Asymmetric Redundancy of Soybean *Nodule Inception (NIN)* Genes in Root Nodule Symbiosis

Mengdi Fu^{1,2}, Jiafeng Sun³, Xiaolin Li¹, Yuefeng Guan^{4,*} and Fang Xie^{1,*}

¹ National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai 200032, China

² University of the Chinese Academy of Sciences, Beijing, China

³ College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou, Fujian, 350002, China

⁴ FAFU-UCR Joint Center for Horticultural Biology and Metabolomics, Fujian Agriculture and Forestry University, Fuzhou, 350002, China

* To whom correspondence should be addressed: fxie@cemps.ac.cn; guan@fafu.edu.cn

AUTHOR CONTRIBUTIONS

M. Fu, X. Li and F. Xie designed the research, M. Fu and X. Li conducted experiments and analyzed data. J. Sun and Y. Guan generated and scored the CRISPR-Cas9 mutants. F. Xie and M. Fu wrote the paper. All authors read and

24 approved the final article.

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32 **ABSTRACT**

33 *NIN* is one of the most important root nodule symbiotic genes as it is required for both
 34 infection and nodule organogenesis in legume. Unlike most legumes with a sole *NIN*
 35 gene, there are four putative *NIN* genes in soybean (*Glycine max*). Whether and how
 36 these orthologs *NIN* genes contribute to soybean-rhizobia symbiotic interaction
 37 remain unknown. In this study, we found that all four *GmNIN* genes are induced by
 38 rhizobia, and that conserved CE and CYC binding motifs in their promoter regions
 39 are required for their expression in the nodule formation process. By generation of
 40 multiplex *Gmnin* mutants, we found that *Gmnin1a nin2a nin2b* triple mutant and
 41 *Gmnin1a nin1b nin2a nin2b* quadruple mutant displayed similar defects in rhizobia
 42 infection and root nodule formation, *Gmnin2a nin2b* produced less nodules but
 43 displayed hyper infection phenotype than wild type, while a *Gmnin1a nin1b* double
 44 mutant nodulated as wild type. Overexpression of *GmNIN1a*, *GmNIN1b*, *GmNIN2a*,
 45 and *GmNIN2b* reduced nodule numbers after rhizobia inoculation, with *GmNIN1b*
 46 overexpression having the weakest effect. In addition, overexpression of *GmNIN1a*,
 47 *GmNIN2a*, or *GmNIN2b*, but not *GmNIN1b*, produced malformed pseudo-nodule like
 48 structures without rhizobia inoculation. In conclusion, *GmNIN1a*, *GmNIN2a* and
 49 *GmNIN2b* play functionally redundant yet complicated roles for soybean nodulation.

50 GmNIN1b, although is expressed at comparable level with other homologs, plays a
51 minor role in root nodule symbiosis. Our work provides insight into the understanding
52 of asymmetrically redundant function of *GmNIN* genes in soybean.

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54 INTRODUCTION

55 The legume-rhizobia symbiosis is the most important symbiotic association in terms
56 of biological nitrogen fixation. In root nodules, endosymbiotic rhizobia use carbon
57 from their legume hosts to fix atmospheric nitrogen into ammonia to provide the host
58 plant with nitrogen nutrition. Understanding the underlying mechanisms of nodulation
59 and nitrogen fixation is very important for improving agricultural production and for
60 insights into natural ecosystems.

61 The legume-rhizobia symbiotic interaction is initiated by mutual recognition of
62 molecular signals. The process is initiated by plant roots secreting flavonoid
63 compounds into the soil, this attracts compatible rhizobia and stimulates them to
64 synthesize and secrete highly specific lipochito-oligosaccharide (LCO) signalling
65 molecules called nodulation factors (NFs) (Liu et al., 2016). Legume plants perceive
66 NF signals via LysM receptor-like kinase expressed in their roots. NF recognition
67 triggers two coordinated plant developmental programs: initiation of the infection
68 process by which the bacteria enter the host cells, and, simultaneously, the elicitation
69 of pericycle and cortical cell division resulting in nodule organ formation (Murray,
70 2011). When the bacteria reach the nodule primordium cells through the infection
71 threads they are released into host-membrane enclosed structures called symbiosomes
72 where they fix nitrogen (Oldroyd et al., 2011).

73 Soybean (*Glycine max*) is one of the most important legume crops in the world,
74 and provides human food, animal feed, nutritional by-products and vegetable oil for
75 human consumption and biofuel production. As a legume, soybean also has the
76 capability to form root nodules through association with *Rhizobium* bacteria. The

soybean genome was sequenced (Schmutz et al., 2010) and transcriptome and proteome analyses after rhizobia or NF inoculation have been performed in a number of studies (Hayashi et al., 2012; Libault et al., 2010; Libault et al., 2009; etc.). Understanding the biological function of the genes involved will help to establish elite cultivars that benefit sustainable farming practices. Forward and reverse genetic methods are available for soybean. However, due to multiple genome duplications that occurred at approximately 59 and 13 million years ago, approximately 75% of soybean genes are present in multiple copies leading to high genetic redundancy (Schmutz et al., 2010). Duplication occurred in segmental regions, accompanied by local genomic as well as sequence divergences. For example, the Nod factor receptor genes *NFR1* and *NFR5* are duplicated in soybean (GmNFR1 α –GmNFR1 β and GmNFR5 α –GmNFR5 β). GmNFR1 α and GmNFR1 β have 92% identity at the nucleotide level, but have different functions (Indrasumunar et al., 2010). On the other hand, GmNFR5 α and GmNFR5 β have 95% nucleotide identity and can functionally complement each other (Indrasumunar et al., 2011). The *Symbiosis Receptor Kinase* (*SymRK*) gene also has two copies in soybean (GmSymRK α and GmSymRK β). RNAi knock down of the *GmSYMRK* genes revealed that *GmSymRK β* is more important for root endosymbiosis than *GmSymRK α* , suggesting it retained its function after duplication (Indrasumunar et al., 2015).

