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1 **A newly-evolved chimeric lysin motif receptor-like kinase in *Medicago truncatula* spp.**
2 ***tricycla* R108 extends its Rhizobia symbiotic partnership**

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Summary

- Rhizobial lipochitooligosaccharidic Nod factors (NFs), specified by *nod* genes, are the primary determinants of host specificity in the legume-Rhizobia symbiosis.
- We examined the nodulation ability of *Medicago truncatula* cv Jemalong A17 and *M. truncatula* ssp. *tricycla* R108 with the *Sinorhizobium meliloti nodF/nodL* mutant, which produces modified NFs. We then applied genetic and functional approaches to study the genetic basis and mechanism of nodulation of R108 by this mutant.
- We show that the *nodF/nodL* mutant can nodulate R108 but not A17. Using genomics and reverse genetics, we identified a newly-evolved, chimeric LysM receptor-like kinase gene in R108, *LYK2bis*, which is responsible for the phenotype and can allow A17 to gain nodulation with the *nodF/nodL* mutant. We found that LYK2bis is involved in nodulation by mutants producing non-*O*-acetylated NFs and interacts with the key receptor protein NFP. Many, but not all natural *S. meliloti* and *S. medicae* strains tested require *LYK2bis* for efficient nodulation of R108.
- Our findings reveal that a newly-evolved gene in R108, *LYK2bis*, extends nodulation specificity to mutants producing non-*O*-acetylated NFs and is important for nodulation by many natural *Sinorhizobia*. Evolution of this gene may present an adaptive advantage to allow nodulation by a greater variety of strains.

Key words: *Medicago truncatula*, legume-Rhizobia symbiosis, LysM receptor-like kinase, Nod factor perception, lipochitooligosaccharide signalling, nodulation specificity

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42 **Introduction**

43 The importance of legumes in agriculture and the environment is largely due to their ability to
44 form a N_2-fixing symbiosis with Rhizobia bacteria, allowing them to grow independently of an
45 additional nitrogen source (Graham & Vance, 2003). This ability has stimulated research on the
46 evolution of such a successful mutualism, and the mechanisms that allow it to develop (Oldroyd
47 *et al.*, 2011; Griesmann *et al.*, 2018). Legumes represent a huge family and show considerable
48 variation in their specificity for their bacterial symbiont (Andrews & Andrews, 2017). Indeed,
49 Rhizobia are polyphyletic, defined primarily by their ability to induce root nodules on their legume
50 host, in which they differentiate into N_2-fixing forms. Different genera/species/strains may show
51 either a narrow or promiscuous host range (Masson-Boivin *et al.*, 2009). Similarly, some legumes
52 are highly promiscuous whereas others show exquisite fidelity to a narrow range of rhizobia
53 (Andrews & Andrews, 2017; Chen *et al.*, 2021).

54 Coevolution has ensured that Legume-Rhizobia couples are highly adapted to each other and has
55 led to improvement of plant and bacterial fitness (Younginger & Friesen, 2019). However, as
56 Rhizobia are horizontally transmitted symbionts, host-partner coevolution would not be possible
57 if there were not signalling mechanisms to select the evolved partner from the external microbial
58 milieu. Thus, partner choice signalling is a crucial step in the evolution of a successful symbiosis
59 (Younginger & Friesen, 2019). Nod factors (NFs) are key rhizobial signals for initiating and
60 maintaining the interaction between most well-studied legume-Rhizobia couples (Krönauer &
61 Radutoiu, 2021). NFs are lipochitooligosaccharides (LCOs) with a basic structure of 3-5 *N*-acetyl
62 glucosamine residues, *N*-acylated on the terminal non-reducing sugar. Various chemical
63 substitutions on the basic structure lead to the primary mechanism of partner specificity (D'Haeze
64 & Holsters, 2002; Oldroyd *et al.*, 2011; Krönauer & Radutoiu, 2021). Although other signals may
65 be important for some legume-Rhizobia interactions, such as cell-surface polysaccharides or
66 protein effectors (Wang *et al.*, 2018; Chen *et al.*, 2021), the genomic linkage of the NF (*nod*) genes
67 to nitrogen fixation (*nif*) genes (generally on a symbiotic megaplasmid or a genomic island),
68 provides a means by which NF signalling can be used by the legume host to select its co-evolved
69 and beneficial nitrogen-fixing partner (Younginger & Friesen, 2019).

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70 On the plant side, lysin motif receptor like kinase (LysM-RLK) genes have evolved as key
71 elements of NF perception and signalling. They encode plasma-membrane located proteins with
72 three lysin motifs in the external region connected through a single transmembrane spanning
73 domain to an internal kinase-like domain. These genes are involved in defence and mycorrhiza
74 signalling in many plants, but the family is particularly expanded in legumes, where two genes
75 show neofunctionalization to play essential roles in NF perception and nodulation (Buendia *et al.*,
76 2018; Chiu & Paszkowski, 2020). One of these genes encodes a receptor with an inactive kinase
77 domain and is known as *NFP/NFR5* (according to the species), whereas the other gene encodes a
78 receptor with an active kinase domain and is known as *LYK3/NFR1* (Krönauer & Radutoiu, 2021).
79 This latter gene evolved by tandem duplication, prior to the speciation of the legume family and it
80 has been suggested that its neofunctionalization was fundamental to the evolutionary gain of a
81 legume-specific NF receptor (De Mita *et al.*, 2014). Further, more recent duplications of this and
82 other genes in the cluster have led to a species-specific source of variation in NF signalling (De
83 Mita *et al.*, 2014; Sulima *et al.*, 2017).

