

Metabolic reprogramming in nodules, roots and leaves of symbiotic soybean in response to iron deficiency

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Running titles

Metabolic reprogramming of soybean under Fe deficiency

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1 **Abstract**

2 To elucidate the mechanism of adaptation of leguminous plants to iron (Fe)-deficient
3 environment, comprehensive analyses of soybean (*Glycine max*) plants (sampled at
4 anthesis) were conducted under Fe-sufficient control and Fe-deficient treatment using
5 metabolomic and physiological approach. Our results show that soybeans grown under
6 Fe-deficient conditions showed lower nitrogen (N) fixation efficiency, however,
7 ureides increased in different tissues, indicating potential N feedback inhibition. N
8 assimilation was inhibited as observed in the repressed amino acids biosynthesis and
9 reduced proteins in roots and nodules. In Fe-deficient leaves, many amino acids
10 increased, accompanied by the reduction of malate, fumarate, succinate and α -
11 ketoglutarate, which implies the N reprogramming was stimulated by the anaplerotic
12 pathway. Accordingly, many organic acids increased in roots and nodules, however,
13 enzymes involved in the related metabolic pathway (e.g., Krebs cycle) showed opposite
14 activity between roots and nodules, indicative of different mechanisms. Sugars
15 increased or maintained at constant level in different tissues under Fe deficiency, which
16 probably relates to oxidative stress, cell wall damage and feedback regulation.
17 Increased ascorbate, nicotinate, raffinose, galactinol and proline in different tissues
18 possibly helped resist the oxidative stress induced by Fe deficiency. Overall, Fe
19 deficiency induced the coordinated metabolic reprogramming in different tissues of
20 symbiotic soybean plants.

21 **Keywords:** carbon and nitrogen metabolism; concerted metabolic change; feedback
22 regulation; organic acids; oxidative stress; metabolome; soybean; symbiotic nitrogen
23 fixation

24 **Introduction**

25 Iron (Fe) is a transition metal and an essential nutrient for the survival of all higher
26 plants. In plants, Fe plays critical roles in metabolic processes, including respiration,
27 electron transport and photosynthesis (López-Millán *et al.* 2013), and as a component
28 of Fe–sulfur (S) cluster or haem-containing enzymes involved in oxydoreduction
29 reaction (Qin *et al.* 2015; Sarah *et al.* 2016). However, despite its relative abundance in
30 soil, plants frequently show symptoms of Fe deficiency, especially under high pH
31 conditions (Donnini *et al.* 2012; Hsieh & Waters 2016). Low Fe availability causes a
32 common nutritional disorder in plants, namely chlorosis, resulting in lower crop yields
33 and quality (Chen *et al.* 2018). Given that more than 30% of the soils in the world are
34 calcareous, Fe deficiency is predicted to be a common problem (Chen & Barak 1982).
35 Fe deficiency induces various responses in plant roots that increase the availability of
36 the Fe³⁺ in the rhizosphere. Strategy I (dicotylednus) plants respond to the shortage of
37 Fe in the soil by increasing the activity of Fe³⁺ chelate reductase (FCR) in roots
38 (Robinson *et al.* 1999), releasing protons which acidifies the rhizosphere and increases
39 Fe ion solubility (Santi & Schmidt 2009).

40 Symbiotic nitrogen fixation (SNF) in legumes is dramatically affected by numerous
41 environmental limitations. Low Fe availability is one of the major factors that limit
42 SNF capacity, because the biosynthesis of some proteins involved in SNF requires Fe,
43 including the highly abundant leghaemoglobin that buffers oxygen, nitrogenase that
44 catalyses nitrogen (N) fixation and cytochromes that are involved in the electron
45 transport chain (López-Millán *et al.* 2000, 2013). Fe deficiency has been shown to limit
46 the growth and SNF of legume crops (Slatni *et al.* 2011, 2014). In addition to the
47 common Fe acquisition strategy of Strategy I plants, leguminous plants improve the
48 activities of FCR, H⁺-ATPase and the phospho*enol*pyruvate carboxylase (PEPC) in
49 nodules to promote Fe uptake from soil by the nodule epidermis (Slatni *et al.* 2008,
50 2014). The altered FCR and H⁺-ATPase activities increase the reducing power (NADH
51 and NADPH) of nodules. The PEPC catalyses the irreversible carboxylation of PEP to
52 oxaloacetate, which connects the Krebs cycle with amino acids biosynthesis by
53 providing both carbon (C) and N source for the biosynthesis of aspartate and malate.
54 Thus, Fe deficiency has complex effects on the primary metabolism of C and N in root
55 nodules. Although low Fe availability is well-documented as a major limiting factor
56 affecting SNF capacity, little information is available on the mechanisms connecting
57 inhibited SNF and primary C and N metabolism.