NIN (*Nodule Inception*) is one of the most important genes in nodulation and is the founder gene for the NIN-like protein (NLP) transcription factor family in plants, which contain a conserved RWP-RK DNA-binding domain and a Phox and Bem1 (PB1) dimerization domain (Griesmann et al., 2018; Schauser et al., 1999; van Velzen et al., 2018). *NIN* has been shown to be required for rhizobial infection and nodule organogenesis in several legumes, including *M. truncatula*, *L. japonicus* and *Pisum sativum* (Marsh et al., 2007; Schauser et al., 1999; Borisov et al., 2003). LjNIN can directly bind to promoters of infection-specific genes and activate their expression, such as *NPL* (*Nodulation pectate lyase*), *SCARN* (*SCAR Nodulation*) and *EPR3* (*EPS receptor 3*) (Xie et al., 2012; Qiu et al., 2015; Kawaharada et al., 2017). *NIN*'s targets

106 include genes which function in cortical cell division for root nodule organogenesis in
 107 *L. japonicus* and *M. truncatula*, including two *Nuclear Factor-Y* (*NF-Y*) subunit genes
 108 (*LjNF-YA1* and *LjNF-YB1*), the cytokinin receptor *MtCRE1*, and *LjLBD16a/ASL18*
 109 (Soyano et al., 2013; Vernie et al., 2015; Soyano et al., 2019). In addition, NIN has an
 110 essential role in the autoregulation of nodulation (AON) signaling pathway through its
 111 direct control of *CLE-Root Signall1/2* (*LjCLE-RS1/2*) or *MtCLE13/35* expression
 112 which act to limit nodule numbers (Soyano et al., 2014; Laffont et al., 2020; Luo et al.,
 113 2021). NIN can interact with other NLPs, including NLP1 which has important role in
 114 regulating nitrate inhibition of nodulation (Lin et al., 2018). Moreover, studies from
 115 non-legume nodulating plants, including *CgNIN* from the actinorhizal tree *Casuarina*
 116 *glauca* and *PaNIN* from *Parasponia*, showed that *NIN* is essential gene for
 117 actinorhizal and rhizobial nodulation, respectively (Clavijo et al., 2015; Bu et al.,
 118 2020). Thus, NIN is important for symbiotic nodulation, including rhizobial infection,
 119 nodule organogenesis, and the dynamic regulation of nodule numbers by nitrate
 120 through AON. In soybean, microarray data showed that several *NIN* genes were
 121 induced by rhizobia, but their function in nodulation has not been studied.

122 In this work, we investigated the expression patterns of the *GmNIN* genes, and
 123 used an RNAi knock downs strategy, CRISPR-Cas9 knock out mutants, and
 124 overexpression to fully analyze their function during the nodulation process. Our
 125 results show that *GmNIN1a*, *GmNIN2a*, and *GmNIN2b* function redundantly to
 126 mediate rhizobia infection and nodule initiation, and that *GmNIN1b* plays only a
 127 minor role.

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130 **RESULTS**

131 ***GmNINs* are Induced by Rhizobia and Have Nodulation-Specific Expression** 132 **Patterns**

133 The *G. max* genome encodes four GmNIN proteins belonging to the same clade as
134 LjNIN and MtNIN (Lin et al., 2018) (Supplemental Figure S1). We then analyzed the
135 gene expression patterns of the *GmNINs* by RT-qPCR after rhizobia inoculation. Our
136 results showed that all four *GmNINs* can be induced by rhizobia and the induction
137 levels were highest after 2 weeks post inoculation (Figure 1, A-D), and highly
138 expressed in nodules (Figure 1, E-H). This result was consistent with previous
139 microarray data that these *GmNINs* are highly induced during nodulation process
140 (Libault et al.,2010).

141 The promoter of *NIN* has a CYC motif (for CYCLOPS binding site) which is
142 required for expression of *NIN* in epidermal cells to regulate rhizobial infection, and a
143 remote conserved region with putative cytokinin response elements (CE region)
144 which is required for expression of *NIN* in the pericycle to initiate nodule primordium
145 formation in *L. japonicus* and *M. truncatula* (Singh et al., 2014; Liu et al., 2019).

146 Analysis of all four *GmNIN* promoter regions revealed CYC motifs within 3 kb
 147 upstream of their start codons (Supplemental Figure S2 and Table S1), and two
 148 (*GmNIN1a* and *GmNIN2a*) or three (*GmNIN1b* and *GmNIN2b*) conserved regions far
 149 upstream of their start codon (Supplemental Table S1). The 2nd region was the most
 150 conserved region, which contains several putative cytokinin response elements in the

151 promoter of *MtNIN* (Liu et al., 2019). Even all the 4 *GmNINs* contains the 2nd CE
 152 region, it was conserved in *GmNIN1a*, *NIN2a* and *NIN2b*, but not in *GmNIN1b*
 153 promoter (Supplemental Figure S3). In order to further analyze the expression
 154 patterns of *GmNINs*, we made two promoter-GUS reporter constructs, one with the
 155 promoter of *GmNIN2a* containing its CYC motif (*pNIN2a_{CYC}*) and a second version

Figure 1. *GmNINs* were induced in soybean roots and nodules by rhizobia inoculation and have nodulation-specific expression patterns. A to H, Expression levels of *GmNIN1a* (A, E), *GmNIN1b* (B, F), *GmNIN2a* (C, G) and *GmNIN2b* (D, H) in *G. max* roots (A-D) and nodules (E-H) after rhizobia inoculation. RT-qPCR were used to measure gene expression levels, normalized relative to uninoculated roots (0 d). Values are means $\pm$ SE. Data shown are from one representative experiment of three biological replicates. Asterisks indicate significant differences between treated and control conditions (0 d) (Student's *t*-test, * *P*<0.05, ** *P*<0.01). I to R, The *pGmNIN2a_{CYC}:GUS* (I-K), *pGmNIN2a_{CE-CYC}:GUS* (L-R) were expressed in *G. max* hairy roots, and the images were taken at 3 days (I, L), 5 days (M) or 14 days (J, N) after rhizobia inoculation. (K, O-R) Section of these nodules stained with ruthenium red. Arrows (O) indicate GUS expression in pericycle cells. Scale bars: 1 mm (J, M and N); 100 μm (K, Q and R); 50 μm (I, L, O and P).

