled, conditions excised leaves from Wt and mutants were exposed to an extended dark
232 treatment (Xing and Last, 2017; Wang et al., 2019). Over 7 days, leaves from Wt and mutants
233 responded to dark-induced senescence similarly with no apparent growth phenotypes
234 (**Supplementary Fig. 6a**). Furthermore, there were no significant differences in α -tocopherol
235 levels between Wt and mutants at any time point analyzed (**Supplementary Fig. 6b**). These data
236 together suggest that the lack of PDH had no substantial effects on Tyr catabolism or metabolism
237 under standard growth conditions and at least under the senescence conditions tested here
238 (**Supplementary Figs. 5 & 6**).

239

240 *Less Tyr is accumulated in pdh mutants following shikimate feeding.*

241 Although the steady-state levels of Tyr were not altered in the mutants (**Fig. 3a**), some of the
242 above data suggest that the PDH enzyme may contribute to Tyr production (**hypothesis I**) at least
243 under some stress conditions (**Supplementary Figs. 1 and 3b**). Thus, we hypothesized that the
244 potential role of PDH in Tyr biosynthesis might be manifested when the carbon flux through the
245 Tyr pathway is elevated. To test this possibility, an intermediate of the shikimate pathway,
246 shikimate, was exogenously fed to Wt and mutants and the levels of AAAs were analysed.

247 Excised leaves were floated in a solution containing shikimate or H₂O for up to 8 hours, rinsed to
248 remove metabolites in the feeding solution, and leaf metabolites were extracted and analyzed
249 using HPLC and GC-MS. As more prolonged feeding with shikimate led to abnormal leaf
250 phenotypes, 8 hours was selected as an optimal time for increased carbon flux without pleiotropic
251 effects. After 8 hours of feeding, shikimate and Phe levels were increased drastically in all
252 genotypes, suggesting that shikimate was taken up and metabolized by the leaves (**Fig. 4**).
253 Tocopherol levels were generally unaffected upon shikimate feeding, likely because the feeding
254 time was not long enough to convert shikimate into tocopherols (**Fig. 4**). Trp levels were reduced
255 in the mutants compared with Wt after feeding with water, and were induced upon shikimate
256 feeding in one mutant (**Fig. 4**). The unexpected differences observed with Trp levels under
257 standard growth conditions (**Fig. 3**) and the feeding experiments (**Fig. 4**) may reflect unknown
258 stress responses during the feeding experiments. Nevertheless, after 8 hours of feeding, Tyr levels
259 increased by >39-fold in Wt, but only by 16- and 13-fold in *pdh1-1* and *pdh1-2*, respectively
260 (**Fig. 4**). Repeated 8 hour shikimate feeding experiments with *pdh1* mutants and Wt yielded
261 similar Tyr accumulation patterns. The difference in the Tyr accumulation suggests that PDH
262 contributes to Tyr biosynthesis when carbon flow through the shikimate pathway is enhanced.

263 To determine if global metabolite changes occurred after shikimate feeding, additional
264 amino acids and TCA pathway metabolites were analyzed by GC-MS. Interestingly, glutamine
265 levels were higher in H₂O-fed Wt, compared with mutants (**Supplementary Fig. 7**), and upon
266 shikimate feeding, glutamine levels were significantly higher in Wt (**Supplementary Fig. 7**). The
267 levels of TCA cycle intermediates may indicate the functionality of the Tyr catabolic pathway;
268 however, those TCA cycle intermediates analyzed here, including fumarate, were not
269 consistently altered after shikimate feeding in Wt and mutants (**Supplementary Fig. 8**). Citrate
270 levels were reduced in *pdh1-1* and *pdh1-2* compared with Wt in H₂O control treatment; however

271 after shikimate feeding these differences were no longer apparent (**Supplementary Fig. 8**). These
272 data did not provide evidence to support the involvement of the PDH enzyme in Tyr catabolism
273 to the TCA cycle intermediate.

274

275 ***PDH has limited role in the legume-rhizobia symbiosis***

276 To test if PDH plays a role in legume-rhizobia symbiosis (**hypothesis IV**), Wt plants and *pdh1-1*
277 mutants were grown side-by-side on low nitrogen Fahræus medium and inoculated with a
278 well-characterized rhizobium of *M. truncatula*, *Sinorhizobium meliloti* Rm1021. Nodules were
279 counted 14, 21, and 28 days post-inoculation (dpi) and acetylene reduction assays (ARA) were
280 performed to measure nitrogenase activity (Wych and Rains, 1978). At all timepoints *pdh1-1*
281 mutants did not display any phenotypic difference from Wt plants, including the number of
282 nodules produced per root (**Fig. 5a**), suggesting that PDH is not essential for nodule
283 development. *M. truncatula* forms indeterminate nodules that displayed the four standard zones:
284 I, meristematic; II, infection; III, fixation; and IV, senescence zones in both Wt and *pdh1-1* (**Fig.**
285 **5b**, Van de Velde et al., 2006). Expression of the bacterial *nifH* gene, which is required for
286 nitrogen fixation, was monitored through the use of a *S. meliloti* strain expressing a *PnifH::uidA*
287 fusion to evaluate nodule maturation on *pdh1-1* and Wt plants (Starker et al., 2006). After 21 and
288 28 dpi, β -glucuronidase (GUS) activity and the overall nodule development were not altered
289 between *pdh1-1* and Wt (**Fig. 5c**). Although nitrogenase activity in *pdh1-1* appeared to be slightly
290 reduced compared with Wt at 21 and 28 dpi when nodule senescence might have been initiated,
291 no significant differences was observed (**Fig. 5d**). ARA were repeated at the 21 dpi timepoint
292 with *pdh1-1* and this time including *pdh1-2*. Nitrogenase activity was not significantly different
293 between genotypes, even though *pdh1-1* showed a slight reduction, similar to the first experiment
294 (**Fig. 5e**).

295 Independently, we also measured the presence and absence of PDH from plant tissue
296 extracts of over twenty legume species, which were sampled across the legume phylogeny (Azani
297 et al., 2017, **Supplementary Fig. 9**) and compared with their capacity to form nodules (Afkhami
298 et al., 2018). All legumes sister to previously analyzed *G. max* (soybean) and *M. truncatula*
299 (Schenck et al., 2015) showed PDH activity including *Arachis ipaensis* (peanut), which possess a
300 TyrA enzyme with similar ADH and PDH activity (Schenck et al, 2017). All of these legumes
301 were previously reported to be able to nodulate (Afkhami et al., 2018). When four legume
302 species in the genistoid crown were analyzed (e.g. *Lupinus polyphyllus*), PDH activity was not
303 detectable in any of them, despite successful detection ADH activity (**Supplementary Fig. 9**).
304 Importantly, these legumes were reported to be able to nodulate, suggesting that legume plants
305 having undetectable levels of PDH activity can still maintain nodulation. Some early diverging
306 legume lineages are known to nodulate. Out of four of these species sampled from the mimosid
307 crown three showed PDH activity (**Supplementary Fig. 9**). In contrast, all four species from
308 caesalpinoid, another early diverging legume clade, are incapable of nodulating (Afkhami et al.,
309 2018), but showed PDH activity (**Supplementary Fig. 9**). The lack of detectable PDH activity
310 (e.g. in the genistoid crown) may be simply due to insufficient sensitivity with HPLC-based assay
311 and/or the use of tissues that do not necessarily express *PDH*. However, detection of PDH
312 activity in species lacking nodulation (e.g. in the caesalpinoid node) strongly support the lack of
313 positive correlation between the presence of PDH activity and nodulation across the legume
314 phylogeny. Phylogenetic sampling (**Supplementary Fig. 9**) together with *pdh1* mutant analysis
315 (**Fig. 5**) together suggest that PDH is not essential for legume-rhizobia symbiosis, though more
316 detailed analyses are needed to fully address its contribution to potentially enhance nitrogen
317 fixation within the nodules in some legumes.