58 The inhibited nodule activity and growth under Fe deficiency mediate by metabolic
59 changes of whole plant. Nodules comprise only a small proportion of the plant weight,
60 however, they consume 13%-28% of the total photoassimilates (Vance *et al.* 2008) to

61 provide the energy for SNF. SNF is limited by ATP availability, which is restricted by
62 Fe availability as Fe is required for electron transfer in bacteroid respiration (Schikora
63 & Schmidt 2001). Additionally, Fe deficiency inhibits the photosynthetic activity of
64 leaves (Atwood *et al.* 2013; Morales *et al.* 1994), which limits the provision of sugars
65 required for nodule metabolism (Fotelli *et al.* 2011). Furthermore, 60% of sugars
66 delivered to nodules are catabolised into organic acids to provide the direct substrates
67 for generating ATP required for nitrogenase activity (Voisin *et al.*, 2003). Thus, the
68 inhibition of photosynthetic activity of leaves due to Fe deficiency limits the function
69 of nodules as well. In addition to leaves, roots are another main storage organ in which
70 the remobilisation of sugars and nitrogenous compound is crucial for regrowth and
71 survival under environmental stress (Aranjuelo *et al.*, 2013; Volence *et al.* 1996).
72 Previous proteomic studies have reported that Fe deficiency causes C and N
73 reprogramming in roots and leaves of cucumber (*Cucumis sativa*) (Barlotti *et al.* 2012),
74 xylem sap and leaves of *Lupinus* (Rellán-Álvarez *et al.* 2010), root tips of beets (*Beta*
75 *vulgaris*) (Rellán-Álvarez *et al.*, 2010) and leaves of soybean (*Glycine max*) (Lima *et*
76 *al.*, 2014). Although these studies characterised the metabolome of specific tissues, the
77 influence of Fe deficiency on the nodule metabolic pathways and the associated
78 metabolic changes between nodules and other plant organs remains uncertain. A
79 thorough understanding of the complex strategies employed by legumes to tackle Fe
80 deficiency can lead to the development of crop cultivars with enhanced symbiotic
81 efficiency via conventional breeding or biotechnological strategies.

82 Grain legumes are an essential source of nutrition and income for a large number of
83 consumers and farmers worldwide (Abdelrahman *et al.* 2018). Soybean, a legume
84 species, contains very high levels of protein and is the main source of vegetable proteins
85 for humans. In this study, to understand the responsive mechanism of leguminous plants
86 to Fe deficiency, we conducted a comprehensive examination of the metabolic and
87 biochemical pathways underlying SNF responses and coordinated metabolic responses
88 in nodules, roots and leaves to Fe deficiency in symbiotic soybean. Our results support
89 the hypothesis that the complex C and N metabolic reprogramming and the coordinated
90 metabolic changes among nodules, roots and leaves were induced in soybean under Fe
91 deficiency.

92 **2 | Methods and Materials**

93 **2.1 | Plant material and growth conditions**

94 Soybean seeds cv. Toyomasari were obtained from Japan, surface-sterilised and
95 germinated in trays containing autoclaved vermiculite for 4 days at 26°C in the dark.
96 After germination, five seedlings were transferred to 2 L pots filled with sterile river
97 sand; only two seedlings with similar growth were retained. Half strength nutrient
98 solution supplemented with 25µM Fe(III)-EDTA as starter Fe was used to water the
99 plants for the first 10 days. Ten-day-old seedlings were inoculated with a suspension of
100 $\sim 10^9$ cells mL⁻¹ of *Bradyrhizobium elkanii* strain BXYD3. The strain was obtained

101 from the NARO Genebank, National Agriculture and Food Research Organization,
102 Tsukuba, Japan.

103 Ten days after inoculation, the seedlings were separated into two plots when the
104 nodules were estimated to appear: one received 2 μM Fe (Fe-deficient treatment; —
105 Fe) and the other received 50 μM Fe(III)-EDTA as control (Fe-sufficient treatment;
106 +Fe). An appropriate concentration of EDTA disodium salt was also added to the Fe-
107 deficient solution to ensure an equal supply of EDTA. Then, the seedlings were watered
108 with nutrient solution containing 1.5 mM KH_2PO_4 , 1.5 mM K_2SO_4 , 0.5 mM NH_4NO_3 ,
109 1 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 200 μM MgSO_4 , 10 μM H_3BO_3 , 1.5 μM
110 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 μM MnSO_4 , 0.5 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.12 μM CoCl_2 and 0.8 μM
111 $\text{Na}_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. Plants were irrigated three times a week with N-free nutrient
112 solution (pH 6.5). Holes in the bottom of the pots allowed run-off of excess nutrient
113 solution, preventing salt accumulation in the sand. Each experiment was performed in
114 quadruplicate, and each replicate consisted of six plants to satisfy the analysis demand.
115 The plants were sampled when most of plants began to bloom, because the anthesis
116 signifies the maturity of nodule (Velde et al. 2006). Forty days after sowing, the shoots,
117 roots and nodules were collected separately and dried at 70 °C until no further changes
118 in weight were observed. The dry weight (DW) of shoots, roots and nodules was
119 measured. In addition, leaf, root and nodule fractions used in assays of enzyme activity
120 were freshly assayed, used in gene expression and metabolite profiling were frozen
121 immediately in liquid N_2 , and stored at —80 °C until the analyses were conducted.

122 **2.2 | Mineral composition analysis**

123 After measuring DW, N concentration on ground dry material of nodules, roots and
124 leaves were determined using Kjeldahl method after digestion in 98% sulphuric acid,
125 as described in previous study (Chu *et al.* 2016b). The concentration of various mineral
126 elements, including phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg),
127 sulphur (S), Fe, manganese (Mn), copper (Cu), zinc (Zn), molybdenum (Mo) and nickle
128 (Ni), were determined after digestion in 61% nitric acid using ICP-MS (Elan, DRC-e,
129 PerkinElmer, Waltham, MA, USA), as described previously (Chu *et al.* 2015, 2016a).