84 *Medicago truncatula* Gaertn. (closely related to alfalfa and pea), together with *Lotus japonicus,* is
85 a widely used model for studying the molecular mechanisms leading to the Rhizobial symbiosis
86 and also, more generally, for traits important for the successful use of legumes in agriculture
87 (Garmier *et al.*, 2017). Initially most work used the genotype *M. truncatula* ssp. *truncatula* cv
88 Jemalong line A17 (referred to here as A17), but more recently, genomic resources have been
89 developed for many other genotypes (see the Hapmap project – https://medicagohapmap2.org/) in
90 order to exploit the considerable genetic variation of this species for these studies (Garmier *et al.*,
91 2017). Of note, *M. truncatula* ssp. *tricycla* line R108-1 (referred to as R108) has achieved
92 considerable success due to the development of a *Tnt1*-insertion population resource and its
93 superior genetic transformation properties (Garmier *et al.*, 2017; Kaur *et al.*, 2021). Studies on
94 A17 have shown that it has a narrow partner specificity, being nodulated primarily by the
95 *Sinorhizobium (*also known as *Ensifer)* species *S. meliloti* and *S. medicae* (Andrews & Andrews,
96 2017; Kazmierczak *et al.*, 2017). Analysis of the widely-used *S. meliloti* strain 2011 has shown
97 that it produces NFs that are 6-*O*-sulphated on the reducing sugar and contain predominantly a
98 C16:2 acyl chain on the terminal non-reducing sugar, which may also be 6-*O*-acetylated. The
99 presence of the sulphate (specified by the *nodH* gene) is essential for nodulation (Roche *et al.*,
100 1991) and the modifications on the terminal non-reducing sugar are also important. For example,

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101 a double *nodF/nodL* mutant which couples mutations in *nodF* (leading to *N*-acylation mainly with
102 a C18:1 fatty acid) and *nodL* (leading to lack of 6-*O*-acetylation), is almost completely Nod$^-$,
103 whereas a *nodF* or a *nodL* mutant induce similar or a reduced number of nodules (Ardourel *et al.*,
104 1994; Smit *et al.*, 2007). On the plant side, *NFP* is required for all NF responses and its role can
105 be partially substituted by *NFP* genes from other species (Bensmihen *et al.*, 2011; Girardin *et al.*,
106 2019). Studies on a weak *lyk3* mutant of A17 (Smit *et al.*, 2007) and recent structural studies
107 (Bozsoki *et al.*, 2020) suggest that *LYK3* is involved in the perception of specific NF features.

108 Comparative studies between A17 and R108 have shown that they are highly divergent members
109 of the *M. truncatula* species (Zhou *et al.*, 2017), show differences in their symbiotic effectiveness
110 with different Rhizobial strains (Kazmierczak *et al.*, 2017) and exhibit differences in partner choice
111 when confronted with a mixed *Sinorhizobium* (*Ensifer*) inoculum (Burghardt *et al.*, 2018). In this
112 article, we report that R108 has an extended NF-related strain specificity compared to A17 and
113 have identified a newly evolved *LysM-RLK* gene, unique to R108, which is required for this
114 phenotype.

115 **Materials and methods**

116 *Seed germination and growth conditions*

117 *Medicago* seeds were scarified in 95% sulphuric acid for 5 min, washed with distilled water two
118 times and surface-sterilized in bleach (3.2% chlorine) for 3 min, then washed three times. After
119 that, the seeds were soaked in water for 1 h and placed on 1% agar, supplemented with 1 μg ml^{-1}
120 GA3. The plates were kept upside-down at 4ºC for 5 d and then put at 16ºC overnight for
121 germination. Seedlings were transferred to pots and grown in a growth chamber at 22ºC with a 16
122 h photoperiod.

123 *cDNA cloning*

124 R108 roots were collected and immediately frozen in liquid N_2. Total RNA was extracted using a
125 Nucleospin plant RNA extraction kit (Marcherey-Nagel GmbH & Co. KG, Germany). cDNA was
126 synthesized using SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific, USA).

127 *LYK2bis* coding sequence (CDS) was amplified with Phusion High Fidelity DNA Polymerase
128 (F530S, Thermo Fisher Scientific, USA) using R108 root cDNA as template and the following

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129 primers (5'-ATGAAACTAAAAAATGGCTTAC-3' and 5'-
130 TCTAGTTGACAACAAATTTATG-3'). The PCR product was cloned into pJet1.2/blunt using
131 CloneJET PCR Cloning Kit (K1231, Thermo Fisher Scientific, USA). The correct clone was
132 selected by sequencing, then used for further cloning steps.

133 *Constructs for* in planta *protein expression*

134 For *M. truncatula* root transformation, full length CDS of *LYK2bis* and of *LYK3* from A17 (LYK3-
135 A17) were fused at the C-terminus with 3xFLAG under the control of the Ubiquitin promoter from
136 *L. japonicus* (ProLjUbi) by Golden gate cloning, using a vector based on pCAMBIA2200
137 expressing DsRED (Fliegmann *et al.*, 2016). For *Nicotiana benthamiana* leaf agro-infiltration, full
138 length CDS of LYK2bis and NFP were fused at the C-terminus with mCherry and GFP tag
139 proteins, respectively, under the control of ProLjUbi. The vector pCambia2200ΔDsRED was used
140 (Fliegmann *et al.*, 2016).