156 containing the 2nd CE region and CYC motif (*pNIN2a_{CE-CYC}*) fusion GUS reporter
 157 gene and expressed them in *G. max* by hairy root transformation. After rhizobia
 158 inoculation, weak GUS expression was detected in curled root hairs and nodules of
 159 *pNIN2a_{CYC}:GUS* transgenic roots (Figure 1, I and J). In contrast, in roots transformed
 160 with the *pNIN2a_{CE-CYC}:GUS* construct the GUS expression was much stronger, and

161 staining was seen in root hairs, nodule primordia and nodules (Figure 1, L-N).
 162 Sectioning of the nodules revealed that both *pNIN2a_{CYC}* and *pNIN2a_{CE-CYC}* expressed
 163 in the nodule fixation zone, but the *pNIN2a_{CE-CYC}* expression was stronger than
 164 *pNIN2a_{CYC}* (Figure 1, K and R). However, only *pNIN2a_{CE-CYC}*:GUS transgenic roots
 165 showed GUS activity in divided pericycle and cortex cells (Figure 1, O-Q). We
 166 further deleted (Δ) the D1, D2 or D3 in the 2nd CE region, and found that Δ D1
 167 displayed normal expression pattern, but only half of the transgenic plants can detect
 168 weaker GUS expression (Supplemental Figure S4, A-F and Table S2). However, Δ D2
 169 or Δ D3 barely affected *GmNIN2a* expression pattern and expression level
 170 (Supplemental Figure S4, G-L, M-R and Table S2). This result was consistent with
 171 *MtNIN*, which showed that *pMtNIN_{CE(ΔD1)-CYC}*:GUS cannot rescue *nin-1* nodulation
 172 phenotype (Liu et al., 2019), and suggested that the D1 motif is essential for *GmNINs*
 173 expression.

174 We then analyzed the GmNINs protein sequence, and found that the C-terminal
 175 RWP-RK and PB1 domain are conserved in the GmNINs, but the GmNIN1b
 176 N-terminal was shorter than other GmNINs (Supplemental Figure S5). Then the
 177 subcellular localization of GmNINs were examined using N-terminal GFP fusion
 178 proteins which were expressed in *N. benthamiana* leaves. Our result showed that
 179 these four GmNIN proteins displayed nuclear localization (Supplemental Figure S6).

180

181 **Silencing of *GmNINs* Using RNAi Reduced Nodule Numbers**

182 In order to investigate the role of *GmNINs* in the root nodule symbiosis, we first used
 183 RNA interference (Ri) to reduce their transcript levels and nodule numbers were
 184 analyzed at 21 days post inoculation (dpi) with rhizobia. We found that *GmNIN1a Ri*
 185 plants produced no nodules; *GmNIN2a Ri* and *GmNIN2b Ri* transgenic roots had
 186 significantly reduced nodule numbers, while *GmNIN1b Ri* nodule numbers were less
 187 than the EV control but more than *GmNIN1a Ri*, *GmNIN2a Ri* and *GmNIN2b Ri*
 188 (Figure 2, A-F). The transcript levels of the *GmNINs* were analyzed by RT-qPCR, and

Figure 2. Nodulation phenotypes in *GmNIN* RNAi hairy roots. A to E, Nodule phenotypes of EV control (A), *GmNIN1a* (B), *GmNIN1b* (C), *GmNIN2a* (D) and *GmNIN2b* (E) RNAi transgenic roots 3 weeks after rhizobia inoculation. Upper panels (A-E) show bright-field images and the lower panels (A'-E') are epifluorescence microscopy images showing GFP expression in the same transgenic roots. Scale bars = 1 mm. F, Nodule numbers of the EV and RNAi transgenic lines. The nodule numbers were scored at 3 weeks post inoculation with *B. japonicum* USDA110. Data from one representative experiment of three biological replicates. Error bars indicate SE, dots represent individual nodule numbers. The different letters indicate significant differences (one-way ANOVA-multiple comparisons). G to J, RT-qPCR analyze of the relative expression levels of *GmNINs* (G and H) and symbiotic reporter genes (I and J) in EV and *GmNINs* RNAi transgenic roots at 7 days post inoculation with *B. japonicum* USDA110. Error bars indicate SE. Data shown are from one representative experiment using three biological replicates. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test, comparisons between EV control and experimental groups).

189 the results showed that *GmNIN1a Ri* reduced expression of all four *GmNINs*, while
 190 *GmNIN1b Ri*, *GmNIN2a Ri* and *GmNIN2b Ri* reduced expression of just two of the
 191 four *GmNINs* (Figure 2, G-H). We then analyzed *GmRIC1*, *GmRIC2* and *GmENOD40*
 192 genes expression levels in the *GmNINs Ri* roots. The RT-qPCR results showed that the
 193 expression of these genes was strongly reduced in the *GmNIN Ri* lines (Figure 2, I-J).

194 These results suggest that these four *GmNINs* act redundantly in root nodule
195 formation.

196

197 ***GmNINs* CRISPR-Cas9 Mutants were Deficient in Rhizobia Infection and** 198 **Nodule Organogenesis**

199 To verify the function of *GmNINs* in the root nodule symbiosis process, we used
200 multiplex mutagenesis via pooled CRISPR-Cas9 to generate high order *Gmnin*
201 mutants (Bai et al., 2020). Vectors with small guide RNAs (sgRNAs) targeting the
202 conserved RWP-RK motif or the N-terminus of *GmNIN1a/1b* or *GmNIN2a/2b* were
203 constructed, respectively. These vectors were pooled transformed into soybean
204 cultivar, Huachun 6, using *Agrobacterium tumefaciens*-mediated transformation. In
205 T2 progenies, *Gmnin1b*, *Gmnin1a nin1b* and *Gmnin2a nin2b* double, *Gmnin1a nin2a*
206 *nin2b* triple and *Gmnin1a nin1b nin2a nin2b* quadruple mutants were successfully
207 obtained. These contained insertion or deletions that led to frameshifts which
208 generated premature stop codons (Supplemental Figure S7).

209 The nodulation phenotypes of these mutants were further analyzed by
210 inoculation with *B. japonicum* USDA110 which constitutively expresses the GUS
211 reporter gene. *Gmnin1b* and *Gmnin1a nin1b* produced a similar number of nodules as
212 wild type, *Gmnin2a nin2b* produced less nodule numbers than wild type, and
213 *Gmnin1a nin2a nin2b* and *Gmnin1a nin1b nin2a nin2b* mutants did not develop any
214 nodules at 21 dpi (Figure 3, A and B). RT-qPCR analyze revealed that *RIC1*, *RIC2* and
215 *ENOD40* expression were reduced in these mutants, and the expression levels were
216 much lower in the *Gmnin1a nin2a nin2b* and *Gmnin1a nin1b nin2a nin2b* mutants
217 (Figure 3, C-E).