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323 **DISCUSSION:**

324 In this study, we sought to understand the function of legume-specific PDH enzymes through
325 analysis of *Tnt1*-transposon mutants of *PDH* in *M. truncatula*. Not only is *M. truncatula* a
326 convenient model system having mutant populations and many genomic/transcriptomic
327 resources, but *M. truncatula* also has a single *PDH* gene unlike some other legumes, i.e.,
328 soybean, having two *PDH* genes (Schenck et al., 2015). Analysis of two independent *pdh1*
329 mutant lines suggests that MtPDH1 is responsible for all the PDH activity in *M. truncatula* (**Fig.**
330 **1**). This observation is consistent with chromatographic separation of PDH from ADH in
331 soybean, which resulted in the detection of a single PDH peak as compared to multiple ADH
332 peaks (Schenck et al., 2015). Our genetic data also support that ncADH, and ADH enzymes do
333 not contribute to PDH activity, in agreement with *in vitro* data of soybean and *M. truncatula*
334 enzymes (Schenck et al., 2015; Schenck et al., 2017a).

335 Surprisingly, the null *pdh1* mutants, displayed no visible aberrant growth phenotypes
336 (**Fig. 1**). A slight bushy phenotype was observed in the *pdh1-2* allele, but not in *pdh1-1*, and thus
337 here we focused on consistent responses observed in both alleles to avoid potential pleiotropic
338 effects specific to *pdh1-2*. The lack of substantial phenotypes in *pdh1* is in contrast to the highly
339 compromised growth and leaf development phenotypes of Arabidopsis *adh2/tyra2* mutant (de
340 Oliveira et al., 2019). In *M. truncatula*, we were also unable to recover homozygous mutants of
341 the canonical *ADH*, which is a single copy gene in *M. truncatula* as compared to two *ADH* copies
342 present in Arabidopsis (Rippert and Matringe, 2002), further suggesting the essential nature of
343 the ADH-mediated Tyr pathway in legumes. Thus, the canonical plastid-localized ADH pathway
344 is the predominant Tyr biosynthetic route and cannot be compensated by the cytosolic PDH
345 pathway in legumes.

346 Using the *pdh1* mutants, this study further systematically evaluated potential roles of

347 PDH in Tyr biosynthesis (**hypothesis I**), biosynthesis of Tyr- and HPP-derived metabolites (**II**),
348 Tyr catabolism and senescence (**III**), and legume-rhizobia symbiosis (**IV**). The obtained data
349 provide evidence for support of **hypothesis I** that the PDH enzyme is involved in Tyr
350 biosynthesis when carbon flux is increased through the shikimate pathway. Although Tyr levels
351 were not altered between Wt and mutants under standard conditions (**Fig. 3a**), upon feeding with
352 shikimate, Wt accumulated over 2-fold more Tyr than *pdh1-1* and *pdh1-2* (**Fig. 4**). Although
353 AAA biosynthesis is localized to the plastids (Bickel et al., 1978; Jung et al., 1986; Maeda and
354 Dudareva, 2012), some isoforms of the shikimate and AAA pathway enzymes were detected in
355 the cytosol (Rubin and Jensen, 1985; Ganson et al., 1986; Ding et al., 2007). Cytosolic CM
356 activities have been detected from various plant species including soybean (Goers and Jensen,
357 1984; Kuroki and Conn, 1989; Mobley et al., 1999; Schenck et al., 2015), which are not
358 regulated by the AAAs, unlike their plastidic counterparts (Eberhard et al., 1996; Mobley et al.,
359 1999; Westfall et al., 2014). Tyr-insensitive PDH enzymes provide a cytosolic route for
360 conversion of prephenate into HPP, which can be further transaminated to Tyr by cytosolic
361 Tyr-AT enzymes (**Fig. 1**) (Wang et al., 2016). Although the PDH-mediated Tyr pathway is not the
362 predominant route for Tyr biosynthesis, it may provide an alternative route for additional Tyr
363 synthesis under some conditions that increase flux through the shikimate pathway.

364 The current data also partly support **hypothesis II** that the PDH enzyme contributes to
365 the production of specialized metabolites derived from Tyr and/or HPP in legumes. Although
366 Tyr/HPP-derived tocopherols were not altered in the *pdh1* mutants under standard growth
367 conditions (Fig. 3b), high light-induced tocopherol accumulation was partially attenuated in the
368 *pdh1* mutant (**Supplementary Figure 3**). Therefore, the PDH enzyme may also contribute to
369 tocopherol biosynthesis under stress conditions. Some specialized metabolic pathways emerged
370 through duplication and neofunctionalization of genes from primary metabolic pathways (Weng

371 et al., 2012; Moghe and Last, 2015). The legume PDH enzyme emerged as the result of a recent
372 gene duplication of an *ADH* gene followed by a shift in substrate specificity from aroenate to
373 prephenate (Schenck et al., 2017a). Thus, legumes may be able to divert the shikimate pathway
374 flux to provide additional HPP or Tyr in the cytosol for the synthesis of downstream specialized
375 metabolites including tocopherols. To further test this possibility, additional metabolomics
376 experiments were conducted, but did not identify any putative HPP-derived compounds that were
377 absent or lower in *pdh1* mutants than Wt (**Supplementary Table 1**). Although there are many Tyr
378 and HPP-derived specialized metabolites produced in plants (e.g., rosmarinic acid, betalains,
379 dhurrin, and benzyloquinoline alkaloids), none appear to be specific to the legume lineage or
380 correlate with the distribution of PDH activity (**Supplementary Fig. 9**) (Schenck and Maeda,
381 2018). Some species of the legume genus *Inga* can accumulate Tyr to 20% of leaf dry weight,
382 which deters insect predation (Lokvam et al., 2006). Further analysis identified *Inga* species with
383 high *PDH* expression that have hyper-accumulation of Tyr-derived specialized metabolites, such
384 as Tyr-gallates (Coley et al., 2019). Therefore, more comprehensive analyses of Tyr-derived
385 metabolites under different conditions, tissues types, and other legume species may identify
386 specialized metabolites derived from the PDH enzyme in legumes.

387 The current data did not provide sufficient support for **hypothesis III** that the PDH
388 pathway potentially contributes to Tyr catabolism and senescence. As Tyr is the most energetic
389 amino acid (Hildebrandt et al., 2015), its catabolism is a crucial process in recovering energy.
390 Mutations in Tyr catabolic genes can have dramatic effects of plant development (Han et al.,
391 2013). Tyr is catabolized into fumarate, a TCA pathway intermediate, first by conversion into
392 HPP via a Tyr-AT enzyme and further through the canonical Tyr catabolism pathway
393 (**Supplementary Fig. 5a**) (Hildebrandt et al., 2015). Knockout of a soybean *HGO* did not cause
394 lethality but led to an increase in vitamin E content by 2-fold, suggesting that a significant

395 amount of carbon flux is diverted into vitamin E production when Tyr catabolism is blocked
396 (Stacey et al., 2016). Furthermore, *MtPDH1* was co-expressed with *HPPD*, which together
397 provide a direct pathway for homogentisate production. This potential coordination of PDH and
398 *HPPD*, and cytosolic localization, can bypass three enzymatic steps of the ADH-mediated Tyr
399 biosynthetic pathway, catalyzed by PPA-AT, ADH, and Tyr-AT to enter into the canonical Tyr
400 catabolism pathway (**Fig. 1 & Supplementary Fig. 5a**). Additionally, *PDH* was co-expressed
401 with many senescence-related genes (**Supplementary Fig. 1**, Van de Velde et al., 2006; Kusaba
402 et al., 2013; Xi et al., 2013) suggesting that *PDH* may function when Tyr catabolism is enhanced.
403 Despite these correlative data, however, experiments designed here to test the potential link
404 between PDH and senescence did not provide evidence to support this hypothesis: upon
405 dark-induced (**Supplementary Fig. 6**) and natural senescence (**Supplementary Fig. 4**)
406 conditions that likely stimulate Tyr catabolism, no phenotypic differences were observed between
407 mutants and Wt. Also, genes involved in Tyr catabolism were not consistently altered in mutants
408 as compared to Wt (**Supplementary Fig. 5b**), and PDH expression and enzymatic activity were
409 not induced upon senescence (**Supplementary Fig. 4c**). Thus, further experiments under
410 different conditions (e.g. specific stress that induces Tyr catabolism) are needed to address the
411 potential role of PDH in Tyr catabolism and senescence.