141 *Mutants and nodulation tests*

142 *Tnt1* insertional mutants, *lyk2-1R* (NF13076), *lyk2bis-1R* (NF15454) and *lyk3-1R* (NF2752) of
143 R108, obtained from the Noble Research Institute (USA), were used for nodulation tests. R108
144 and A17 were used as wild-type (WT) controls. Note that "*R*" designates a R108 mutant.

145 Germinated seedlings were transferred into pots (8x8x8 cm^3 in size), filled with sterilized
146 attapulgite clay granules (Oil Dri, UK). To each pot, 80 ml of Fahraeus medium supplemented
147 with 0.2 mM NH_4NO_3, was added. After 3 d, plants were inoculated with approximately 10,000
148 bacteria/plant of *S. meliloti* 2011 WT or mutant strains (Table **S1**). The number of nodules per
149 plant was counted at 21 dpi. When appropriate, bacterial infection was determined by LacZ
150 straining with X-gal. All results were confirmed in separate biological experiments.

151 For the nodulation test with natural strains, germinated seedlings were transferred into test tubes
152 with 20 ml agar slants of Fahraeus medium with 13 g/l of agar Kalys HP 696 (Kalys SA, Bermin,
153 France), supplemented with 0.2 mM NH_4NO_3. After 5 d, plants were inoculated with
154 approximately 10,000 bacteria/plant of different rhizobial strain. The lower part of the tubes was
155 covered by brown paper to avoid excessive light access. The number of nodules per plant was
156 analysed at 28 dpi. Results presented are from two separate biological replicates.

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157 *Acetylene reduction assays*

158 Assays were performed on inoculated plants at 28 dpi as described by Hardy *et al.*, (1968). Briefly,
159 1 ml of acetylene was injected into a test tube containing one single inoculated plant and closed
160 with a septum. Tubes were incubated in a growth chamber for two hours. Then, 400 µl of gas
161 samples were analysed on an Agilent 7020 gas chromatograph, equipped with a flame ionization
162 detector. Activity was normalised with the number of nodules per plant.

163 *Complementation and gain-of-function assays*

164 Using *Agrobacterium rhizogenes*-mediated transformation (Boisson-Dernier *et al.*, 2001),
165 seedlings of *lyk2bis-1R* and A17 were transformed using strains containing either empty vector
166 (EV) or ProLjUb:LYK2bis-3xFLAG or ProLjUb:LYK3-A17-3xFLAG constructs, and
167 transformants were selected on medium containing 25 µg ml^{-1} kanamycin, and after two-weeks
168 growth, by expression of the DsRed marker. R108 seedlings transformed with EV were used as
169 control. Nodulation was analysed in pots as above after 4 wk. The number of nodulated plants and
170 the number of nodules/nodulated plant were analysed.

171 *Agro-infiltration and Fluorescence Lifetime Imaging on Nicotiana benthamiana leaves*

172 *Agrobacterium tumefaciens LBA4404* strains containing ProLjUb:LYK2bis-mCherry or NFP-GFP
173 fusion constructs were used to agro-infiltrate the three to four oldest leaves of each *N.*
174 *benthamiana* plant in which the OD_{600} of LYK2bis-mCherry was five times higher than that of
175 NFP-GFP in order to have similar expression levels.

176 Two days post infiltration, the protein expression in leaves was assessed by confocal microscopy.
177 Forster resonance energy transfer (FRET) between the fluorophores was analysed by Fluorescence
178 Lifetime Imaging Measurements (FLIM) on a Leica TCS SP8 SMD which consists of an inverted
179 LEICA DMi8 microscope equipped with a TCSPC system from PicoQuant. The excitation of the
180 FITC donor at 470 nm was carried out by a picosecond pulsed diode laser at a repetition rate of 40
181 MHz, through an oil immersion objective (63×, N.A. 1.4). The emitted light was detected by a
182 Leica HyD detector in the 500-550 nm emission range. Images were acquired with acquisition
183 photons of up to 1500 per pixel.

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184 From the fluorescence lifetime images, the decay curves were calculated per pixel and fitted (by
185 Poissonian maximum likelihood estimation) with either a mono- or double-exponential decay
186 model using the SymphoTime 64 software (PicoQuant, Germany). The mono-exponential model
187 function was applied for donor samples with only GFP present. The double-exponential model
188 function was used for samples containing GFP and mCherry. Experiments were repeated at least
189 three times. The efficiency of energy transfer (*E*) based on the fluorescence lifetime (τ) was
190 calculated as $E = 1 - (\tau_{D+A}/\tau_{D-A})$, where τ_{D+A} is donor fluorescence lifetime in the presence of
191 acceptor while τ_{D-A} is the donor fluorescence lifetime in the absence of acceptor.