218 We further analyzed the root hair deformation and rhizobial infection thread
219 formation phenotypes of these *Gmnin* mutants. In wild type and *Gmnin1a nin1b*, the
220 root hairs were deformed and curled (Figure 4, A and B), and similar infection events

Figure 3. Nodulation phenotypes of *Gmnin* CRISPR-Cas9 mutants and gene expression levels in these mutants. A, Nodule phenotypes of *G. max* wild type (Huachun 6) and *Gmnin* CRISPR-Cas9 mutants 3 weeks after inoculation of *B. japonicum* USDA110. Scale bars= 1 mm. B, Nodule numbers of *G. max* wild type and *Gmnin* CRISPR-Cas9 mutants at 3 wpi. Error bars show SE, dots represent individual nodule numbers. Data from one representative experiment of two or three independent experiments. The different letters indicate significant differences (one-way ANOVA-multiple comparisons). C to E, RT-qPCR analyze of the relative expression levels of symbiotic reporter genes in wild type and *Gmnin* mutants. Error bars indicate SE. Data from one representative experiment of three biological replicates. Asterisks indicate significant differences (Student's *t*-test, **P* < 0.05, ***P* < 0.01, comparisons between WT control and experimental groups).

221 including infection foci and infection threads can be produced 7 days after inoculation
 222 (Figure 4, E-H and M). *Gmnin1a nin2a nin2b* and *Gmnin1a nin1b nin2a nin2b*
 223 showed excessive root hair curling in response to rhizobia (Figure 4, C and D), no
 224 infection threads developed, and very rarely a few infection foci formed (Figure 4, K
 225 and L). Surprisingly, *Gmnin2a nin2b* showed hype infection phenotype, produce more
 226 infection foci and infection threads than wild type (Figure 4, I-J and N). Based on

Figure 4. Root hair deformation and infection phenotypes of *Gmnin* mutants. A to D, Root hair deformation phenotypes of wild type (A), *Gmnin1a nin1b* (B), *Gmnin1a nin2a nin2b* (C) and *Gmnin1a nin1b nin2a nin2b* (D). E to L, Wild type (E, F), *Gmnin1a nin1b* (G, H) and *Gmnin2a nin2b* (I, J) plants have normal infection foci (E, G and I) and infection threads (F, H and J), but *Gmnin1a nin2a nin2b* (K) and *Gmnin1a nin1b nin2a nin2b* (L) only occasionally developed a few infection foci after rhizobia inoculation. Arrowheads represent deformed root hairs. Scale bars = 100 μ m. M to N, Infection events in wild type and *Gmnin1a nin1b* (M), *Gmnin2a nin2b* (N) 7 days after inoculation of *B. japonicum* USDA110 carrying a GUS reporter gene. Error bars show SE, dots represent individual numbers. Data from one representative experiment of two independent experiments. Foci: infection foci; IT: infection thread. ns: no significant difference (Student's *t*-test).

these results we concluded that the *Gmnin* triple or quadruple mutants have similar phenotypes as reported for *Ljnin* and *Mtnin* mutants, which showed excessive root hair deformation, developed occasional infection foci, and were unable to form nodules.

Overexpression of *GmNINs* Blocked Nodulation but Produced Spontaneous Nodule Primordium-Like Structures

It has been shown that overexpression of *NIN* can reduce nodule numbers after

Figure 5. *GmNIN* overexpression lines suppress nodulation. A to C, Nodule phenotypes of EV (A), *p35S: GmNIN1a* (B) and *p35S: GmNIN1b* (C) lines at 3 wpi. Scale bars= 1 mm. Upper panels (A-C) represent bright-field images, and the lower panels (A'-C') represent fluorescence images show mCherry expression in the same transgenic roots. D, Nodule numbers of *GmNIN* overexpression transgenic roots at 3 wpi with *B. japonicum*. Error bars show SE, dots represent individual numbers. Data from one representative experiment of three independent experiments. Different letters indicate significant differences (one-way ANOVA-multiple comparisons). E to K, RT-qPCR analyze transcription levels of *GmNIN1a* (E), *RIC1* (F), *RIC2* (G), *ENOD40a* (H), *ENOD40b* (I), *NF-YA1a* (J) and *NF-YA1b* (K) in EV and *GmNIN1a*-OE hairy roots after rhizobia inoculation. Error bars indicate SE. Data from one representative experiment of three biological replicates. Asterisks indicate significant differences (Student's *t*-test, **P* < 0.05, ***P* < 0.01, comparisons between EV control and *GmNIN1a*-OE hairy roots).

235 rhizobia inoculation (Soyano et al., 2014)¹⁶ and that it can result in formation of some

236 root nodule-like structures in the absence of rhizobia inoculation (Soyano et al., 2013;
237 Vernie et al., 2015). We then overexpressed *GmNINs* using 35S promoter driven
238 *GmNIN* cDNA in *G. max* hairy roots. The transgenic roots were inoculated with
239 rhizobia and the nodulation phenotypes were scored at 21 dpi with *B. japonicum*. We
240 found that overexpression of *GmNIN1a*, *GmNIN2a* or *GmNIN2b* dramatically reduced
241 nodule numbers; while with overexpression *GmNIN1b*, the nodule numbers were less
242 than EV control but more than *GmNIN1a* and *GmNIN2a* overexpression lines (Figure
243 5, A-D). The expression of symbiotic reporter genes were examined in the *GmNIN1a*
244 overexpression roots after rhizobia inoculation. The results showed that *GmNIN1a*
245 was highly induced after rhizobia inoculation and in *GmNIN1a* overexpression hairy
246 roots (Figure 5E). Symbiotic reporter genes such as *RIC1*, *RIC2*, *ENOD40* and
247 *NF-YA1* were induced by rhizobia in the EV control and the *GmNIN1a* overexpression
248 transgenic roots (Figure 5, F-K).

249 We next examined whether overexpression of *GmNINs* can induce the formation
250 of nodule primordium-like structures without rhizobia inoculation. The composite
251 plants with transgenic hairy roots were transferred into vermiculite: perlite mixture
252 (1:1) and the phenotype was scored 35 days after transfer. We found that
253 overexpression of *GmNIN1a*, *GmNIN2a* and *GmNIN2b* produced abnormal root
254 growths, including white nodule-like structures, small bumps and short but enlarged
255 lateral roots (Figure 6, A-D, I and Supplemental Figure S8, C-H). Nearly all the
256 *GmNIN1a* overexpression roots, 60% of *GmNIN2a* overexpression roots, and 80% of
257 *GmNIN2b* overexpression roots showed these abnormal architectures, but none of EV
258 and *GmNIN1b* overexpression roots developed this kind of malformed structures, in
259 three independent experiments (Figure 6I and Supplemental Figure S8, A-H).
260 Sectioning of the *GmNINs*-induced abnormal structures revealed that the nodule-like
261 structures and white bumps were formed *via* cortical cell division and were
262 anatomically similar to root nodule primordia (Figure 6, E-F and Supplemental Figure
263 S8, I-J, M-N); the malformed nodule-like structures have central vascular systems
264 similar to lateral roots but also have undergone extensive cortical cell divisions and