412 The data obtained in this study failed to directly support the **hypothesis IV** that the PDH
413 pathway plays a role in legume-rhizobia symbiosis. Legumes initiate a symbiotic relationship
414 with soil-dwelling rhizobia when there is insufficient nitrogen (Oldroyd et al., 2011). A chemical
415 communication ensues that ultimately results in compatible rhizobia invading legume roots and
416 formation of a new organ, the root nodule (Oldroyd, 2013). In the nodule, rhizobia fix
417 atmospheric dinitrogen into ammonium, which is assimilated by the plant through the glutamine
418 synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) cycle, in which glutamine and

419 glutamate are key amino acid carriers (Krapp, 2015). Mutations in *MtPDH1* did not alter nodule
420 numbers (**Fig. 5a**) or the developmental progression of the nodules (**Fig. 5c**). Furthermore,
421 nitrogenase activity was not significantly affected in mutants at any stage during the symbiotic
422 interaction even when senescence might have been initiated (**Figs. 5d,e**). Alterations in nitrogen
423 assimilation can lead to reduced nodulation in legumes (Streeter and Wong, 1988; Matamoros et
424 al., 1999): for example, plants treated with a GS inhibitor phosphinothricin (PPT) result in loss of
425 nodulation (Seabra et al., 2012) and also stimulate *MtPDH1* expression (**Supplementary Fig. 1**)
426 as well as many other genes. Interestingly, *pdh1-1* and *pdh1-2* had reduced glutamine levels in
427 the H₂O-treated control leaves after 8 hours (**Supplementary Fig. 7**), which also persisted after
428 shikimate feeding (**Supplementary Fig. 7**). Reduced glutamine levels in the *pdh1* mutants may,
429 in turn, affect the symbiotic efficiency with rhizobia, although no statistically-significant
430 reduction in nitrogenase activity was observed (**Figs. 5d,e**). Furthermore, PDH activity, which
431 was measured in a phylogenetically diverse group of legumes, does not correlate with ability to
432 nodulate (Azani et al., 2017; Afkhami et al., 2018, **Supplementary Fig. 9**). Thus, although PDH
433 may provide an adaptive advantage to some legumes, it is likely not directly involved in and
434 essential for the legume-rhizobia symbiosis.

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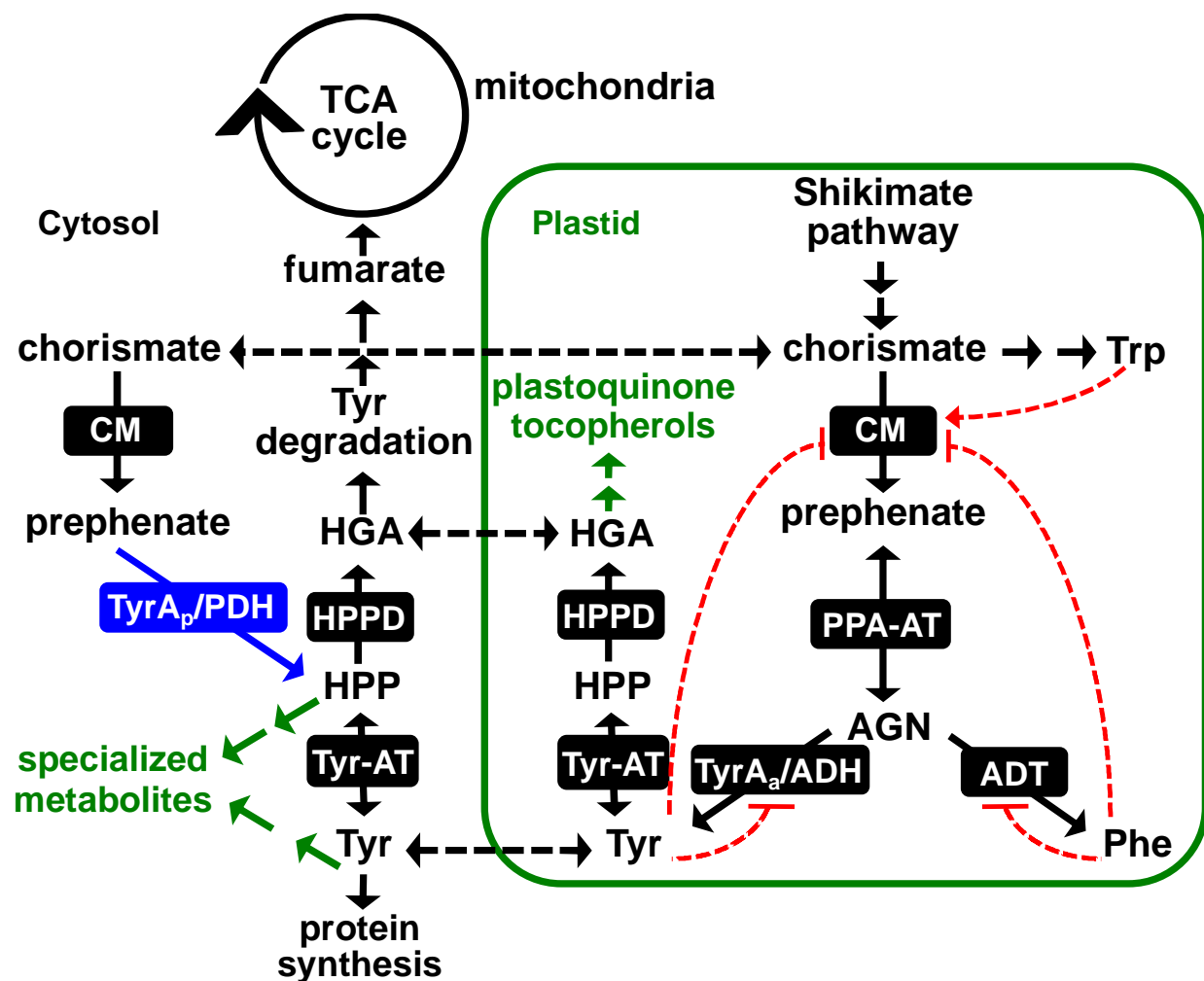
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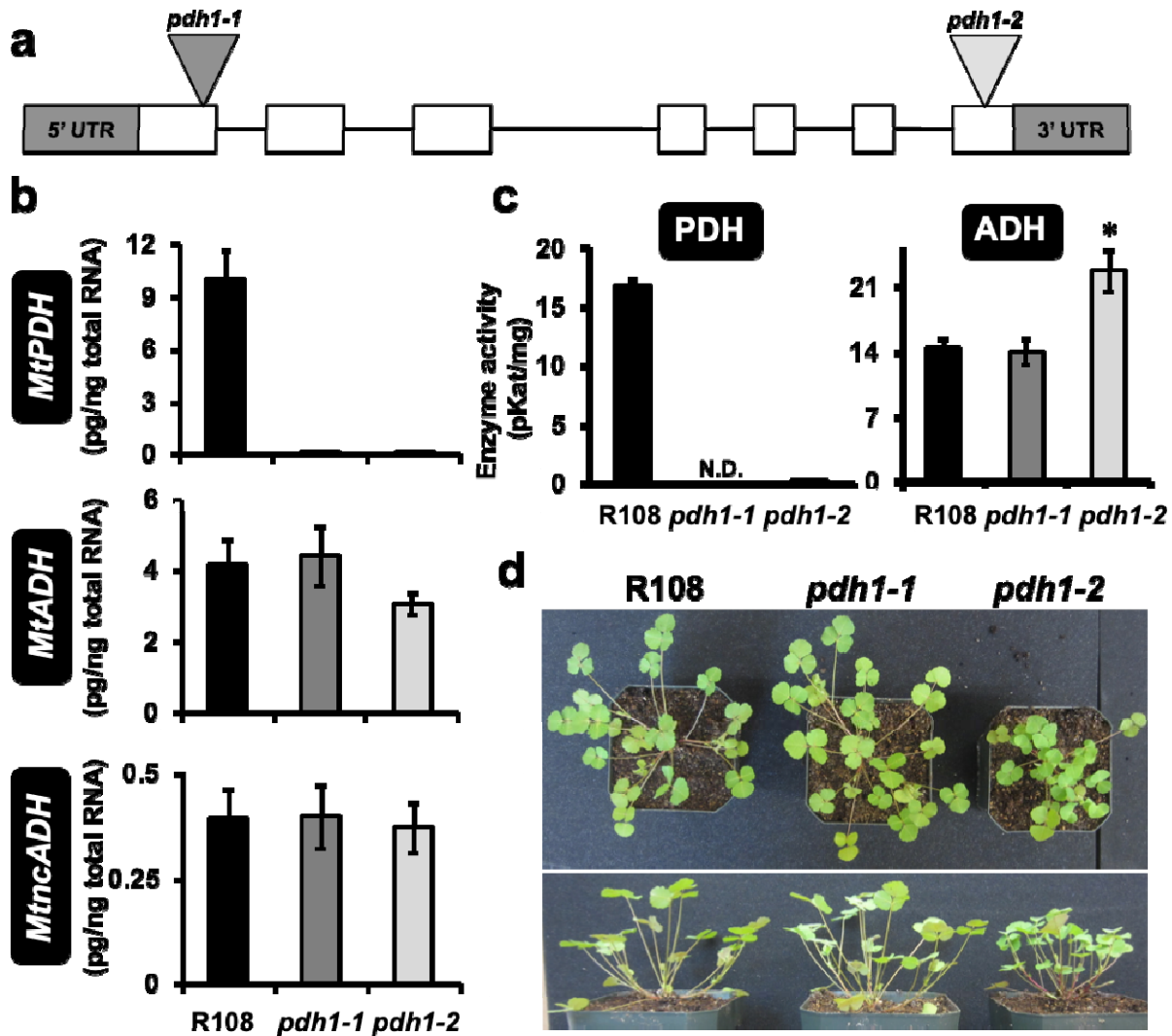
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441 **FIGURES:**