130 **2.3 | Analysis of free ammonium concentration and nitrogenase activity**

131 Frozen nodule samples were ground to a fine powder in liquid N. Free ammonium
132 was extracted from these samples, and its concentration was determined
133 spectrophotometrically at 620 nm, as described by Luo *et al.* (2013). The fresh nodule
134 samples were used for nitrogenase activity assay using the acetylene (C₂H₄) reduction
135 assay, as described by Gordon *et al.* (1999).

136 **2.4 | RNA extraction, reverse transcription and real-time quantitative PCR (qRT- 137 PCR) analysis**

138 Total RNA was extracted from frozen nodule samples (*ca.* 50 mg) using an
139 RNAqueous column with Plant RNA Isolation Aid (Ambio, Austin, TX, USA),
140 according to the manufacturer's protocol. RNA quality and concentration were

141 determined via absorbance measurement at 260 and 280 nm (A_{260}/A_{280}) using a
142 Nanodrop ND-1000 spectrophotometer (Saveen 1 Werner, Malmö, Sweden). Total
143 RNA (1 μ g) was used for cDNA synthesis using QuantiTech Reverse Transcription Kit
144 (Qiagen, Hilden, Germany). qRT-PCR was carried out on four biological replicates as
145 described previously (Libault *et al.* 2010) and each biological replicate was run in
146 duplicate, using previously reported gene-specific primers (Collier & Tegeder 2012;
147 Sarah *et al.* 2016), including those encoding ureide transporters (*GmUSPI-1* and
148 *GmUSPI-2*) and subunits of the bacterial nitrogenase complex, *nifH* (Fe-S subunit) and
149 *nifD* [Component I of the Fe-Mo subunit]. Relative quantification of gene expression
150 was normalised according to the $2^{-\Delta\Delta Ct}$ method. mRNA abundances of targeted genes
151 were evaluated by the relative standard curve method using *Gmcons6* as internal
152 standard for *GmUSPI-1* and *GmUSPI-2* (Libault *et al.* 2010) and 16S ribosomal RNA
153 (rRNA) gene for *nifH* and *nifD*.

154 **2.5 | Assays for starch, total soluble protein and primary C and N metabolism-** 155 **related enzymes**

156 Starch was determined in leaf, root and nodule samples by enzymatic analysis using
157 a Starch Assay Kit (Sigma Aldrich) according to the manufacturer's instructions.

158 Leaf, root and nodule samples were extracted as described previously (Nasr Esfahani
159 *et al.* 2014). The concentration of total soluble protein concentration was determined
160 by using the dye-binding method described by Bradford (1976), with γ -globulin as a

161 standard. Activities of sucrose synthase (SS), alkaline invertase (AI), glutamine
162 synthetase (GS), aspartate amino transferase (AAT) and PEPC were measured
163 spectrophotometrically as described by Gordon & Kessler. 1990. The glutamate
164 dehydrogenase (GDH) and NADH-dependent glutamate synthase (NADH-GOGAT)
165 were measured spectrophotometrically, monitoring the absorbance of NADH at 340
166 nm, as described by Groat & Vance (1981). Activities of NAD⁺-dependent malate
167 dehydrogenase (NAD-MDH) and NAD⁺-dependent malic enzyme (NAD-ME) were
168 measured as described by Apples & Haaker (1988). Activities of aconitase (ACO),
169 citrate synthase (CS), carbonic anhydrase (CA), fumarase (FUM) and NADP⁺-
170 dependent isocitrate dehydrogenase (NADP-ICDH) were analysed as described
171 previously (López-Millán *et al.* 2000).

172 **2.6 | Lipid peroxidation assay**

173 Lipid peroxidation in nodules, roots and leaves was assayed as described by Hodges
174 *et al.* (1999), measuring the concentration of malondialdehyde (MDA), the major 2-
175 thiobarbituric acid-reacting substance (TBA). Modifications were carried out to correct
176 interference generated by non-specific turbidity, thiobarbituric acid-sugar complexes,
177 and other non-TBA compounds absorbing at 532 nm.

178 **2.7 | Analyses of ureides and ureide metabolism-related enzymes**

179 To analyse ureides, the lyophilised and ground nodule, root and leaf sample (*ca.* 5-
180 10 mg) were homogenised in 1 ml 0.2 M NaOH for ureide determination. The

181 homogenates were treated and analysed as described previously (Gil-Quintana *et al.*
182 2013). In this method, allantoin is transformed to allantoate by an alkaline hydrolysis
183 and determined using the high-performance capillary electrophoresis (P/ACE 5500;
184 Beckman Coulter Instruments, Fullerton, CA, USA). Activities of ureides metabolism-
185 related enzymes were measured as described by Gil-Quintana *et al.* (2013). Fresh plant
186 samples were ground to a fine powder under liquid nitrogen and 4 ml extraction buffer
187 per gram of tissue were added. The activity of urate oxidase (UO) was determined from
188 the decrease in absorbance at 292 nm due to the aerobic oxidation of urate. The activity
189 of allantoate-degrading enzyme (ADE), including allantoinase and allantoate
190 amidohydrolase, was determined using a colorimetric assay based on the determination
191 of glyoxylate.