Figure 6. *GmNINs* overexpression triggers the formation of spontaneous nodule-like structures. A to D, *p35S: GmNIN1a* expressed in wild type hairy roots and cultured for 5 weeks without rhizobia inoculation, produced nodules (A), small white bumps (B) and malformed nodule-like structures (C and D). Upper panels (A-D) showed bright-field images, and lower panels (A'-D') are epifluorescence microscopy images show mCherry expression in the same transgenic roots. Scale bars = 1 mm. E to H, Transverse sections of spontaneous nodules (E), small white bumps (F) and malformed nodule-like structures (G and H) in *GmNIN1a-OE* hairy roots in the absence of rhizobia inoculation. Scale bars = 100 μ m. I, Nodule numbers in the overexpressed *GmNINs* hairy roots 5 weeks after culture. Dots represent individual numbers. Data from one representative experiment of three independent experiments. J to L, RT-qPCR analyze transcription levels of *GmNIN1a* (J), *ENOD40a* (K) and *ENOD40b* (L) in EV and *p35S:GmNIN1a* hairy roots. *p35S: NIN1a* - represent no spontaneous nodule-like structures hairy roots, and *p35S: NIN1a* + represent have spontaneous nodule-like structures hairy roots. Error bars indicate SE. Data from one representative experiment of three biological replicates. Asterisks indicate significant differences (Student's *t*-test, **P* < 0.05, ***P* < 0.01, comparisons between EV control and *GmNIN1a-OE* hairy roots).

265 show over-proliferation of pericycle and cortical cells (Figure 6, G-H and
266 Supplemental Figure S8, K-L, O-P). RT-qPCR analysis showed that *GmNIN1a* was

267 highly expressed in the transgenic hairy roots, but the early nodulin gene *ENOD40*
 268 was highly expressed in the roots showing abnormal architecture, while was not
 269 changed in normal roots (Figure 6, J-L).

270 LjNIN systemically suppresses nodulation through the AON pathway (Soyano et
 271 al., 2014). We expressed *35S:GmNIN1a* in wild type or *nark* mutant plants, and the
 272 nodulation phenotype was analyzed in transformed or untransformed hairy roots at 21
 273 dpi. The mean number of nodules was significantly suppressed in both
 274 mCherry-positive transgenic and mCherry-negative untransformed roots in wild type
 275 background, but the repressive level was stronger in mCherry-positive transgenic
 276 roots (Figure 7, A-B and E). However, the suppression effect was not present in *nark*
 277 mutant background either in transgenic or non-transgenic hairy roots (Figure 7, C-D
 278 and E). This result indicates that ectopic expression of *GmNIN1a* systemically inhibits
 279 nodulation *via* the AON pathway.

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Figure 7. Systemic inhibition of nodulation by overexpression of *GmNIN1a*. A to D, Nodulation on mCherry-negative untransformed roots and mCherry-positive transformed roots on wild type carrying EV (A) or *p35S-GmNIN1a* (B), and on *nark* carrying EV (C) or *p35S-GmNIN1a* (D) at 3 wpi. Upper panels (A-D) show composite bright-field images. Lower panels (A'-D') show corresponding fluorescent images with mCherry as a transformation marker for transgenic hairy roots. White dash in lower panels represent mCherry-negative roots. Scale bars= 1 mm. E, Nodule numbers on mCherry-positive and -negative roots that were transformed with the EV or *p35S-GmNIN1a* in wild type (WT) or *nark* mutants. Dots represent individual numbers. Asterisks indicate significant differences between mCherry-negative untransformed roots and mCherry-positive transformed roots on wild type carrying EV or *p35S-GmNIN1a* (Student's *t*-test, **P*<0.05, ** *P*<0.01, ns: no significant difference).

282 DISCUSSION

283 Soybean is an ancient polyploid due to genome duplications, resulting in a nearly 75%
 284 of genes being present in multiple copies (Schmutz et al., 2010). This makes it more
 285 difficult to identify recessive inheritance in this duplicated genome. Using a
 286 CRISPR-Cas9 approach, we were able to make higher order mutants, and then
 287 analyzed their biological function. NIN is essential for all processes in root nodule
 288 formation, and *G. max* contains four *NIN* genes due to genome duplications, but
 289 which genes are required for nodulation was not studied. In this work, we carefully
 290 examined *GmNIN* expression patterns and nodulation phenotypes in CRISPR-Cas9
 291 mutants or *GmNIN* overexpression lines. The results suggested that GmNIN1a,
 292 GmNIN2a and GmNIN2b are essential for nodulation in a functionally redundant
 293 manner, but that GmNIN1b likely has only a minor role in root nodule symbiosis.

294 *NIN* is specifically induced and expressed in nodules (Marsh et al., 2007;
 295 Schauser et al., 1999). In *G. max*, all these four *GmNINs* were induced by rhizobia

and were highly expressed in nodules. In all these *GmNIN* promoters, contains the CYC motif, but the most important 2nd CE motif was not conserved in *GmNIN1b* promoter. The CYC motif is required for *NIN*'s expression in epidermal cells where infection threads are initiated. Compared with p*MtNIN_{CYC}*:GUS, which is expressed in epidermal and pericycle cells (Liu et al., 2019), p*GmNIN2aCYC*:GUS was only detected in epidermal cells and nodules. This difference might result from shorter promoter we used, and the GUS expression was weaker than detective. Or this might also suggests that another motif in *NIN* promoter besides the CYC motif may be required for *NIN* expression in pericycle cells. However, p*GmNIN2a_{CE-CYC}*:GUS was found to express in epidermal cells, pericycle cells, divided cortical cells, and in nodules, suggesting that this conserved 2nd CE motif is essential and sufficient for *GmNIN2a* expressed in pericycle cells.