442
 443 **Figure 1. Tyr biosynthesis in legumes.** In addition to a highly regulated plastid localized ADH pathway
 444 for Tyr biosynthesis, legumes possess a Tyr-insensitive, cytosolic PDH enzyme (blue). Tyr synthesized in
 445 the plastids is exported into the cytosol where it can be incorporated into proteins, enter the Tyr degradation
 446 pathway to the TCA cycle or serve as a precursor to specialized metabolism. Black dotted lines denote
 447 known or potential transport steps, red dotted lines represent feedback regulation with arrows meaning
 448 induction and hashes inhibition. AGN, arogenate; ADT, arogenate dehydratase; CM, chorismate mutase;
 449 HGA, homogentisate; HPP, 4-hydroxyphenylpyruvate; HPPD, HPP dioxygenase; PPA-AT, prephenate
 450 aminotransferase; TyrA_a/ADH, arogenate dehydrogenase; TyrA_p/PDH, prephenate dehydrogenase;
 451 Tyr-AT, tyrosine aminotransferase.

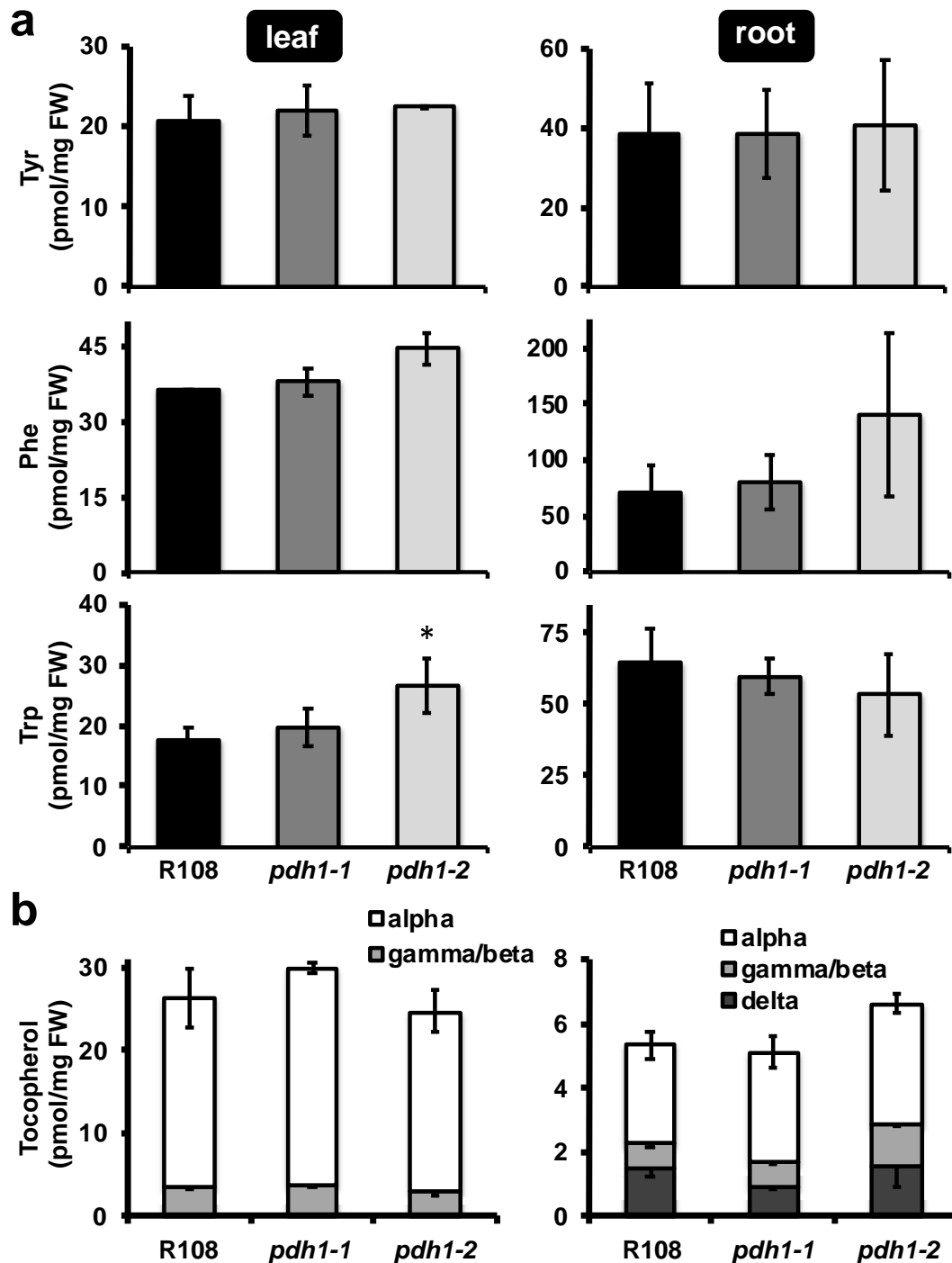
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 455 **Figure 2. Isolation of *MtPDHI* mutants.** (a) *MtPDHI* (Mt3g071980) genomic structure, exons (white
 456 boxes) and introns (lines), 5' and 3' untranslated regions (UTR, gray boxes). Two *Tnt1*-transposon mutants
 457 were isolated with insertions in exon one (*pdh1-1*) and seven (*pdh1-2*). (b) *MtPDHI* transcripts were nearly
 458 abolished in *pdh1-1* and *pdh1-2*, without effecting expression of either *ADH* homolog. Bars represent
 459 average absolute mRNA levels (pg/ng total RNA) \pm s.e.m of n = 3 biological replicates. (c) PDH and ADH
 460 activity from mutants and wild-type (Wt, R108). Bars represent average enzymatic activity (pKat mg⁻¹) \pm
 461 s.e.m of n = 3 biological replicates. Significant differences to R108 control are indicated; **P* \leq 0.05. (d)
 462 Phenotype of R108 and mutants after 6-weeks growth under standard conditions.