192 **2.8 | Plant metabolites extraction**

193 Plant metabolites were extracted from nodule, root and leaf samples from -Fe and
194 +Fe treatments and analysed via gas chromatography coupled with time-of-flight mass
195 spectrometry (GC-MS) as reported previously (Hernández *et al.* 2009). Frozen leaf,
196 root and nodule tissues (*ca.* 10-35 mg) were ground in liquid N using a mortar and
197 pestle to ensure the samples are metabolically inactive. Frozen powder was
198 homogenised in ice-cold solution of methanol: chloroform: water (3:1:1), with the
199 addition of ribitol (0.2 mg ml⁻¹ of methanol) as an internal standard. The slurry was
200 mixed for 5 min using a microtube mixer. Approximately 160 µL of distilled water was

201 added to the extraction solution to separate the polar and nonpolar phases. After
202 centrifugation, only the upper layer (polar phase) was used for further analysis.
203 Derivatisation was performed as described previously (Watanabe *et al.* 2012).

204 **2.9 | GC-MS analysis**

205 After derivatisation, samples (1 μ L) were injected randomly in splitless mode with
206 a cold injection system (Gerstel, Mülheim an der Ruhr, Germany) into GC (Agilent
207 GC6890, San Jose, CA, USA) and analysed as described previously (Watanabe *et al.*
208 2012). Raw data of the detected metabolites were transferred from the chromaTOF
209 software in NetCDF format to MATLAB software 7.0 (MathWorks). The numerical
210 analyses of metabolome were based on the peak height values of the response values.
211 Chromatograms were processed using high-throughput data analysis method. These
212 values were normalised by the sample fresh weight and ribitol (internal standard), using
213 the cross-contribution-compensating multiple standard normalisation algorithm
214 (Jansson *et al.* 2005; Redestig *et al.* 2009). The data of fold-changes of $-Fe/+Fe$
215 treatment were calculated.

216 **2.10 | Statistical analyses**

217 Four biological replicates from each $+Fe/-Fe$ treatment and tissue were used to
218 evaluate the growth parameter, enzymes activity, metabolite analyses and level of gene
219 expression. A one-way ANOVA with Bonferroni test was applied to analyse the effect
220 of the Fe deficiency on the whole dataset with a significant threshold of $P \leq 0.05$. The

221 dataset of metabolome, ionome and enzymes activity in nodules, roots and leaves was
222 submitted to orthogonal partial least squares-discriminant analysis (OPLS-DA) carried
223 out with Simca-P (MKS Umetrics, Malmo, Sweden), respectively. Prior to OPLS-DA,
224 data were standardised using unit variance scaling by Simca-P to remove the unit
225 difference of different variables. The OPLS model was evaluated using the
226 determination coefficient R^2 , the predictive power Q^2 by the cross-validated
227 determination coefficient. The significance of the statistical OPLS model was tested
228 using a chi-square comparison with a random model (average \pm random error), and the
229 associated P -value ($P_{CV-ANOVA}$) is reported. A biplot co-charting scores of treatments
230 and loadings of variables was made to interpret the difference of treatments in terms of
231 the variables. To enable the score vectors can be re-scaled into the -1 and $+1$
232 numerical range in biplot, the scaling factor was based on a ratio of the sum of squares
233 of the loadings to the sum of squares of the scores. A volcano-plot was made to identify
234 the important variables to discriminate $+Fe/ -Fe$ treatment using the $-\log_{10}P$,
235 whereby P was obtained in one-way ANOVA with Bonferroni test, plotting against the
236 rescaled loading of variables (P_{corr}) obtained in the OPLS-DA.

237 **3 | Results**

238 **3.1 | Plant growth and nodulation**

239 The DW of shoot and root of soybean grown under Fe-deficiency was significantly
240 reduced compared with the control samples (**Table. S2**). Shoot DW was more severely

241 affected than the root DW under Fe-deficient treatment because the ratio of root to
242 shoot was 1.35-fold higher than that in control. Fe-deficient stress did not affect the
243 nodule number, however, the nodule DM in Fe-deficient treatment was only 35.02% of
244 that in the control. This suggests that Fe-deficiency did not alter nodule formation but
245 rather inhibit nodule development.

246 **3.2 | Mineral elements concentration in nodules, roots and leaves of soybean**

247 The concentration of Fe in nodules, roots and leaves grown under Fe-deficient
248 treatment was significantly reduced compared with the control samples (**Fig. 1**). With
249 Fe deprivation in growth medium, the Fe concentration in nodules, roots and leaves
250 was decreased by 1.63-, 4.41- and 2.26-fold, respectively. Moreover, Fe deficiency
251 affected significantly the concentration of other elements in different tissues as well
252 (**Fig. S1**). In nodules, Fe deficiency was accompanied by a reduced level of N, S, Cu,
253 Mo and Ni and compensated for an increase of P and K. In roots, N, K, Mg, S, Mn and
254 Zn concentration decreased and P, Cu, Mo and Ni increased upon Fe deficiency.
255 Growth of plants under Fe deficiency also affected mineral elements in leaves, with
256 markedly decreased S, Mo and Ni and increased K, Mg, Mn, Zn and Cu concentration.

257 **3.3 | Ammonium concentration, SNF capacity and SNF products (ureides) in** 258 **nodules**

259 Data of ammonium concentration and nitrogenase activity in nodules are shown in
260 **Fig. 2**. Ammonium is the immediate product of SNF. Fe deficiency increased

261 ammonium concentration in nodules by 1.70-fold relative to control. The nitrogenase
262 catalyses the reduction of N₂ to ammonium. The activity of nitrogenase was greatly
263 reduced under Fe-deficient conditions and was only 18.63% of that in control. Also,
264 Fe-deficient stress resulted in the downregulation of *nifD* and *nifH* transcription, 2.30-
265 and 3.71-fold lower compared with Fe-sufficient condition, respectively (**Fig. 3**). The
266 decreased nitrogenase activity and downregulation of *nifD* and *nifH* reflects the
267 inhibition of Fe deficiency on SNF capacity.