Compared with other species, *G. max* contains four *NIN* genes and all of them were induced and expressed in nodules. Several studies including ours, showed that RNAi knock down *GmNIN1a* showed a nodule-minus phenotype (He et al., 2020 and this study), suggesting that *GmNIN1a* is sufficient for *NIN*'s function in root nodule symbiosis. However, after careful analysis of *GmNIN* expression levels in *GmNIN1a* RNAi plants, we found that not only the expression of *GmNIN1a*, but also the expression of other three *GmNINs*, was reduced, suggesting that our *GmNIN1a* RNAi plants phenocopy a *GmNIN* quadruple mutant. Moreover, knock down other *GmNINs* reduced nodule numbers, and gene expression showed these RNAi lines, also knock down other *GmNINs*. Our RNAi analysis of *GmNINs* suggest that they act in a functionally redundant manner to regulate the root nodule symbiosis. To further validate this hypothesis, we made a series of *GmNINs* mutants using a CRISPR-Cas9 approach. Unexpectedly, we found that *Gmnin1b* and *Gmnin1a nin1b* showed a wild type nodulation phenotype, including root hair deformation, rhizobial infection and nodule numbers. *Gmnin2a nin2b* mutant exhibited an intermediate phenotype with reduced nodule number. *Gmnin1a nin2a nin2b* triple and *Gmnin1a nin1b nin2a nin2b* quadruple mutants showed a typical *Ljnin* and *Mtnin* nodulation phenotype, including

325 excessive root hair deformation, nearly no rhizobial infection events and no nodules.
326 These results suggested that GmNIN1a, GmNIN2a, GmNIN2b are required for
327 nodule organogenesis in a dosage dependent manner, while GmNIN1b is not able to
328 compensate their function in *Gmnin1a nin2a nin2b* triple mutant.

329 Unexpectedly, *Gmnin2a nin2b* mutant showed a hyper infection phenotype that
330 was similar to *Ljdephne* or *Mtdephne-like* mutant (Yono et al., 2014; Liu et al., 2019).
331 Considering the non-infection phenotype in triple and quadruple mutants and normal
332 infection in *Gmnin1a nin1b* double mutant, it is less likely that GmNIN2a and
333 GmNIN2b simply play a negative role in rhizobial infection. One possibility is that
334 the *GmNINs* expressions at cellular level might be differentiated with diversified
335 biological implications. For instance, we speculate that *GmNIN2a* and *GmNIN2b* in
336 nodule cortex cells might non-cell-autonomously modulate *GmNIN1a* expression in
337 epidermal cells. In *Gmnin2a nin2b* mutant, *GmNIN1a* in epidermal cells might be
338 induced and lead to more infection events. So far, we failed to clone *GmNIN1a*
339 promoter for detailed expression analysis, and further studies are required to validate
340 these hypotheses. Taken together, our results show that *GmNINs* play functionally
341 redundant yet complicated roles in the root nodule symbiosis.

342 Due to multiple genome duplications, approximately 75% of soybean genes are
343 duplicated leading to the perspective of high genetic redundancy (Schmutz et al.,
344 2010). In some cases, duplicated genes are asymmetrically redundant, as mutation in
345 one gene may cause a phenotype while in the other gene showed no or weak
346 phenotype (Vaddepalli et al., 2019). Such asymmetric genetic redundancy is usually
347 explained by diverged or unequal gene expression (Vaddepalli et al., 2019). Here we
348 show that *GmNIN1b* plays a minor role in root symbiosis, even though its expression
349 is comparable to the other three *GmNIN* homologs, but the most important 2nd CE
350 region was not conserved in *GmNIN1b*, implying its expression pattern may different
351 with other NINs. Such divergence may also be attributed to variations in protein
352 sequences, GmNIN1b was shorter than other GmNINs, and overexpression of

353 *GmNIN1b* lead to marginal phenotypes. Elucidation of the protein sequences among
354 *GmNIN* homologs may provide further sights into the evolution of NIN genes in
355 legumes.

356 Consistent with *LjNIN*, overexpression of *GmNINs* reduced nodule numbers
357 after rhizobia inoculation, and this suppression nodulation was systemic and
358 dependent on AON pathway (Soyano et al., 2013; Soyano et al., 2014). However,
359 *GmNIN1a* and its putative target genes (e.g. *GmRIC1*, *GmRIC2* and *GmNF-YA1*) were
360 also induced in *GmNIN1a* overexpression plants. This result was different with *LjNIN*
361 ectopic expression which down-regulated *NIN* expression and NIN activity (Soyano
362 et al., 2014). This difference might have resulted from our use of the constitutive 35S
363 promoter to drive *GmNIN1a* expression, rather than the DEX-inducible expression
364 system used for the study of *LjNIN*. Moreover, we found that the overexpression of
365 *GmNIN1a*, *GmNIN2a* or *GmNIN2b*, but not *GmNIN1b*, can form spontaneous
366 malformed root nodule-like structures in the absence of rhizobia inoculation. We
367 noted some cases where the structures that formed appeared to resemble hybrids
368 between lateral roots and nodules, with proliferation of cortical cells around a central
369 vascular bundle. The formation of nodules with central vascular bundles has been
370 reported in several plant and rhizobial nodulation mutants (Imaizumi-Anraku et al.,
371 2000; Guan et al., 2013), and better understanding of this phenomenon might give
372 insights into how the peripheral vasculature particular to nodules forms. All these
373 results support that GmNINs have similar important roles in root nodule symbiosis,
374 and *GmNIN1a*, *GmNIN2a* and *GmNIN2b* function redundantly to regulate
375 nodulation.

376

377 MATERIAL AND METHODS

378 Biological Materials and Growth Conditions

379 *Glycine max* Williams 82 and Huachun 6, rhizobial strain *B. japonicum* USDA110
380 was used in this study. *nark* mutant in Huachun 6 genetic background was previously

generated by CRSIPR-Cas9 (Bai et al., 2020). For hairy root transformation, *Agrobacterium rhizogenes* strain K599 was used. *A. tumefaciens* strain EHA105 was used for expressing in *N. benthamiana*, and *A. tumefaciens* strain GV3101 was used for generating CRISPR-Cas9 stable mutants. Plasmids were transformed into *Escherichia coli* DH10B or DH5a for cloning.

Analysis of Promoter of *GmNINs*

The sequence of *GmNINs*' promoters were extracted from Phytozome 12.1 (<http://phytozome.jgi.doe.gov/pz/portal.html>) and multiple alignment using website (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and the conserved regions and putative CYCLOPS binding site in *GmNINs*' promoters were searched in their promoter regions.