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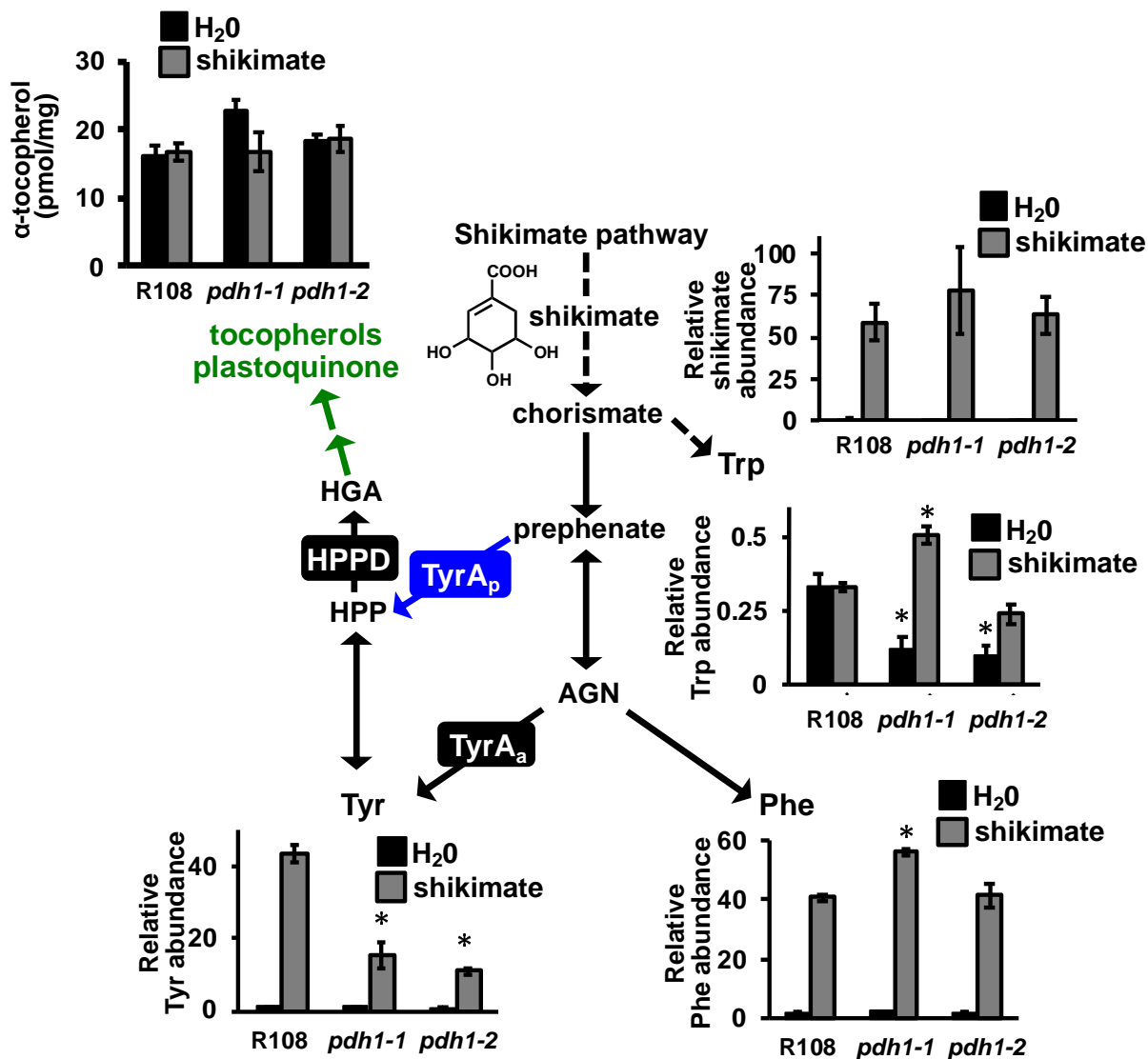


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466 **Figure 3. Targeted metabolite analysis of *mtdh1* mutants and Wt.** Leaf and root tissue from
 467 6-week-old plants, grown under standard conditions were used for metabolite extraction. Bars represent
 468 average absolute metabolite levels (pmol/mg fresh weight (FW)) \pm s.e.m of $n = 3$ biological replicates. (a)
 469 Aromatic amino acid levels in leaf and root tissue. Significant differences to Wt (R108) control are
 470 indicated; * $P \leq 0.05$. (b) Tocopherol composition and content from leaf and root tissue.

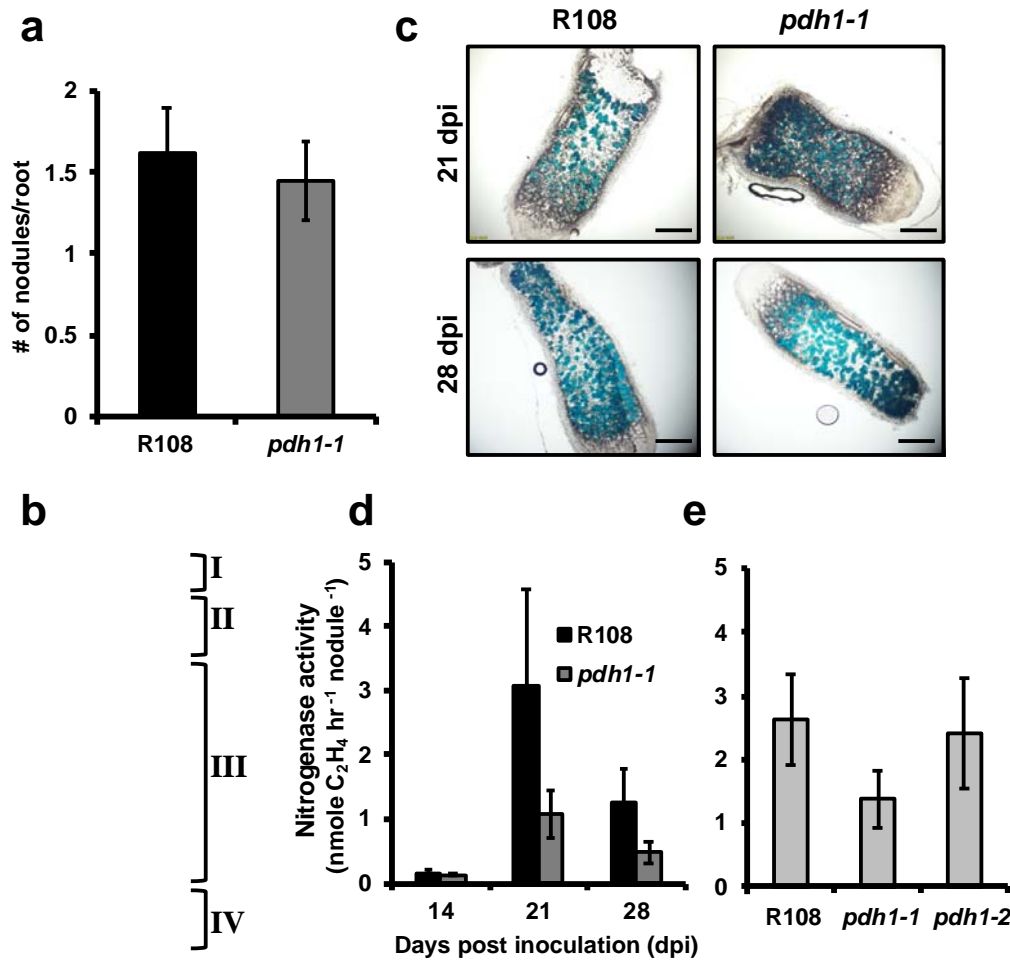
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474 **Figure 4. Metabolite analysis after shikimate feeding in Wt and *mtpdh1* mutants.** Excised leaves from
 475 6-week-old plants were floated on a solution containing H₂O (black bars) or 25 mM shikimate (gray bars) for
 476 8 hours under constant light. Leaves were then used for metabolite analysis using GC-MS (Tyr, Trp, Phe
 477 and shikimate) or HPLC (α -tocopherol). Bars represent average relative metabolite abundance of Tyr, Phe,
 478 Trp and shikimate \pm s.e.m of n = 3 biological replicates. α -tocopherol is shown as the average absolute
 479 metabolite abundance in pmol/mg FW \pm s.e.m of n = 3 biological replicates. Additional metabolites after
 480 shikimate feeding are shown in **Supplementary Figures 8 & 9**. Significant differences to Wt (R108)
 481 control are indicated; * $P \leq 0.05$.