268 In most tropical leguminous species such as soybean, fixed N is initially incorporated
269 into the asparagine, aspartate, glutamine and glutamate and is then further metabolised
270 in nodules to form ureides, including allantoin and allantoic acid, which are exported
271 to and transported in the xylem stream. In this study, Fe deficiency caused an increase
272 of ureides in nodules, 2.08-fold higher than in control, despite inhibited SNF capacity.
273 Nevertheless, the activity of UO and ADE, enzymes involved in ureides biosynthesis,
274 was both reduced by 68.2% and 81.1%, respectively, under Fe-deficient conditions
275 compared with Fe-sufficient conditions (**Fig. 4**). Ureides content was significantly
276 reduced in Fe-deficient roots and leaves by 36.8% and 43.1%, respectively (**Fig. 4**).
277 Additionally, the expression levels of *GmUPSI-1* and *GmUPSI-2* genes, responsible
278 for ureides transport, were significantly downregulated under Fe-deficient conditions
279 compared with Fe-sufficient conditions (**Fig. 3B**).

280 **3.4 | Lipid oxidation**

281 The lipid oxidation was measured through the determination of MDA content as a
282 marker of oxidative damage in nodules, roots and leaves (**Fig. 5**). Fe deficiency did not
283 cause significant changes of MDA content in leaves, however, caused significant
284 increase in roots and nodules, suggesting Fe deficiency provoked a generalised
285 oxidative damage in roots and nodules.

286 **3.5 | Multivariate analysis of metabolome, enzyme activity and elements in** 287 **nodules**

288 In total, 77, 77 and 74 metabolites were identified in nodules, roots and leaves
289 through peak comparison with mass spectra libraries (**Table. S3**). The OPLS-DA was
290 conducted to identify important variables that discriminate the effect of +Fe/-Fe
291 treatment using the dataset consisting of metabolome, mineral elements and enzymes
292 activity in nodules (**Fig. 6A**). The variables were clearly discriminated along the
293 horizontal axis between +Fe/-Fe sample groups in the multivariate analysis (**Fig. 6A**).
294 The statistical model was highly significant ($P_{CV-ANOVA} < 10^{-6}$) and predictive ($R^2 =$
295 0.827 , $Q^2 = 0.893$). The volcano-plot (**Fig. 6B**) was made to screen out the most
296 important variables (biomarker) to reflect the effects of Fe deficiency. Biomarkers of
297 enzymes are shown in **Fig. 6C** and metabolites in **Fig. 6D**.

298 It was noting that all of the assayed enzymes were screened out except AI (**Fig. 6C**).
299 Fe deficiency inhibited the activity of enzymes catalysing CO₂ fixation and organic
300 acids production, including CA, ME, GDH, FUM, ACO and ICDH, while increased

301 the organic acids content, including citrate, fumarate, α -ketoglutarate, malate and
302 succinate (**Fig. 6D**). These results suggest that Fe deficiency inhibited Krebs cycle but
303 still increased the accumulation of organic acids (**Fig. 6E**). Also, Fe deficiency
304 inhibited the activity of enzymes catalysing amino acids biosynthesis, including AAT,
305 GS and GOGAT, however, improved the activity of GDH. Accordingly, alanine, serine,
306 glutamate, glycine, lysine, threonine and aspartate showed significant decrease under
307 Fe deficiency, while proline, asparagine, tyrosine and phenylalanine showed significant
308 increase. Fe-deficiency caused the reduction of N-storage compounds as well,
309 including proteins, urea, spermidine and putrescine. Moreover, Fe deficiency inhibited
310 the decomposition of sucrose by repressing the SS activity and improved the sugars
311 accumulation in nodules, including sucrose, ribose, galactonate, glucuronate, arabinose
312 and raffinose. Some marker of oxidative stress, including MDA, ascorbate and
313 threonate, were increased in Fe-deficient nodules.

314 **3.6 | Multivariate analysis of metabolome, enzyme activity and elements in roots**

315 The OPLS-DA was conducted to identify important variables that discriminate the
316 effect of +Fe/-Fe treatment in roots (**Fig. 7A**). The variables in roots were clearly
317 discriminated along the horizontal axis between +Fe/-Fe sample groups (**Fig. 7A**). The
318 statistical model was highly significant ($P_{CV-ANOVA} < 10^{-6}$) and predictive ($R^2 = 0.895$,
319 $Q^2 = 0.936$). From volcano plot ((**Fig. 7B**), PEPC, CA, GDH, ACO, FUM and CS were
320 screened out as the biomarker enzymes in Fe-deficient roots (**Fig. 7C**).

321 Improved activity of ACO, CS and FUM was consistent with the increased content
322 of citrate, α -ketoglutarate, succinate, maleate and malate in Krebs cycle (**Fig. 7D&E**).
323 Fe deficiency inhibited PEPC activity but still increased pyruvate content. Moreover,
324 ascorbate, threonate and many sugars showed similar increasing trend both in roots and
325 nodules. In addition, nicotinate, relating to the production of reactive oxygen species
326 (ROS) (Pétriacq *et al.* 2012, 2016), was increased in roots under Fe deficiency. For N
327 metabolism, Fe deficiency caused the decrease of alanine, aspartate, lysine, glycine,
328 valine, tyrosine and serine, however, the activity of amino acids biosynthesis was not
329 affected except GDH. Fe deficiency also affected shikimate pathway, reducing
330 shikimate and tyrosine content but increasing phenylalanine (**Fig. 7E**). Notably,
331 nicotianamine, an effective Fe-chelator (Curie *et al.* 2009), was increased under Fe
332 deficiency.