The *GmNIN2a* CE and CYC were amplified from Williams 82 leaf genomic DNA as template, the primer sequences are shown in Table S3. The PCR products and binary vector pK7FWG2-R which was modified by adding GUS gene before GFP, then using ClonExpress® multis One Step Cloning Kit (Vazyme), to generate p*GmNIN2a*_{CE-CYC}:GUS, p*GmNIN2a*_{CYC}:GUS and the ΔD1, D2 or D3 in p*GmNIN2a*_{CE-CYC}:GUS. The constructs were confirmed by DNA sequencing and expressed in *A. rhizogenes* strain K599 for hairy root transformation. The transgenic roots were transferred into a vermiculite:perlite mixture (1:1) and inoculated with *B. japonicum* USDA110 5-7 days after transfer. The roots were harvested for GUS staining at indicated timepoints after inoculation. The stained nodules were sectioned and images were taken by light microscope (Nikon Eclipse Ni).

Transient Expression of GFP-*GmNINs* in *N. benthamiana* Epidermal Cells

The *GmNINs* cDNA were amplified from *G. max* Williams 82 root cDNA using the

primers shown in Table S2. The PCR products were cloned into pDONR207 and then recombined into the vector pK7WGF2 to generate 35S-*GmNINs*, and then recombined this product into pUb-3xflag and p35S:mCherry (in order to replace its pUb promoter). The constructs were confirmed by DNA sequencing and transformed into *A. tumefaciens* strain EHA105, then co-infiltrated into *N. benthamiana* leaves plus p19. The images were taken by confocal microscopy with DAPI staining and protein expression levels were analyzed by western blot 60 hrs after infiltration.

RNAi and Overexpression of *GmNINs*

For knock down *GmNINs* by RNAi strategy, specific fragments of *GmNINs* (about 150-200 bp) were amplified by PCR using *G. max* cDNA as template. The PCR products were cloned into pDONR207 and then recombined into pUb-GWS-GFP. For overexpression *GmNINs*, full-lengths *GmNINs* were amplified by PCR using *G. max* cDNA as template and cloned into pDONR207, then recombined into pK7WGF2. The primers used are showed in Table S3 and all the constructs in pDONR207 were sequenced. These constructs were introduced into *A. rhizogenes* K599 by electroporation and then expressed in *G. max* Williams 82 by hairy root transformation. The transgenic hairy roots were planted into a vermiculite:perlite (1:1) mixture and inoculated by *B. japonicum* USDA110 5-7 days after transfer. *GmNINs* overexpression hairy roots also were planted in a vermiculite:perlite (1:1) mixture without rhizobial inoculation. And the phenotypes were analyzed at the indicated time points.

Generation of *Gmnin* CRISPR-Cas9 Mutants

To generate *Gmnin* mutants by CRISPR-Cas9, two small-guide RNAs targeting different regions of *GmNINs* were designed and cloned into the pGES201 vector. The pooled constructs were introduced into *A. tumefaciens* strain GV3101, which was then

transformed into soybean cultivar Huachun 6 by *Agrobacterium*-mediated transformation as described (Bai et al., 2020). The mutant genotypes were identified using the Hi-TOM platform (Liu et al., 2019).

To observe *Gmnin* CRISPR-Cas9 infection phenotype, the mutants seedling were grown in a vermiculite:perlite mixture (1:1) and inoculated with *B. japonicum* USDA110 contains GUS reporter. The inoculated roots were harvested 7 days or 21 days after inoculation and the roots were stained by GUS (7 dpi), and phenotypes were observed under a light microscope (Nikon Eclipse Ni).

Gene Expression Analysis

The *G. max* roots were harvested and total RNA was extracted using an RNAprep Plant plus Kit (Tiangen), and 1st strand cDNA was synthesized using Transcript One-Step gDNA Removal and cDNA Synthesis super Mix Kit (TransGen Biotech.). RT-qPCR was performed on three biological replicates using an ABI StepOne PCR detection system with SYBR Green (Takara). *GmUbiquitin* (*SUBI-2.2*) expression was used as an internal control.

Statistical Analysis

Statistical significance was analyzed by Student's *t*-test ($*P < 0.05$, $**P < 0.01$) or one way ANOVA (non-parametric or mixed) and error bars indicate SE. Histograms were generated using GraphPad Prism 8.0 software.

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463 CONFLICTS OF INTEREST

464 The authors declare that they have no conflicts of interest.

465

466

467 Figure Legends

468 **Figure 1.** *GmNINs* were induced in soybean roots and nodules by rhizobia inoculation
469 and have nodulation-specific expression patterns. A to H, Expression levels of
470 *GmNIN1a* (A, E), *GmNIN1b* (B, F), *GmNIN2a* (C, G) and *GmNIN2b* (D, H) in *G.*
471 *max* roots (A-D) and nodules (E-H) after rhizobia inoculation. RT-qPCR were used to
472 measure gene expression levels, normalized relative to uninoculated roots (0 d).
473 Values are means $\pm$ SE. Data shown are from one representative experiment of three
474 biological replicates. Asterisks indicate significant differences between treated and
475 control conditions (0 d) (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$). I to R, The
476 *pGmNIN2a_{CYC}:GUS* (I-K), *pGmNIN2a_{CE-CYC}:GUS* (L-R) were expressed in *G. max*
477 hairy roots, and the images were taken at 3 days (I, L), 5 days (M) or 14 days (J, N)
478 after rhizobia inoculation. (K, O-R) Section of these nodules stained with ruthenium
479 red. Arrows (O) indicate GUS expression in pericycle cells. Scale bars: 1 mm (J, M
480 and N); 100 μ m (K, Q and R); 50 μ m (I, L, O and P).

481 **Figure 2.** Nodulation phenotypes in *GmNIN* RNAi hairy roots. A to E, Nodule
482 phenotypes of EV control (A), *GmNIN1a* (B), *GmNIN1b* (C), *GmNIN2a* (D) and
483 *GmNIN2b* (E) RNAi transgenic roots 3 weeks after rhizobia inoculation. Upper panels
484 (A-E) show bright-field images and the lower panels (A'-E') are epifluorescence

485 microscopy images showing GFP expression in the same transgenic roots. Scale bars
486 = 1 mm. F, Nodule numbers of the EV and RNAi transgenic lines. The nodule
487 numbers were scored at 3 weeks post inoculation with *B. japonicum* USDA110. Data
488 from one representative experiment of three biological replicates. Error bars indicate
489 SE, dots represent individual nodule numbers. The different letters indicate significant
490 differences (one-way ANOVA-multiple comparisons). G to J, RT-qPCR analyze of
491 the relative expression levels of *GmNINs* (G and H) and symbiotic reporter genes (I
492 and J) in EV and *GmNINs* RNAi transgenic roots at 7 days post inoculation with *B.*
493 *japonicum* USDA110. Error bars indicate SE. Data shown are from one representative
494 experiment using three biological replicates. Asterisks indicate significant differences
495 (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test, comparisons between EV control and
496 experimental groups).