482
 483 **Figure 5. The role of PDH in legume-rhizobia symbiosis.** (a) Wt and *pdh1-1* were grown on plates with
 484 low nitrogen Fahræus medium, and 14 days post inoculation (dpi) with *S. meliloti* nodules were counted.
 485 Bars represent average number of nodules per root \pm s.e.m. with $n > 11$ plants. (b) Indeterminate nodules
 486 from *M. truncatula* consist of four developmental zones. I meristem, II infection, III fixation, and IV
 487 senescence located closest to the root. These four developmental zones are shown on a characteristic nodule
 488 (enlarged for visualization) from 28 dpi. GUS staining with *PnifH::UidA* shows expression of bacterial *nifH*
 489 gene localized to the bacteroids (blue). (c) Thin sections of nodules from 21 and 28 dpi showing expression
 490 of *PnifH::UidA*, highlight bacteroid and nodule development in R108 and *pdh1-1*. Approximately equal
 491 staining was observed in all nodule developmental zones, suggesting *pdh1-1* is not affected in nodule
 492 development or bacteroid number, scale bar = 1 mM. (d) Nitrogen fixation efficiency was measured using
 493 an acetylene reduction assay (ARA). Plants were grown under the same conditions as in a, 14, 21 and 28 dpi
 494 ethylene production was measured and expressed as the average \pm s.e.m with $n > 7$. (e) Nitrogen fixation
 495 efficiency assay performed in the same way as in (d) however, only at 21 dpi and with both mutant alleles
 496 and Wt. Activity is expressed as the average \pm s.e.m with $n > 4$.

497

498 **Acknowledgements:**

499 This work was supported by grants from the National Science Foundation IOS-1354971 to
500 H.A.M. and NSF#1331098 and NSF#1546742 to JMA. LWS is supported in part by NSF awards
501 1340058, 1743594, 1139489, and 1639618. The University of Missouri, Office of Research
502 provided initial instrumental and personnel funding for the MU Metabolomics Center.
503 Development of *M. truncatula Tnt1* mutant population was, in part, funded by the National
504 Science Foundation, USA (DBI-0703285 and IOS-1127155) to KSM. We thank the Germplasm
505 Resources Information Network (GRIN) for providing some of the legume seeds used in this
506 study.

507

508 **Conflict of Interest Statement:**

509 The authors declare no conflicts of interest.

510

511 **Author Contribution:**

512 CAS, JW, DJ, and KG carried our experiments and interpreted results. CAS, JMA, LWS, and
513 HAM designed experiments. KSM and JW developed *Tnt1* mutant lines. CAS and HAM wrote the
514 manuscript. All authors read and edited the manuscript.

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- 723

724 **MATERIALS AND METHODS:**

725 ***Plant Materials and Growth Conditions***

726 *Medicago truncatula* seeds were scarified in concentrated hydrochloric acid for eight minutes
727 and repeatedly washed with water. Seeds were then surface sterilized with bleach for 1.5 minutes
728 followed by repeated water washes. Seeds were placed in sterile water for 16 hours at 4°C and
729 then transferred to germination media (0.5x MS media, 0.8% agar, 1 μM GA₃, pH 7.6), wrapped
730 in aluminum foil and placed at 4°C. After 48 hours plates with sterilized seeds were moved to
731 22°C. After 24 hours the aluminum foil was removed and the germinated seedlings were
732 transferred to standard potting soil and placed in a growth chamber (Conviron). Pots were
733 watered with 1x Hoaglands solution when dry, and grown under 12 hour light:dark cycles with
734 200 μE light intensity and ~60% humidity.

735 ***Genotyping***

736 A single young leaf from 6-week-old plants was placed in 1.7 mL microcentrifuge tube with 600
737 μL of DNA extraction buffer (10 mM Tris-HCl pH 8.0, 25 mM ethylenediaminetetraacetic acid
738 (EDTA), and 0.5% SDS), pulverized with a mini blue pestle, and incubated at 55°C for 15
739 minutes. The solution was cooled to room temperature and 200 μL of 5 M ammonium acetate
740 was added, vortexed for 20 seconds and centrifuged at 14,000 g for 3 minutes. The supernatant
741 was transferred to a fresh tube and 600 μL of isopropanol was added, followed by centrifugation

742 at 14,000 g for 1 minute. The supernatant was decanted and the pellet washed with 400 μ L of
743 70% ethanol followed by centrifugation at 14,000 g for 1 minute. The supernatant was decanted
744 and the pellet dried for 1 hour in a sterile hood. The resulting DNA was dissolved in 50 μ L of
745 H₂O. Genotyping PCR reactions contained 1 μ M gene and insertion specific primers
746 (**Supplementary Table 2**), 1x EconoTaq PLUS master mix (Lucigen), and genomic DNA. DNA
747 was amplified in a thermocycler with the following conditions: an initial denaturation at 95°C for
748 5 min, 35 cycles of amplification at 95°C for 20 s, 60–65°C for 20 s, 72°C for 60 s, with a final
749 extension at 72°C for 5 min.

750 *Quantitative reverse transcription PCR (qRT-PCR)*

751 RNA was extracted from leaves of 6-week-old plants. About 50mg of tissue was pulverized in
752 liquid N₂ using a mini pestle. RNA extraction buffer (68mM sodium citrate, 132mM citric acid,
753 1mM EDTA and 2% SDS) was added and vortexed immediately for 10 seconds, the tubes were
754 then placed on their sides for 5 minutes at 22°C. The solution was centrifuged at 12,000g for 2
755 minutes and 400 μ L of the supernatant was transferred to a fresh tube. 100 μ L of 1M NaCl was
756 added and mixed by pipetting, followed by addition of 300 μ L of chloroform, inverted multiple
757 times and centrifuged at 4°C for 10 minutes at 12,000g. The upper phase was transferred to a new
758 tube and an equal volume of isopropanol was added, mixed by inverting and placed at 4°C for 10
759 minutes. The solution was then centrifuged at 4°C for 10 minutes at 12,000g and the supernatant

760 decanted. The pellet was washed with 70% ethanol, followed by centrifugation for 3 minutes at
761 12,000g and the supernatant decanted and the pellet dried in a sterile hood until all residual
762 ethanol was evaporated. The resulting pellet was redissolved in 25 μ L of nuclease free H₂O
763 (Promega). To remove DNA, the RNA solution was treated with DNase (Turbo DNase, Fisher)
764 following the manufacturer's protocol. The remaining RNA was quantified using a nano-drop
765 spectrophotometer (Thermo) and diluted to a 20 ng/ μ L concentration. RNA was converted into
766 cDNA using reverse transcriptase (Applied biosystems) with an oligo d(T) primer.

767 For qPCR, cDNA was diluted to 5 ng/ μ L and additional 5-fold dilutions were made to
768 calculate primer efficiency. All primer pair efficiencies were between 90-100%. cDNA was
769 mixed with GoTaq qPCR master mix (Promega) containing SYBR green and 300 nM of each
770 primer. Reactions were placed in an Stratagene Mx3000P (Agilent) thermocycler using the
771 following PCR cycle an initial denaturation at 95°C for 10 min, 45 cycles of amplification at
772 95°C for 15 s, 60°C for 30 s, 72°C for 30 s.

773 For relative quantification, Ct values were extracted for each reaction and used to
774 quantify initial cDNA concentration using $2^{-\Delta\Delta C(t)}$ method normalized to a housekeeping gene
775 (*MtPI4K* Kryvoruchko et al., 2016, **Supplementary Table 2**) using the LinRegPCR program
776 (Ruijter et al., 2013). For absolute quantification, pET28a vectors carrying *MtPDH1*, *MtncADH*
777 and *MtADH* were used as the template to amplify a fragment of the corresponding genes. The

778 resulting fragments were purified from 0.8% agarose gels using a QIAquick gel extraction kit
779 (Qiagen) following manufacturer's protocol. The DNA concentration was quantified and repeated
780 5-fold dilutions were made and used to obtain a standard curve in qRT-PCR with gene-specific
781 primers (**Supplementary Table 2**). Ct values were extracted for each qRT-PCR reaction and used
782 to quantify initial cDNA concentration by using the linear range created as above for the
783 respective gene.