333 **3.7 | Multivariate analysis of metabolome, enzyme activity and elements in leaves**

334 The OPLS-DA was conducted to identify important variables that discriminate the
335 effect of +Fe/-Fe treatment in roots (**Fig. 8A**). The variables in roots were clearly
336 discriminated along the horizontal axis between +Fe/-Fe sample groups (**Fig. 8A**). The
337 statistical model was highly significant ($P_{CV-ANOVA} < 10^{-4}$) and predictive ($R^2 = 0.849$,
338 $Q^2 = 0.952$). From volcano plot (**Fig. 8B**), among enzymes participating in the
339 production of organic acids, only ACO was screened out. Fe deficiency inhibited ACO
340 activity in leaves (**Fig. 8C**). Moreover, succinate, fumarate, malate and α -ketoglutarate

341 were reduced (**Fig. 8D**). PEPC and CA were both inhibited by Fe deficiency and
342 pyruvate content reduced accordingly (**Fig. 8E**). SS and AI were inhibited and fructose
343 content was reduced. However, Fe deficiency caused the increase of many sugars,
344 including starch, arabinose, glucose, galactinol and raffinose in leaves, showing a
345 similar trend with roots and nodules. Moreover, Fe deficiency repressed GOGAT and
346 GS activity, however, increased the content of many amino acids and two nitrogenous
347 metabolites, GABA and nicotianamine. Notably, the increase of aspartate affected by
348 Fe deficiency resulted in the decrease of nicotinate, which shows an opposite varying
349 trend between leaves and roots.

350 **4 | Discussion**

351 **4.1 | Fe deficiency inhibits SNF and N assimilation**

352 The present study shows that Fe deficiency inhibited SNF capacity, as revealed by
353 inhibited nitrogenase activity and expression levels of *nifD* and *nifH* (Nasr Esfahani *et*
354 *al.* 2014; 2016; Sarah *et al.* 2016), but increased content of direct product ammonium
355 (**Fig. 2**) and final product ureides in nodules (**Fig. 4**), which is consistent with previous
356 study by Rotaru & Sinclair (2009). The imbalance between SNF capacity and products
357 could be explained by inhibited export of ureides to xylem sap, as detected in
358 downregulated *GmUPS1-1* and *GmUPS1-2* (**Fig. 3B**) and reduced ureides content in
359 roots and leaves. Repressed UO and ADE (**Fig. 4**) indicated that ureide accumulation
360 was irrelevant with the ureides biosynthesis in nodules. According to previous studies,

361 the anomalous ureide accumulation in nodules under abiotic stress may be a N-feedback
362 regulation on nitrogenase activity (Gil-Quintana *et al.* 2013; King & Purcell 2005).

363 The deleterious effect of Fe deficiency on SNF was accompanied by impeded N
364 assimilation in nodules, as reflected on the inhibited GS, GOGAT and AAT activity
365 (**Fig. 6C**) and the reduction of amino acids (**Fig. 6D**). Negative effects of Fe deficiency
366 on GS/GOGAT cycle have been reported in common bean (Slatni *et al.* 2008) and
367 cucumber (Barlotti *et al.* 2012). By contrast, the GDH activity was improved in nodules,
368 which suggests that GDH possibly helped prevent the accumulation of toxic ammonium
369 (**Fig. 2**) and played an alternative role to the GS/GOGAT cycle in ammonium
370 assimilation (Robinson *et al.* 1991; Slatni *et al.* 2008). Additionally, as organic acids
371 provide C-skeleton for amino acids biosynthesis, the imbalance between increased
372 organic acids and reduced amino acids in nodules (**Fig. 6D**) suggests the inhibited N
373 assimilation. α -ketoglutarate, the C-skeleton of glutamine and lysine, increased but
374 glutamine and lysine reduced under Fe deficiency. Glyceric-acid-3-phosphate
375 increased and its receptor serine and glycine reduced. Moreover, the inhibition of Fe
376 deficiency on N assimilation was also revealed by the cleavage of N-storage compound,
377 including protein, urea, 5,6-dihydrouracil, spermidine and putrescine (**Fig. 6D**). The
378 cleavage of N-storage compounds may play a vital role in the accelerating C and N
379 rearrangement in nodules, and explained the abnormal increase of some amino acids.
380 For instance, asparagine still increased although AAT activity was repressed.

381 In roots, Fe deficiency led to the reduction of many amino acids, showing a similar
382 trend with nodules (**Fig. 7D**). The reduced amino acids in root was probably transported
383 to leaves (**Fig. 8D**), because in Fe-deficient leaves, repressed GS and GOGAT activity
384 reflected the inhibited N assimilation but the amino acids still increased (**Fig. 8C**). The
385 increased amino acids content in Fe-deficient leaves is consistent with previous studies
386 (Barlotti *et al.* 2012; Donnini *et al.* 2010; Lima *et al.* 2014; Rellán- Álvarez *et al.* 2011).
387 The lower shoot biomass and higher root/shoot biomass ratio (**Table S1**) implied that
388 soybean decelerated growth to maintain N metabolism, which resembles the adaptation
389 of plants to N deficiency (Luo *et al.* 2013). Moreover, increasing evidence has also
390 suggested that shoot high amino acids content regulates the SNF capacity via a whole-
391 plant N feedback mechanism (Gil-Quintana *et al.* 2013; Nasr Esfahani *et al.* 2016). In
392 the present study, Fe deficiency led to the increase of GABA in in leaves (Fig. 8D),
393 which agrees with previous study by Lima *et al.* (2014). GABA plays an important role
394 in N feedback regulation as a signal molecule (Sulieman 2011) and usually generated
395 under abiotic stress (Aranjuero *et al.*, 2013; Nasr Esfahani *et al.*, 2014; 2016).