497 **Figure 3.** Nodulation phenotypes of *Gmnin* CRISPR-Cas9 mutants and gene
498 expression levels in these mutants. A, Nodule phenotypes of *G. max* wild type
499 (Huachun 6) and *Gmnin* CRISPR-Cas9 mutants 3 weeks after inoculation of *B.*
500 *japonicum* USDA110. Sale bars= 1 mm. B, Nodule numbers of *G. max* wild type and
501 *Gmnin* CRISPR-Cas9 mutants at 3 wpi. Error bars show SE, dots represent individual
502 nodule numbers. Data from one representative experiment of two or three
503 independent experiments. The different letters indicate significant differences (one
504 -way ANOVA-multiple comparisons). C to E, RT-qPCR analyze of the relative
505 expression levels of symbiotic reporter genes in wild type and *Gmnin* mutants. Error
506 bars indicate SE. Data from one representative experiment of three biological
507 replicates. Asterisks indicate significant differences (Student's *t*-test, * $P < 0.05$, ** P
508 < 0.01 , comparisons between WT control and experimental groups).

509 **Figure 4.** Root hair deformation and infection phenotypes of *Gmnin* mutants. A to D,
510 Root hair deformation phenotypes of wild type (A), *Gmnin1a nin1b* (B), *Gmnin1a*
511 *nin2a nin2b* (C) and *Gmnin1a nin1b nin2a nin2b* (D). E to L, Wild type (E, F),
512 *Gmnin1a nin1b* (G, H) and *Gmnin2a nin2b* (I, J) plants have normal infection foci (E,

513 G and I) and infection threads (F, H and J), but *Gmnin1a nin2a nin2b* (K) and
 514 *Gmnin1a nin1b nin2a nin2b* (L) only occasionally developed a few infection foci
 515 after rhizobia inoculation. Arrowheads represent deformed root hairs. Scale bars =
 516 100 μ m. M to N, Infection events in wild type and *Gmnin1a nin1b* (M), *Gmnin2a*
 517 *nin2b* (N) 7 days after inoculation of *B. japonicum* USDA110 carrying a GUS
 518 reporter gene. Error bars show SE, dots represent individual numbers. Data from one
 519 representative experiment of two independent experiments. Foci: infection foci; IT:
 520 infection thread. ns: no significant difference (Student's *t*-test).

521 **Figure 5.** *GmNIN* overexpression lines suppress nodulation. A to C, Nodule
 522 phenotypes of EV (A), *p35S: GmNIN1a* (B) and *p35S: GmNIN1b* (C) lines at 3 wpi.
 523 Scale bars= 1 mm. Upper panels (A-C) represent bright-field images, and the lower
 524 panels (A'-C') represent fluorescence images show mCherry expression in the same
 525 transgenic roots. D, Nodule numbers of *GmNIN* overexpression transgenic roots at 3
 526 wpi with *B. japonicum*. Error bars show SE, dots represent individual numbers. Data
 527 from one representative experiment of three independent experiments. Different
 528 letters indicate significant differences (one-way ANOVA-multiple comparisons). E to
 529 K, RT-qPCR analyze transcription levels of *GmNIN1a* (E), *RIC1* (F), *RIC2* (G),
 530 *ENOD40a* (H), *ENOD40b* (I), *NF-YA1a* (J) and *NF-YA1b* (K) in EV and
 531 *GmNIN1a-OE* hairy roots after rhizobia inoculation. Error bars indicate SE. Data
 532 from one representative experiment of three biological replicates. Asterisks indicate
 533 significant differences (Student's *t*-test, **P* < 0.05, ***P* < 0.01, comparisons between
 534 EV control and *GmNIN1a-OE* hairy roots).

535 **Figure 6.** *GmNINs* overexpression triggers the formation of spontaneous nodule-like
 536 structures. A to D, *p35S: GmNIN1a* expressed in wild type hairy roots and cultured
 537 for 5 weeks without rhizobia inoculation, produced nodules (A), small white bumps
 538 (B) and malformed nodule-like structures (C and D). Upper panels (A-D) showed
 539 bright-field images, and lower panels (A'-D') are epifluorescence microscopy images
 540 show mCherry expression in the same transgenic roots. Scale bars = 1 mm. E to H,

541 Transverse sections of spontaneous nodules (E), small white bumps (F) and
 542 malformed nodule-like structures (G and H) in *GmNIN1a-OE* hairy roots in the
 543 absence of rhizobia inoculation. Scale bars = 100 μ m. I, Nodule numbers in the
 544 overexpressed *GmNINs* hairy roots 5 weeks after culture. Dots represent individual
 545 numbers. Data from one representative experiment of three independent experiments.
 546 J to L, RT-qPCR analyze transcription levels of *GmNIN1a* (J), *ENOD40a* (K) and
 547 *ENOD40b* (L) in EV and *p35S:GmNIN1a* hairy roots. *p35S: NIN1a* - represent no
 548 spontaneous nodule-like structures hairy roots, and *p35S: NIN1a* + represent have
 549 spontaneous nodule-like structures hairy roots. Error bars indicate SE. Data from one
 550 representative experiment of three biological replicates. Asterisks indicate significant
 551 differences (Student's *t*-test, **P* < 0.05, ***P* < 0.01, comparisons between EV control
 552 and *GmNIN1a-OE* hairy roots).

553 **Figure 7.** Systemic inhibition of nodulation by overexpression of *GmNIN1a*. A to D,
 554 Nodulation on mCherry-negative untransformed roots and mCherry-positive
 555 transformed roots on wild type carrying EV (A) or *p35S-GmNIN1a* (B), and on *nark*
 556 carrying EV (C) or *p35S-GmNIN1a* (D) at 3 wpi. Upper panels (A-D) show
 557 composite bright-field images. Lower panels (A'-D') show corresponding fluorescent
 558 images with mCherry as a transformation marker for transgenic hairy roots. White
 559 dash in lower panels represent mCherry-negative roots. Scale bars= 1 mm. E, Nodule
 560 numbers on mCherry-positive and -negative roots that were transformed with the EV
 561 or *p35S-GmNIN1a* in wild type (WT) or *nark* mutants. Dots represent individual
 562 numbers. Asterisks indicate significant differences between mCherry-negative
 563 untransformed roots and mCherry-positive transformed roots on wild type carrying
 564 EV or *p35S-GmNIN1a* (Student's *t*-test, **P*<0.05, ** *P*<0.01, ns: no significant
 565 difference).

566

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