784 *Enzyme extraction and ADH and PDH assays*

785 Leaf tissue from 6-week-old plants were ground to a fine powder in a prechilled mortar and
786 pestle under liquid N₂. Extraction buffer (25 mM HEPES, pH 7, 50 mM KCl, 10% ethylene
787 glycol, 1% polyvinylpyrrolidone (PVP) and 1 mM dithiothreitol (DTT)) was added in a 1:3 ratio
788 of tissue to buffer (w/v). The slurry was centrifuged for 20 min at 4°C, at 20,000 g and the
789 resulting supernatant desalted using a gel filtration column (Sephadex G50-80 resin,
790 Sigma-Aldrich) equilibrated with extraction buffer without DTT and PVP. Protein concentrations
791 were determined by a Bradford assay (Bio-Rad Protein Assay, Bio-Rad).

792 The desalted crude enzyme extracts were used in ADH and PDH reactions that
793 contained 25 mM HEPES (pH 7.5), 50 mM KCl, 10% ethylene glycol, 1 mM NADP⁺, 1 mM
794 substrate (L-arogenate or prephenate, respectively). Arogenate was prepared by enzymatic
795 conversion from prephenate (Sigma-Aldrich), as previously reported (Maeda et al., 2011).

796 Reactions were initiated by addition of enzyme from various sources and incubated at 37°C for
797 45 minutes. The resulting assays were injected into HPLC equipped with a ZORBAX SB-C18
798 column (Agilent) to directly detect the final product of the assay as described previously
799 (Schenck et al., 2015).

800 For PDH and ADH activities from various legumes (**Supplementary Fig. 9**), leaf
801 material was obtained from the University of Wisconsin-Madison Botany Department
802 greenhouse or from identified trees on campus. Plants were grown under varying light and
803 temperature conditions and leaf material was collected at different developmental stages. Enzyme
804 extractions and ADH and PDH assays were performed as described above.

805 *Metabolite extraction and detection*

806 Tissue from 6-week-old plants was added into 1.7 mL microcentrifuge tubes with 400 µL of
807 extraction buffer (2:1 (v/v) methanol:chloroform, 0.01 % butylated hydroxytoluene (BHT), 100
808 µM norvaline and 1.25 µg/mL tocol as previously described (Collakova and DellaPenna, 2003),
809 with 3 glass beads (3 mm). Samples were vigorously shaken for 3 minutes at 1000 r.p.m. using a
810 genogrinder (MiniG 1600, SPEX SamplePrep). Additional chloroform (125 µL) and water (300
811 µL) were added and vortexed for 30 seconds. Samples were centrifuged for 10 minutes at 20,000
812 rpm and the polar and non-polar phases were transferred to new tubes and dried using a vacuum
813 concentrator (Labconco).

814 For tocopherol analysis, the dried nonpolar phase was resuspended in methanol with
815 0.01% BHT. Samples were injected into a HPLC (Agilent 1260) equipped with a ZORBAX
816 SB-C18 column (Agilent) using a 30 minute isocratic elution of 95 % methanol, 5 % water.
817 Tocopherols in the extractions were visualized using fluorescence detection excitation at 290 nm
818 and emission at 330 nm and compared with authentic standards (Sigma) and normalized to an
819 internal control (tocol).

820 For anthocyanin detection, 1 M HCl of methanol was added to the polar phase in a 1:1
821 ratio. A spectrophotometer was used to measure absorbance at 520 nm. Absolute levels were
822 estimated using an extinction coefficient of anthocyanin absorbance (Lee et al., 2005) of 33,000
823 $L \times M^{-1} \times cm^{-1}$.

824 The polar and non-polar phases were analyzed with GC-MS. The dried polar phase was
825 redissolved in pyridine with 15.0 mg/mL methoxyamine-HCl. Samples were vortexed for 30
826 seconds followed by sonication for 10 minutes and incubated for 60 minutes at 60°C. This was
827 repeated once more, then the samples were derivatized with an equal volume of
828 *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide + 0.1 % *tert*-butyldimethylchlorosilane
829 (MTBSTFA + 0.01 % *t*-BDMCS, Sigma-Aldrich) and incubated for 60 minutes at 60°C. Samples
830 were then injected into GC-MS (Trace 1310, ISQ LT, Thermo Scientific). The dried non-polar
831 phase was redissolved in 800 μ L of chloroform with 50 ppm BHT and 500 μ L of 1.25M HCl in

832 methanol. Following incubation at 50°C for 4 hours, samples were completely dried under
833 nitrogen gas. The dried non-polar phase was resuspended in 70 µL of pyridine and derivatized
834 with 30 µL of MTBSTFA + 0.01 % t-BDMCS (Sigma-Aldrich). Following transfer to a glass
835 vial, 1 µL of the polar and non-polar phases were injected onto a 30 m column (TG-5MS,
836 Thermo Scientific) using a 10:1 split ratio, and an oven ramp method of 5°C per minute for 46
837 minutes and held at 300°C for 10 minutes. Detected compounds were compared with library
838 matches from NIST and peak areas based on ion abundance were normalized to an internal
839 standard (norvaline).

840 *Acetylene reduction assay (ARA)*

841 Plants used for ARA were scarified and germinated as described previously. Seedlings were
842 transferred to 12" square plates containing modified solid Fahræus medium supplemented with
843 0.5 mM NH₄NO₃ (Catoira et al., 2000). Plates were grown under the same conditions as pot
844 grown plants, however plates were placed at a ~60° angle so that the roots would not penetrate
845 the agar. After 5 days of growth on plates, each plant was inoculated with 1 mL of water
846 containing *Sinorhizobium meliloti* Rm1021 at an OD₆₀₀ 0.02. Plants were then allowed to grow
847 for 14, 21 and 28 dpi at which point nodules were counted and plants were moved to 10 mL glass
848 jars with 1 mL of sterile water at the bottom. Glass jars were sealed with a rubber stopper, then
849 injected with 1 mL of acetylene gas (10% acetylene final). After 48 hours of incubation at 37°C,

850 1 mL of the gas from the glass jars was injected into a gas chromatograph (GC-2010, Shimadzu)
851 to measure the production of ethylene and and ARA activity was calculated as described in Hardy
852 et al., 1968.

853 ***Histochemical staining of lignin composition***

854 For cell wall composition staining, thin cross sections of stem tissue from 6-week-old plants were
855 prepared using a razor blade. For Mäule staining stem sections were placed in a 1% potassium
856 permanganate solution for 5 minutes, then rinsed with water followed by addition of a 12% HCl
857 (V/V) for 5 minutes and rinsed again with water. A 1.5% solution of sodium bicarbonate was
858 added to facilitate a color change to dark red and visualized using an epifluorescence microscope
859 (Olympus BX60).

860 For phloroglucinol staining, similar stem cross sections were placed in a well plate with
861 1 mL of 10 % phloroglucinol (w/v) solution in 95 % ethanol with 500 μ L of 10 N HCl and
862 incubated for 5 minutes. Stem sections were transferred to a glass slide and washed with water
863 and visualized using a epifluorescence microscope (Olympus BX60).