396 Taken together, Fe deficiency markedly inhibited SNF in nodules and N assimilation
397 in the whole plant. Soybean plant regulated N metabolism to acclimate to Fe-deficient
398 stress, possibly by decelerating shoot growth, cleavage of N-storage compounds, N
399 feedback regulation and coordinating the amino acids allocation between below- and
400 above-ground tissues.

401 **4.2 | Impacts of Fe deficiency on C metabolism**

402 A striking common effect of Fe deficiency on plant development is the inhibition of
403 photosynthesis (Atwood *et al.* 2013; Morales *et al.* 1994). However, in this study, most
404 of sugars were unexpectedly increased or maintained at a relatively stable level under
405 Fe-deficient treatment, irrespective of tissues. In nodules, the energy for SNF mainly
406 originates from the sucrose (Gordon *et al.* 1999). The inhibited SS activity and over-
407 accumulation of sucrose in nodules was probably attributed to the reduced energy
408 demand by inhibited SNF capacity, as reported in P-deprived stress (Nasr Esfahani *et*
409 *al.* 2016) and drought stress (Aranjuelo *et al.* 2013). Also, Fe deficiency caused the
410 increase of starch content in leaves (**Fig. 8D**), which may be attributed to the inhibited
411 phloem transport of C out of leaves due to the inhibited leaf growth. This agrees with
412 earlier studies in cucumber (De Nisi *et al.* 2012). Moreover, Fe deficiency led to the
413 increase of arabinose, raffinose, ribose and galactonate in nodules and roots, which
414 might be attributed to the inhibited cell wall biosynthesis because all of these sugars
415 are precursors of polysaccharide constituents of the cell wall (Zablackis *et al.* 1995).
416 Limited nodules and roots growth under Fe-deficient stress (**Table S2**) likely led to the
417 restricted cell extension. Taken together, the C metabolism was inhibited under Fe-
418 deficiency treatment although sugars content increased; the simultaneous increase of
419 sugars in different tissues may be an adaptive strategy to Fe-deficient stress through
420 feedback regulation and modulating cell wall development.

421 Moreover, Fe deficiency promoted organic acids accumulation in nodules and roots
422 but inhibited in leaves. In nodules, Fe deficiency inhibited the activity of enzymes

423 involved in the Krebs cycle but increased the intermediates content (organic acids). The
424 inconsistent results suggested that the increased organic acids accumulation might be
425 more attributed to reduced C-skeleton requirement by inhibited N assimilation (as
426 mentioned in 4.1). Moreover, Fe deficiency stimulated PEPC activity in nodules, which
427 agrees with previous study (Slatni *et al.* 2008, 2011) and plays crucial role in metabolic
428 fluxes through Krebs cycle (Arabjuero *et al.*, 2013; Fischinger *et al.*, 2010). Stimulated
429 PEPC might tend to catalyse the formation of oxaloacetate at the expense of PEP and
430 thus prevented pyruvate production (**Fig. 6E**). Additionally, reduced alanine might be
431 remobilised to regenerate pyruvate by Cahill cycle and therefore the acetyl-CoA
432 production was maintained. The citrate synthesis was thus improved with the stimulated
433 CS activity. In Fe-deficient roots, increased organic acids showed similar trend as in
434 nodules, but the mechanism was different because they could be coupled to the
435 stimulated enzymes activity in Krebs cycle (**Fig. 7D**). Fe deficiency led to increase of
436 pyruvate and citrate, and decrease of alanine and valine (**Fig. 7D**), suggesting the
437 decrease of pyruvate amination at the expense of increased pyruvate utilization by
438 Krebs cycle. Moreover, the increased production of organic acids in roots may help to
439 1) potentially release into the soil to improve Fe solubility (Rellán-Álvarez *et al.* 2010,
440 2011; Rodríguez-Celma *et al.* 2013); 2) improve Fe uptake and translocation because
441 some organic acids are effective Fe carrier, such as citrate (Takahashi *et al.* 2013); 3)
442 provide C skeleton to the anaplerotic reaction for synthesising amino acids to export to
443 nodules and shoots (Barlotti *et al.* 2012; Rellán-Álvarez *et al.* 2011). In addition, PEPC