192 *Kinase assays*

193 Glutathione-S-transferase (GST) tagged proteins of the predicted intracellular region of LYK2bis
194 (termed the KD) were expressed in *E. coli* DH5α and the proteins purified using glutathione resin
195 (GE Healthcare, USA) as described (Fliegmann *et al.*, 2016). LYK2bis-KD was released from the
196 resin using PreScission Protease (GE27-0843-01, Sigma Aldrich, Germany). LYK2bis-KD was
197 incubated with kinase buffer containing ^{32}P-ATP alone or with purified GST/NFP-KD,
198 GST/LYR2-KD, GST/LYR3-KD, GST/LYR4-KD, GST/LYK3-deadKD (G334E mutation),
199 Myelin Basic Protein (MyBP) or GST at 25ºC for 1 h and the proteins analysed by SDS-PAGE,
200 followed by Coomassie staining and Phosphor Imaging.

201 *Mycorrhization tests*

202 Mycorrhization tests were performed using the gridline intersect method as described in Gibelin-
203 Viala *et al.* (2019). Briefly, *lyk2bis-1R* and R108 seedlings were inoculated with 200 spores per
204 plant of *Rhizophagus irregularis* DAOM197198 (Agronutrition, Toulouse, France) and
205 colonisation was assessed at 3- and 5-wk post inoculation (wpi) using 15 plants/genotype/time-
206 point.

207 *Bioinformatic analysis*

208 The 1st exon encoding the whole LysM domain of *LYK2* (640 bases), *LYK3* (637 bases) and
209 *LYK2bis* (640 bases) from R108 were used with blastn to screen all *M. truncatula* genomes
210 available in the Medicago BLAST Service of the Hapmap2 database
211 (https://medicagohapmap2.org/). The first hit for each query was extracted. The percentages of

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212 identity (%ID) between hits found with the *LYK3-R108* probe and the *LYK2bis* sequence were
213 calculated by EMBOSS Needle.

214 **Results**

215 *R108 shows an extended nodulation specificity with a S. meliloti nodF/nodL mutant strain*

216 To determine the nodulation specificity of the *M. truncatula* R108 genotype, seedlings of R108
217 and A17 were inoculated with either *S. meliloti* 2011 WT or its *nodF/nodL* mutant. The number
218 of nodules was counted at 21 dpi. No nodules were found on A17 plants inoculated with
219 *nodF/nodL,* which is consistent with the results on *M. truncatula* cv. Jemalong obtained previously
220 (Ardourel *et al*., 1994) (Fig. **1**). However, R108 showed a similar nodulation capacity with both
221 WT and *nodF/nodL* strains (Fig. **1**). Most (> 95%) of the R108 nodules with both strains were pink
222 (suggesting expression of leghaemoglobin) and LacZ staining showed that they were well-infected
223 with bacteria. Also, acetylene reduction assays suggest that the nodules from the two strains exhibit
224 a similar ability to fix N_2 (Fig. **S1**). These results suggest that R108 has an extended nodulation
225 specificity, compared to A17, which includes the *nodF/nodL* mutant.

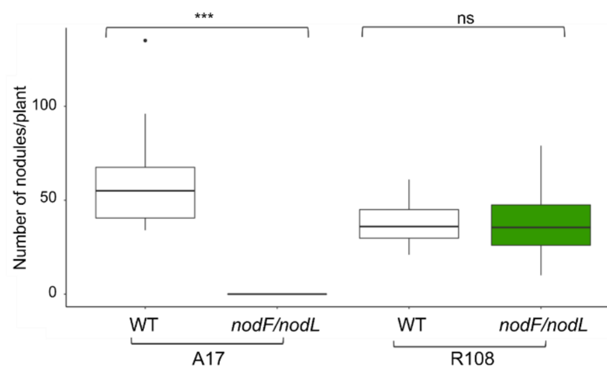

Fig. 1. *Medicago truncatula* R108 is able to nodulate with the *Sinorhizobium meliloti nodF/nodL* strain. The number of nodules in 20 plants/genotype/inoculation was analysed at 21dpi. Statistical analyses were performed using Student's *t*-test (ns, not significant; ***, $P < 0.001$). White shading is for plants inoculated with the WT strain; green shading is for plants inoculated with the *nodF/nodL* strain.

226 *A newly-evolved gene in R108, LYK2bis, is required for nodulation with the nodF/nodL strain*

227 The perception of NF and nodulation in *M. truncatula* have been shown to require two LysM-
228 RLKs, which are NFP and LYK3 in A17 (Arrighi *et al.*, 2006; Smit *et al.*, 2007). In the R108
229 genotype, NFP is reported to play essential roles for these processes (Feng *et al.*, 2019), therefore,

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230 to understand the extension of nodulation in this ecotype, we focused on the *LYK* gene cluster,
231 close to *LYK3*.

232 Using the genome sequence of R108 v1.0 (Kaur *et al.*, 2021), we located the *LYK* gene cluster on
233 chromosome 5, using the sequences of *LYK2* (MtrunA17Chr5g0439701) and *LYK3*
234 (MtrunA17Chr5g0439631) from A17. Between *LYK2* and *LYK3*, an additional *LysM-RLK* gene
235 was found, which is not present in A17, and is designated as *LYK2bis* (Fig. **2a**). Notably, the
236 exon/intron organization is conserved among these genes with 12 exons in each (Fig. **2a**). The
237 predicted amino acid sequence alignment between LYK2, LYK2bis and LYK3 proteins of A17
238 and R108 indicates that LYK2bis is a chimera with a similar extracellular domain to LYK2 up to
239 the middle of LysM3 and then shares a highly conserved sequence with LYK3 (Fig. **2b**, Fig. **S2**
240 and Table **S2**). In order to examine the presence of *LYK2bis* in *M. truncatula* ecotypes, the
241 nucleotide sequence of the first exon of *LYK2*, *LYK3* and *LYK2bis* (containing the whole LysM
242 region) were used to screen the 23 other *M. truncatula* genomes available for BLAST screening
243 in the Hapmap project (https://medicagohapmap2.org/). Separate sequences corresponding to
244 *LYK2* and *LYK3* were identified in all cases (Table **S3**). However, in genotype HM026, the
245 available *LYK3* sequence seems to be not complete. The *LYK2bis* probe identified primarily the
246 *LYK2* gene in all the genomes, with a lesser homology of this exon to the *LYK3* sequence. We did
247 not find any evidence for an additional *LYK2* or *LYK3*-like gene in these genomes. These results
248 suggest that *LYK2bis* is unique to R108 in these *M. truncatula* Hapmap genomes.