864 GUS staining was performed on nodules developed after inoculation with *S. meliloti*
865 Rm1021 carrying a *PnifH::UidA* fusion, to localize expression of the bacterial *nifH* gene, which
866 is required for nitrogen fixation. Nodules were embedded in 4% agarose and 50-100 μ M sections
867 were made with a vibratome[®] 1000 plus (Leica). Sections were immersed in a staining solution

868 (2.5% 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronic (X-Gluc), 0.2M sodium phosphate buffer
869 (pH 7), 0.1 M potassium ferricyanide, 0.1 M potassium ferrocyanide, 0.25 M Na₂EDTA, and
870 10% Triton X-100) and vacuum infiltrated for 10 minutes, incubated in the dark at 37°C for 30
871 minutes, and rinsed with phosphate buffer. Sections were visualized using bright field
872 microscopy.
873

874 **SUPPLEMENTARY RESULTS:**

875 **Supplementary Table 1. Non-targeted metabolite analysis from leaf tissue of Wt and**

876 ***mtpdh1-1***

877

Metabolite	retention time (min)	Ion (m/z)	pdh1-1/R108	pdh1-2/R108	T-test pdh1-1: R108	T-test pdh1-2:R108
Beta-Alanine	21.5809	248.1	NS	1.49638	NS	0.04629
beta-D-glucoside	32.1517	204.1	1.18350	2.20999	0.01116	0.00016
Citric Acid	30.5877	273.1	NS	1.51560	NS	0.00027
D-(+)-Melibiose	43.5201	204.1	0.72128	NS	0.03323	NS
Ferulic Acid	36.1830	338.2	NS	0.51230	NS	0.01121
Gentisic Acid	29.4116	355.2	NS	2.85714	NS	0.00001
Glucuronic Acid	29.1234	292.2	NS	1.58440	NS	0.00001
Glycolic Acid	11.8422	205.1	1.42874	1.92904	0.01487	0.00037
L-Alanine	19.8168	188.1	NS	2.39806	NS	0.00005
Lauryl Alcohol	22.0027	243.1	1.27167	NS	0.01445	NS
Maleic Acid	18.2630	245.0	NS	0.68722	NS	0.04895
Malic Acid	23.0106	233.1	NS	0.73291	NS	0.04836
Malonic Acid	15.4680	233.1	0.75424	NS	0.02370	NS
Pinitol	30.8088	260.2	1.33875	NS	0.01147	NS
Propionic Acid	18.8585	292.2	NS	2.08458	NS	0.01253
Saccharic Acid	35.0341	333.2	0.74697	1.32846	0.00476	0.00815
Sucrose	44.9165	361.2	NS	1.99803	NS	0.02979
Tartaric Acid	25.6516	305.2	1.43056	NS	0.03744	NS
Trehalose	43.9602	361.2	NS	18.99309	NS	0.00048
Tyramine	32.7431	174.1	NS	0.59383	NS	0.00075
Unknown	33.6590	204.1	1.26433	NS	0.00363	NS
Unknown	17.5759	186.0	1.79713	2.62694	0.00452	0.01497
Unknown	13.6890	220.1	1.41853	NS	0.00884	NS
Unknown	30.8657	217.1	1.32711	NS	0.00953	NS
Unknown	9.2975	184.1	1.52453	NS	0.01137	NS
Unknown	47.8363	204.2	1.43690	1.35843	0.01504	0.04118
Unknown	47.2028	204.1	1.21975	1.20442	0.03006	0.04871
Unknown	32.5721	221.2	1.21109	NS	0.03112	NS
Unknown	49.3385	217.1	1.31458	1.40117	0.03151	0.01702
Unknown	36.3824	434.3	0.70531	NS	0.03570	NS
Unknown	21.0322	189.1	1.20140	NS	0.04070	NS
Unknown	23.6949	217.1	NS	3.31769	NS	0.00007
Unknown	32.7980	205.1	NS	3.21406	NS	0.00002
Unknown	27.4875	422.2	NS	3.08602	NS	0.01943
Unknown	17.5759	186.0	NS	2.48604	NS	0.01419
Unknown	24.6017	217.1	NS	2.38962	NS	0.00001
Unknown	22.0509	307.2	NS	2.33226	NS	0.00220
Unknown	19.1101	184.1	NS	2.02055	NS	0.04568
Unknown	30.6073	150.1	NS	1.50572	NS	0.00020

Unknown	24.9767	313.3	NS	1.43477	NS	0.04725
Unknown	31.7135	275.2	NS	1.31801	NS	0.00069
Unknown	38.4361	331.1	NS	1.27114	NS	0.00650
Unknown	33.7685	270.2	NS	0.76019	NS	0.03895
Unknown	41.0710	274.1	NS	0.70608	NS	0.01860
Unknown	37.2316	204.1	NS	0.67959	NS	0.02482
Unknown	25.7858	331.2	NS	0.65537	NS	0.01843
Unknown	31.0367	292.2	NS	0.54155	NS	0.00025
Xylose	26.8968	160.1	NS	0.60672	NS	0.00493

886
887
888 Only compounds are shown that were significantly different from Wt with abundance > 1.2 or <
889 0.8 fold-change and a P-value ≤ 0.05 . Ratio of average relative metabolite abundance is shown
890 for *pdh1-1* and *pdh1-2* compared with Wt with N = 5. NS, not significant. Unknowns are
891 compounds that were detected with unique ions, but did not have a confident library match (<
892 75%).
893
894

895 **Supplementary Table 2. Primer sequences used in this study.**

896	Name	Use	Sequence (5'-3')
897	PDH1-1	<i>mtpdh1-1</i> genotyping	GAGCACTATTTCCATTGTAAAC
898	Tnt1R	<i>mtpdh1-1</i> genotyping	CAGTGAACGAGCAGAACCTG
899	PDH1-2	<i>mtpdh1-2</i> genotyping	ATGAGACTGGAGGGGGAGAT
900	Tnt1F	<i>mtpdh1-1</i> genotyping	GAACATATGGCAGGGGTTACAAG
901	MtPDHqF1	<i>mtpdh1-1</i> qPCR	AAACAAGGTCATACTCTAACTGCAA
902	MtPDHqR1	<i>mtpdh1-1</i> qPCR	CAGCATCAAGGAATGCTGTAA
903	MtPDHqF2	<i>mtpdh1-2</i> qPCR	CAACAGATTCCGCCAGACAAGAGC
904	MtPDHqR2	<i>mtpdh1-2</i> qPCR	CTGGGTTCTGTCCTTCATCGA
905	MtADHqF	<i>MtADH</i> (Medtr4g115980) qPCR	GACCTGAGAGTGGAAAGCAGT
906	MtADHqR	<i>MtADH</i> (Medtr4g115980) qPCR	TTCTCACACCTCGAAACCCT
907	MtncADHqF	<i>MtncADH</i> (Medtr5g083530) qPCR	GCTAGTGAGGGTTGTAAGATGC
908	MtncADHqR	<i>MtncADH</i> (Medtr5g083530) qPCR	GCGGGTAATTCTGTATTATT
909	MtPI4KF ¹	housekeeping gene (<i>MtPI4K</i>) qPCR	GCAGATAGACACGCTGGGA
910	MtPI4KR ¹	housekeeping gene (<i>MtPI4K</i>) qPCR	AACTCTTGGGCAGGCAATAA
911	MtHPPD1F	<i>MtHPPD1</i> (Medtr5g091060) qPCR	CCCACCAACACCACTTCTCT
912	MtHPPD1R	<i>MtHPPD1</i> (Medtr5g091060) qPCR	GGTGCTGGGTTACAGCATTT
913	MtHGOF	<i>MtHGO</i> (Medtr8g463280) qPCR	AGGCACGGGTTCCCTTCTAAT
914	MtHGOR	<i>MtHGO</i> (Medtr8g463280) qPCR	TCAATGAAATCCGTTGGTGA
915	MtMAAIF	<i>MtMAAI</i> (Medtr4g134370) qPCR	CTTCCATGGGTCCAGAGTGT
916	MtMAAIR	<i>MtMAAI</i> (Medtr4g134370) qPCR	CCGCCATGAAAACCTTCATCT
917	MtFAHF	<i>MtFAH</i> (Medtr2g025640) qPCR	ACTTCGGACCCACATTGAAG
918	MtFAHR	<i>MtFAH</i> (Medtr2g025640) qPCR	TCCACAGGTTTTCCCAGTTC
919	MtVPEF ²	<i>MtVPE</i> (Medtr1g016780) qPCR	AGTTCTGCCTGTTGTGGAATGTC
920	MtVPER ²	<i>MtVPE</i> (Medtr1g016780) qPCR	GGTAGCTCCTGTCTGCCAATTAC

921

922 ¹ Housekeeping gene (Kryvoruchko et al., 2016) used in normalization of qPCR data.

923 ² Cysteine protease (vacuolar processing enzyme; VPE) that serves as a senescence marker gene

924 (Pérez Guerra et al., 2010).

925