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

1. Key Laboratory of Agro-Environment in Downstream of Yangtze Plain, Ministry of Agriculture, Institute of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
2. Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589, Japan
3. Centre of Integrated Water-Energy-Food Studies, School of Animal, Rural and Environmental Sciences, Nottingham Trent University, Brackenhurst Campus, Nottinghamshire, NG25 0QF, UK
4. Graduate School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, 200240, China
Information for corresponding author:
*Qingnan Chu, Key Laboratory of Agro-Environment in Downstream of Yangtze Plain, Ministry of Agriculture, Institute of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
Fax: +44 1158485296
E-mail: qingnan.chu@ntu.ac.uk

Running titles
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Email address for each author:
Qingnan Chu: qingnan.chu@ntu.ac.uk
Zhimin Sha: zhiminsha@hotmail.com
Hayato Maruyama: myhayato@res.agr.hokudai.ac.jp
Linzhang Yang: lzyang@issas.ac.cn
Gang Pan: gang.pan@ntu.ac.uk
Lihong Xue: njxuelihong@gmail.com
Toshihiro Watanabe: nabe@chem.agr.hokudai.ac.jp

Abstract

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

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

Introduction

Iron (Fe) is a transition metal and an essential nutrient for the survival of all higher plants. In plants, Fe plays critical roles in metabolic processes, including respiration, electron transport and photosynthesis (López-Millán *et al*. 2013), and as a component of Fe–sulfur (S) cluster or haem-containing enzymes involved in oxydoreduction reaction (Qin *et al*. 2015; Sarah *et al*. 2016). However, despite its relative abundance in soil, plants frequently show symptoms of Fe deficiency, especially under high pH conditions (Donnini *et al*. 2012; Hsieh & Waters 2016). Low Fe availability causes a common nutritional disorder in plants, namely chlorosis, resulting in lower crop yields and quality (Chen *et al*. 2018). Given that more than 30% of the soils in the world are calcareous, Fe deficiency is predicted to be a common problem (Chen & Barak 1982). Fe deficiency induces various responses in plant roots that increase the availability of the Fe^{3+} in the rhizosphere. Strategy I (dicotylednus) plants respond to the shortage of Fe in the soil by increasing the activity of Fe^{3+} chelate reductase (FCR) in roots (Robinson *et al*. 1999), releasing protons which acidifies the rhizosphere and increases Fe ion solubility (Santi & Schmidt 2009).

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

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

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

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

2 | Methods and Materials

2.1 | Plant material and growth conditions

Soybean seeds cv. Toyomasari were obtained from Japan, surface-sterilised and germinated in trays containing autoclaved vermiculite for 4 days at 26°C in the dark. After germination, five seedlings were transferred to 2 L pots filled with sterile river sand; only two seedlings with similar growth were retained. Half strength nutrient solution supplemented with 25μM Fe(Ⅲ)-EDTA as starter Fe was used to water the plants for the first 10 days. Ten-day-old seedlings were inoculated with a suspension of ~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 50μM 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 $NaH_2PO_4 \cdot 2H_2O$, 2 mM $CaSO_4 \cdot 2H_2O$, 200 μM $MgSO_4$, 10 μM H_3BO_3, 1.5μM
110 $ZnSO_4 \cdot 7H_2O$, 1.5μM $MnSO_4$, 0.5 μM $CuSO_4 \cdot 5H_2O$, 0.12 μM $CoCl_2$ and 0.8 μM
111 $Na_6Mo_7O_{24} \cdot 4H_2O$. 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.

2.2 | Mineral composition analysis

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

2.3 | Analysis of free ammonium concentration and nitrogenase activity

Frozen nodule samples were ground to a fine powder in liquid N. Free ammonium was extracted from these samples, and its concentration was determined spectrophotometrically at 620 nm, as described by Luo *et al*. (2013). The fresh nodule samples were used for nitrogenase activity assay using the acetylene (C_2H_4) reduction assay, as described by Gordon *et al*. (1999).