249 To determine the roles of these genes in nodulation, *Tnt1* insertional mutants of *LYK2*, *LYK2bis*
250 and *LYK3* in the R108 background were identified from the Noble Foundation resources (Fig. **2b**).
251 The mutants were inoculated with either *S. meliloti* WT or *nodF/nodL* strains and phenotyped at
252 21 dpi. All tested lines formed a similar number of nodules with the WT strain (Fig. **2c**) indicating
253 that, unlike the crucial role of *LYK3* in A17, none of those genes plays an essential role in
254 nodulation of R108 with the *S. meliloti* WT strain. Similarly, there was no significant difference
255 in the number of nodules formed in *lyk2-1R* and *lyk3-1R* mutants with the *nodF/nodL* strain,
256 compared to R108. In contrast, no nodules formed on *lyk2bis-1R* plants inoculated with *nodF/nodL*
257 suggesting that the *LYK2bis* gene is responsible for nodulation of R108 with the *nodF/nodL* mutant
258 strain.

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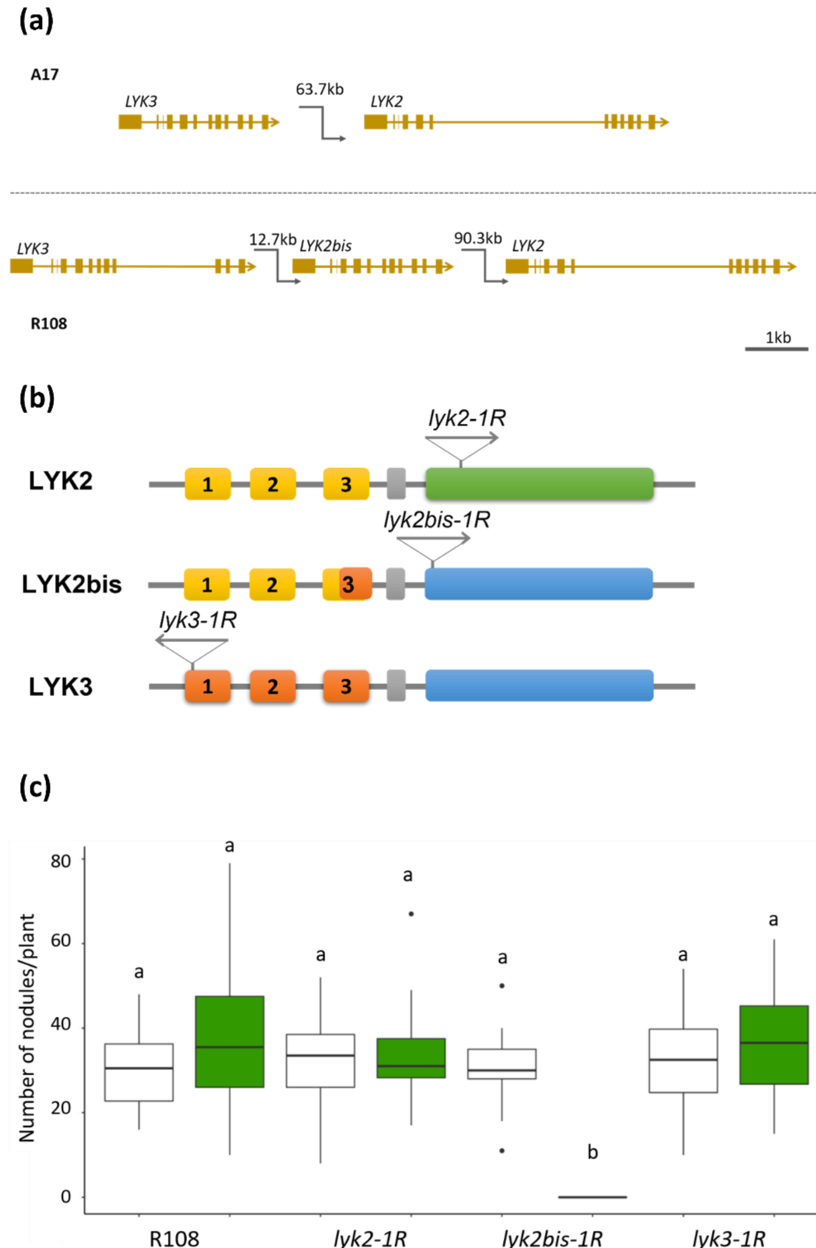