444 activity in Fe-deficient roots was inhibited, which accords with previous study in
445 soybean (Zocchi *et al.* 2007) but disaccords with the results in tomato and sugar beet
446 that PEPC activity increased up to several folds (López-Millán *et al.* 2000; Li *et al.*
447 2008). This paradox may be explained by upregulated shikimate pathway, as revealed
448 by reducing shikimate and increasing phenylalanine (**Fig. 7E**). Shikimate production
449 and reaction catalysed by PEPC both require PEP as substrate. More PEP might enter
450 shikimate pathway due to the repressed PEPC activity. The stimulated shikimate
451 pathway could help to ameliorate the production of phenolics which potentially exudate
452 to the rhizosphere to acquire Fe (Donnini *et al.* 2012; Zocchi *et al.* 2007). In leaves, Fe
453 deficiency inhibited PEPC and CA activity, which indicates the limitation of CO₂
454 fixation. Accordingly, the organic acids content, including succinate, fumarate, malate
455 and α -ketoglutarate, was reduced in Fe-deficient leaves as well. Taken together, PEPC
456 may be an important indicator for the adaptation of soybean to Fe deficiency: the
457 stimulated PEPC activity in nodules and inhibited activity in leaves reflected whether
458 promoted the metabolic fluxes through Krebs cycle to produce organic acids; the
459 inhibited PEPC activity in roots reduced the expense of PEP and indirectly stimulated
460 the shikimate pathway.

461 In nodules, among the enzymes involved in Krebs cycle, only CS activity was
462 improved by Fe deficiency. Also, the similar increase of CS activity and citrate was
463 observed in roots. This special metabolic change may be related to stimulated Fe
464 transport under Fe-deficient stress, because citrate is an effective Fe-carrier both in

465 roots and nodules (Moreau *et al.* 1995; Shi *et al.* 2012; Takahashi *et al.* 2013). In
466 addition to citrate, nicotianamine, complexing Fe in a soluble form suitable for long-
467 distance transport (Curie *et al.* 2009), was increased in Fe-deficient roots and leaves.
468 The stimulated production of citrate and nicotianamine is a possible mechanism to
469 stimulate Fe uptake and transport under Fe-deficient stress.

470 Furthermore, Fe deficiency induced the oxidative stress in roots and nodules, which
471 is reflected by the increased MDA content (**Fig. 5**). Oxidative stress induced by Fe
472 deficiency has been reported because of the inactivity of Fe-containing superoxide
473 dismutase (SOD) and catalase (López-Millán *et al.* 2013). Also, Fe deficiency caused
474 the increase of ascorbate and threonate in nodules and roots (**Fig. 7D&7E**), which
475 possibly suggests the upregulation of ascorbate-glutathione cycle to resist the oxidative
476 stress (Aranjuero *et al.* 2013; Becana *et al.* 2010). In addition, Fe deficiency caused the
477 increase of raffinose and galactinol in different organs, which coincides with the
478 previous study in Fe-deficient roots of cucumber (Li *et al.* 2008). These sugars have
479 been demonstrated to protect plant cells from oxidative damage by scavenging
480 hydroxyl radicals (Nishizawa *et al.* 2008). Moreover, NAD has been demonstrated to
481 positively correlate with the production of reactive oxygen species (ROS) (Pétriaccq *et*
482 *al.* 2012, 2016). In this study, Fe deficiency caused the decrease of aspartate and
483 increase of nicotinate in roots (**Fig. 7D**) and totally opposite changes in leaves (**Fig.**
484 **7E**), suggesting that Fe deficiency also affected NAD metabolism. Higher pool of
485 nicotinate, which is the derivative of NAD, was detected in Fe-deficient roots. This

486 suggests that the catabolism of NAD was possibly induced to avoid stimulating the
487 ROS production. Additionally, increased proline in nodules may be another indicator
488 of resisting oxidative stress because proline can promote the activity of catalase and
489 SOD to protect against ROS (Aranjuelo *et al.* 2011; Saibi *et al.* 2015).

490 **4.3 | Conclusions and perspectives**

491 We show here that Fe-deficient stress inhibited SNF capacity and N assimilation,
492 caused oxidative stress and led to an extensive C and N reprogramming of various
493 metabolic pathways in nodules, roots and leaves (**Fig. S2**, the proposed model). The
494 concerted metabolic change between different tissues may be a compromise between
495 tissues to acclimate to Fe-deficient stress, as reflected by feedback regulation of ureides
496 accumulation in nodules, GABA generation, simultaneous sugars accumulation in
497 different tissues. To our knowledge, this work is the first integrative analyses of
498 metabolic, biochemical and molecular analyses of leguminous nodules under Fe
499 deficiency, with an aim to gain important insights into the response of legume plants to
500 Fe deficiency. Our results may be used for screening Fe-deficiency-tolerant soybean
501 genotypes and perhaps other leguminous crops, to increase the symbiotic efficiency of
502 crop plants under Fe-deficient conditions; this involved targeting key biochemical
503 responses identified in this study.

504 We recognise that in present study we could not dissect all of the important metabolic
505 changes due to the limited analysis. The oxidative stress induced by Fe deficiency may

506 be closely related to the C and N metabolites; generated GABA under Fe-deficient
507 leaves may have important regulatory role on SNF and N assimilation. Information
508 generated here combined with future studies, including proteomic and microarray
509 analyses in nodules and other organs may help in improving the acclimation of grain
510 legumes to Fe-deficient environment.

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Supplementary data

Supplementary data are available at *PCE* online.

Table S1. Target genes and primers in quantitative RT-PCR

Table S2. Growth parameter and nodulation levels in soybean plants under Fe-deficient stress

Table S3. Normalised values of metabolites in nodules, roots and leaves of soybean plants

Fig S1. Mineral elements concentration in roots, nodules and leaves of soybean plants grown under sufficient or deficient Fe condition.

Fig S2. Summary of speculated primary metabolic changes in nodules, roots and leaves of soybean in response to Fe deficiency.