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

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

determined via absorbance measurement at 260 and 280 nm (A_{260}/A_{280}) using a Nanodrop ND-1000 spectrophotometer (Saveen 1 Werner, Malmö, Sweden). Total RNA (1 μg) was used for cDNA synthesis using QuantiTech Reverse Transcription Kit (Qiagen, Hilden, Germany). qRT-PCR was carried out on four biological replicates as described previously **(**Libault *et al*. 2010**)** and each biological replicate was run in duplicate, using previously reported gene-specific primers (Collier & Tegeder 2012; Sarah *et al*. 2016), including those encoding ureide transporters (*GmUSP1-1* and *GmUSP1-2*) and subunits of the bacterial nitrogenase complex, *nifH* (Fe-S subunit) and *nifD* [Component I of the Fe-Mo subunit]. Relative quantification of gene expression was normalised according to the $^{2^-}\Delta\Delta$Ct method. mRNA abundances of targeted genes were evaluated by the relative standard curve method using *Gmcons6* as internal standard for *GmUSP1-1* and *GmUSP1-2* (Libault et al. 2010) and 16S ribosomal RNA (rRNA) gene for *nifH* and *nifD*.

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

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

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

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

2.6 | Lipid peroxidation assay

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

2.7 | Analyses of ureides and ureide metabolism-related enzymes

To analyse ureides, the lyophilised and ground nodule, root and leaf sample (*ca.* 5-10 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 chromoatography 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

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

2.9 | GC-MS analysis

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

2.10 | Statistical analyses

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

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

3｜Results

3.1｜Plant growth and nodulation

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

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

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

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

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

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

ammonium concentration in nodules by 1.70-fold relative to control. The nitrogenase catalyses the reduction of N_2 to ammonium. The activity of nitrogenase was greatly reduced under Fe-deficient conditions and was only 18.63% of that in control. Also, Fe-deficient stress resulted in the downregulation of *nifD* and *nifH* transcription, 2.30- and 3.71-fold lower compared with Fe-sufficient condition, respectively (**Fig. 3**). The decreased nitrogenase activity and downregulation of *nifD* and *nifH* reflects the inhibition of Fe deficiency on SNF capacity.

In most tropical leguminous species such as soybean, fixed N is initially incorporated into the asparagine, aspartate, glutamine and glutamate and is then further metabolised in nodules to form ureides, including allantoin and allantoic acid, which are exported to and transported in the xylem stream. In this study, Fe deficiency caused an increase of ureides in nodules, 2.08-fold higher than in control, despite inhibited SNF capacity. Nevertheless, the activity of UO and ADE, enzymes involved in ureides biosynthesis, was both reduced by 68.2% and 81.1%, respectively, under Fe-deficient conditions compared with Fe-sufficient conditions (**Fig. 4**). Ureides content was significantly reduced in Fe-deficient roots and leaves by 36.8% and 43.1%, respectively (**Fig. 4**). Additionally, the expression levels of *GmUPS1-1* and *GmUPS1-2* genes, responsible for ureides transport, were significantly downregulated under Fe-deficient conditions compared with Fe-sufficient conditions (**Fig. 3B)**.

3.4 | Lipid oxidation

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

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

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

It was noting that all of the assayed enzymes were screened out except AI (**Fig. 6C**). Fe deficiency inhibited the activity of enzymes catalysing CO_2 fixation and organic 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 ($P_{CV\text{-}ANOVA} < 10^{-6}$) 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)**.

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

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

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

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

4 | Discussion

4.1 | Fe deficiency inhibits SNF and N assimilation

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

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

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

4.2 | Impacts of Fe deficiency on C metabolism

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

Moreover, Fe deficiency promoted organic acids accumulation in nodules and roots 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 CO_2
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

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

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

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

4.3 | Conclusions and perspectives

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

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

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