Fig. 2. *LYK2bis* is a newly-evolved gene in *Medicago truncatula* R108 and is responsible for nodulation with the *Sinorhizobium meliloti nodF/nodL* strain. (a) Schematic representation of the *LYK3-LYK2* region in *M. truncatula* A17 and R108. Coding sequences are shown in filled blocks; Non-coding sequences are shown in lines. kb = kilobases. (b) Schematic representation of LYK2, LYK2bis and LYK3 proteins of R108, showing the structure of the proteins and the *Tnt1* insertional sites in *lyk2-1R*, *lyk2bis-1R* and *lyk3-1R mutants*. The proteins are predicted to have three extracellular LysM domains (yellow/orange), a transmembrane domain (grey) and an intracellular kinase domain (blue/green). (c) *LYK2bis* is responsible for nodulation with the *nodF/nodL* mutant in R108. *lyk2-1R, lyk2bis-1R* and *lyk3-1R* mutants were phenotyped for nodulation with either *S. meliloti* WT or the *nodF/nodL* mutant strain. 20 plants/genotype/inoculation were analysed at 21dpi. Statistical analyses were performed using One-way ANOVA ($P < 0.05$). Lowercase letters indicate significant difference. White shading is for plants inoculated with WT; Green shading is for plants inoculated with its *nodF/nodL* mutant.

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259 To validate the involvement of *LYK2bis* in nodulation with the *nodF/nodL* strain, we performed a
260 complementation experiment of the *lyk2bis-1R* mutant. Using *A. rhizogenes*-mediated
261 transformation, constructs of *LYK2bis*, *LYK3-A17* and empty vector (EV) were expressed in the
262 *lyk2bis-1R* mutant. As shown in Fig. **3**, while neither *LYK3* nor EV transformed plants formed
263 nodules with the *nodF/nodL* strain, nodulation was restored in most plants transformed with
264 *LYK2bis*. This result confirms the essential role of *LYK2bis* in nodulation of R108 with the
265 *nodF/nodL* mutant.

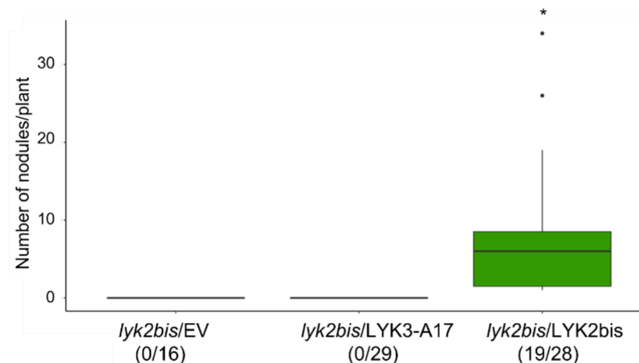

Fig. 3. Complementation analysis confirms the role of *LYK2bis* in nodulation of *Medicago truncatula* R108 with the *Sinorhizobium meliloti nodF/nodL* strain. *lyk2bis-1R* roots were transformed with constructs of empty vector (EV), LYK3-A17 and LYK2bis using *A. rhizogenes*. Transformed plants were analyzed at 4 wpi. Statistical analyses were performed using Student's *t*-test (*, $P < 0.05$). Numbers below indicate number of nodulated plants/total transformed plants.

266 *LYK2bis allows A17 to gain the ability to nodulate with the nodF/nodL strain*

267 As A17 is not able to nodulate with the *nodF/nodL* strain and does not contain a *LYK2bis* gene in
268 the genome, we performed a gain-of-function assay in A17 to determine whether introduction of
269 *LYK2bis* is sufficient to gain nodulation with the *nodF/nodL* strain in this genotype. The
270 experiment was done using a similar approach to the *lyk2bis-1R* complementation. Most of the
271 A17 plants (29/36) transformed with the *LYK2bis* construct nodulated with *nodF/nodL* while none
272 of the ones transformed with *LYK3-A17* and EV formed nodules (Fig. **4**). In a similar experiment
273 but using the A17 *lyk3-1* mutant, 11/18 transformed plants formed nodules with the *nodF/nodL*
274 mutant (data not shown). This evidence reveals that *LYK2bis* is central for interacting and
275 nodulating with the *nodF/nodL* strain and can transfer this ability to another genotype,
276 independently of *LYK3*.

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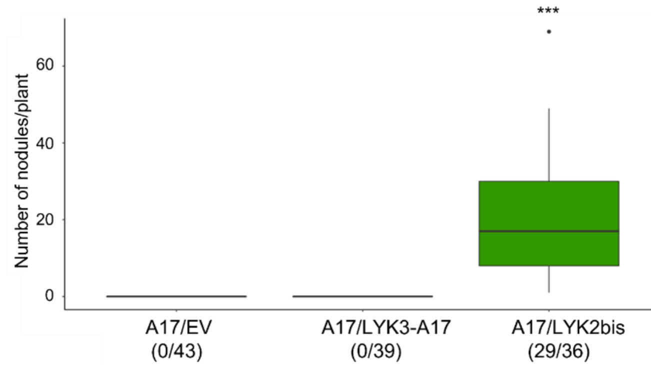

Fig. 4. *LYK2bis* allows *Medicago truncatula* A17 to gain the ability to nodulate with the *Sinorhizobium meliloti nodF/nodL* strain. A17 roots were transformed with constructs of EV, LYK3-A17 and LYK2bis using *A. rhizogenes*. Transformed plants were analysed at 4 wpi. Statistical analyses were performed using Student's *t*-test (***, *P*-value < 0.001). Numbers below indicate number of nodulated plants/total transformed plants.

277 *LYK2bis is involved in the perception of specific NF decorations leading to successful nodulation*
278 *in R108*

279 *S. meliloti nodF/nodL* produces NFs that differ from those of the WT strain in lacking the *O*-
280 acetylation and containing C18:1 fatty acid chains rather than C16 on the terminal non-reducing
281 sugar (Ardourel *et al.*, 1994). The *nodL* mutant produces non-*O*-acetylated NFs (Ardourel *et al.*,
282 1995), whereas mutants in *nodF* and the neighbouring *nodE* lead to NFs with the C18:1 acyl chains
283 (Ardourel *et al.*, 1994). We found that both these strains could form nodules on R108 as expected
284 (Fig. **5**). However, while a similar number of nodules formed on *lyk2bis-1R* and R108 with the
285 *nodFE* strain, no nodules were found on *lyk2bis-1R* inoculated with the *nodL* mutant (Fig. **5**).

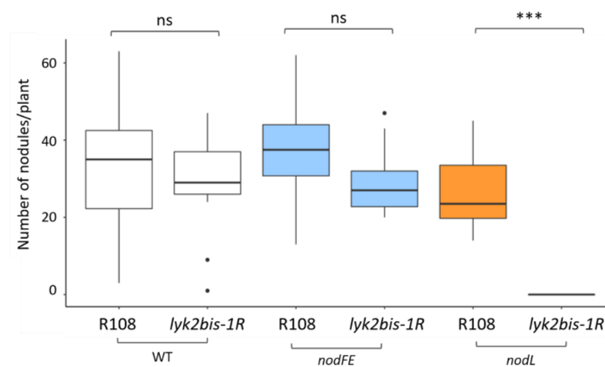

Fig. 5. Nodulation specificity of the *Medicago truncatula lyk2bis-1R* mutant with different *Sinorhizobium meliloti* mutant strains. The *lyk2bis-1R* mutant was tested for nodulation with *nodF/nodE* and *nodL* mutant strains. The number of nodules were counted at 21 dpi. Statistical analyses were performed using Student's *t*-test (ns, not significant; ***, *P* < 0.001). White shading is for plants inoculated with WT; Light blue shading is for plants inoculated with *nodF/nodE;* Orange shading is for plants inoculated with *nodL*.

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286 Our results suggest that while other protein(s) can recognize rhizobia producing C18:1 NFs in
287 R108, LYK2bis is particularly involved in the perception of rhizobia producing non-*O*-acetylated
288 NFs.

289 *LYK2bis allows R108 to successfully nodulate with many natural rhizobial strains*

290 To examine the ecological role of the newly-evolved gene *LYK2bis*, we performed nodulation tests
291 on R108 and the *lyk2bis-1R* mutant with twenty-two natural *Sinorhizobium* strains (Table **S4** and
292 Fig. **6**). The strains were selected as being representative of different multi-locus sequence types,
293 or isolated from different *Medicago* species (Sugawara *et al.*, 2013), including ones trapped from
294 a soil in the South of France (Bailly *et al.*, 2006). They also include strains from different
295 geographical origins, particularly around the Mediterranean basin and including the Middle East.
296 A preliminary experiment in tubes using a small number of plants showed that 9/14 of the *S.*
297 *meliloti* strains and 5/8 of the *S. medicae* strains produced less nodules on the *lyk2bis* mutant than
298 on R108 ($P < 0.05$). No correlation was seen between geographical origin and dependence on
299 *LYK2bis*. To confirm the results, two replicate experiments were set up using a subset of 8 of these
300 strains. All of the strains showed good nodulation of R108 but with some variability (from 8 to 20
301 nodules per plant in the tube system). Four of the strains however could barely nodulate the *lyk2bis*
302 mutant (less than 1 nodule per plant) whereas 3 strains, like Sm 2011, showed no significant
303 difference between the *lyk2bis* mutant and R108, in terms of nodule number. It is notable that
304 strains of *S. meliloti* and *S. medicae* were represented in both the *LYK2bis*-dependent and -
305 independent classes. These results show that *LYK2bis* is very important for nodulation by many
306 but not all *S. meliloti* and *S. medicae* strains tested.

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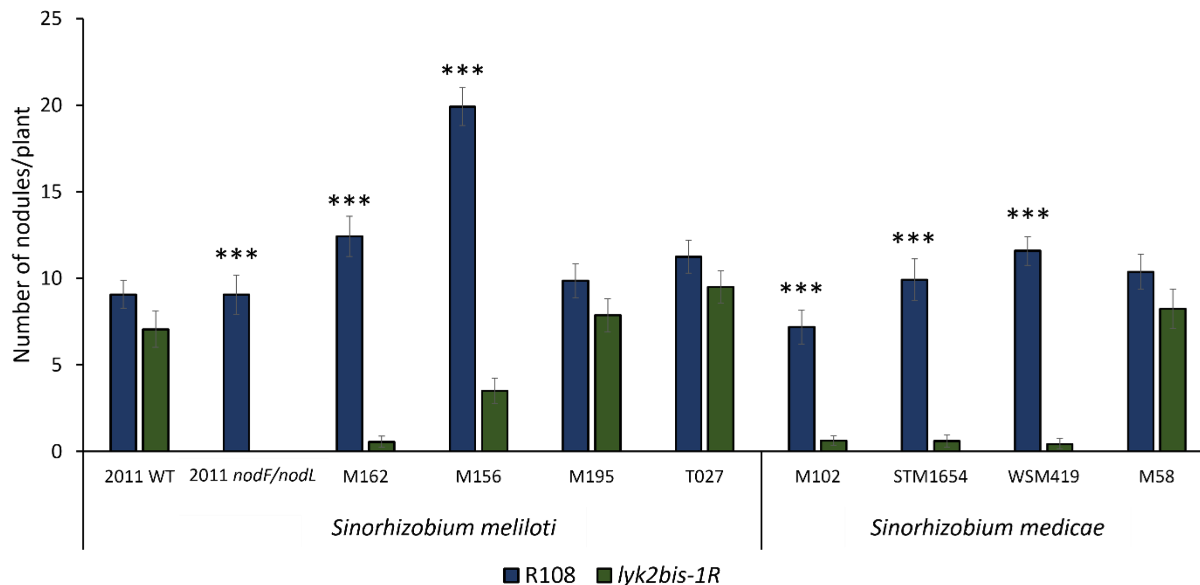

Fig. 6. *LYK2bis* is required for R108 to successfully nodulate with some but not all natural *Sinorhizobium* strains. Seedlings of R108 and *lyk2bis-1R* mutant were inoculated with different strains in a tube system. 16 plants/strain for each genotype were analysed at 28 dpi. Statistical analyses between each R108/*lyk2bis* pair for each strain were performed using Student's *t*-test (***, $P < 0.001$). Dark blue shading is for the R108 genotype and dark green shading is for *lyk2bis-1R*.

307 *LYK2bis does not play an important role in mycorrhization*

308 As some Myc-factors have an identical structure to the major *nodF/nodL* NFs (Maillet *et al.*, 2011),
309 we also tested the role of *LYK2bis* in forming arbuscular mycorrhiza (AM). Seedlings of *lyk2bis-*
310 *1R* and R108 were inoculated with spores of *Rhizophagus irregularis* and phenotyped for AM
311 colonization at 3 wpi and 5 wpi. Both genotypes showed good mycorrhization at both time points
312 (Fig**. 7**), indicating that *LYK2bis* is not essential for mycorrhization.

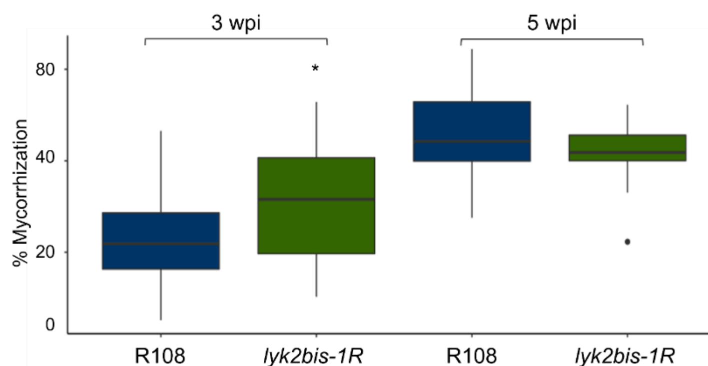

Fig. 7. *LYK2bis* does not play an essential role in mycorrhization. Seedlings were inoculated with *Rhizophagus irregularis* and examined at 3 and 5 wpi. 15 plants/genotype at each time point were analysed. Statistical analyses were done using Students' *t*-test (*, $P < 0.05$). Dark blue shading is for the R108 genotype and dark green shading is for *lyk2bis-1R*.

313

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314 *LYK2bis physically interacts with NFP*

315 NFP is required for nodulation in R108 (Feng *et al.*, 2019) as well as in A17 (Arrighi *et al.*, 2006),
316 therefore, to test whether LYK2bis could be part of an NFP receptor complex, we investigated
317 whether the two proteins interact using FRET-FLIM technology.

318 A construct of the LYK2bis protein fused with mCherry was co-expressed with a construct of a
319 NFP-GFP fusion in *N. benthamiana* leaves using *A. tumefaciens*-mediated expression.
320 Interestingly, at 3 dpi, all leaves expressing both proteins showed strong cell-death (Fig. **8a**) similar
321 to that observed in the co-expression of NFP and LYK3-A17 (Pietraszewska-Bogiel *et al*., 2013).
322 This suggests that LYK2bis may functionally interact with NFP in a similar mechanism to LYK3.

323 At 2 dpi, the cell death response was much reduced and confocal microscopy revealed that the two
324 proteins co-localise at the periphery of the cells (Fig. **8b**). As NFP was previously reported to
325 localise at the plasma membrane of *N. benthamiana* leaves (Lefebvre *et al.*, 2012), this evidence
326 indicates that LYK2bis co-localises with NFP at the plasma membrane.

327 Similar leaf samples were used for FRET-FLIM analysis in which NFP-GFP was used as the donor
328 and LYK2bis-mCherry was the acceptor. A highly significant decrease in lifetime of NFP-GFP
329 was obtained when co-expressed with LYK2bis-mCherry (Fig. **8c**). This data not only supports
330 the co-localization of LYK2bis and NFP at the plasma membrane but also provides strong
331 evidence for the physical interaction between the two proteins.

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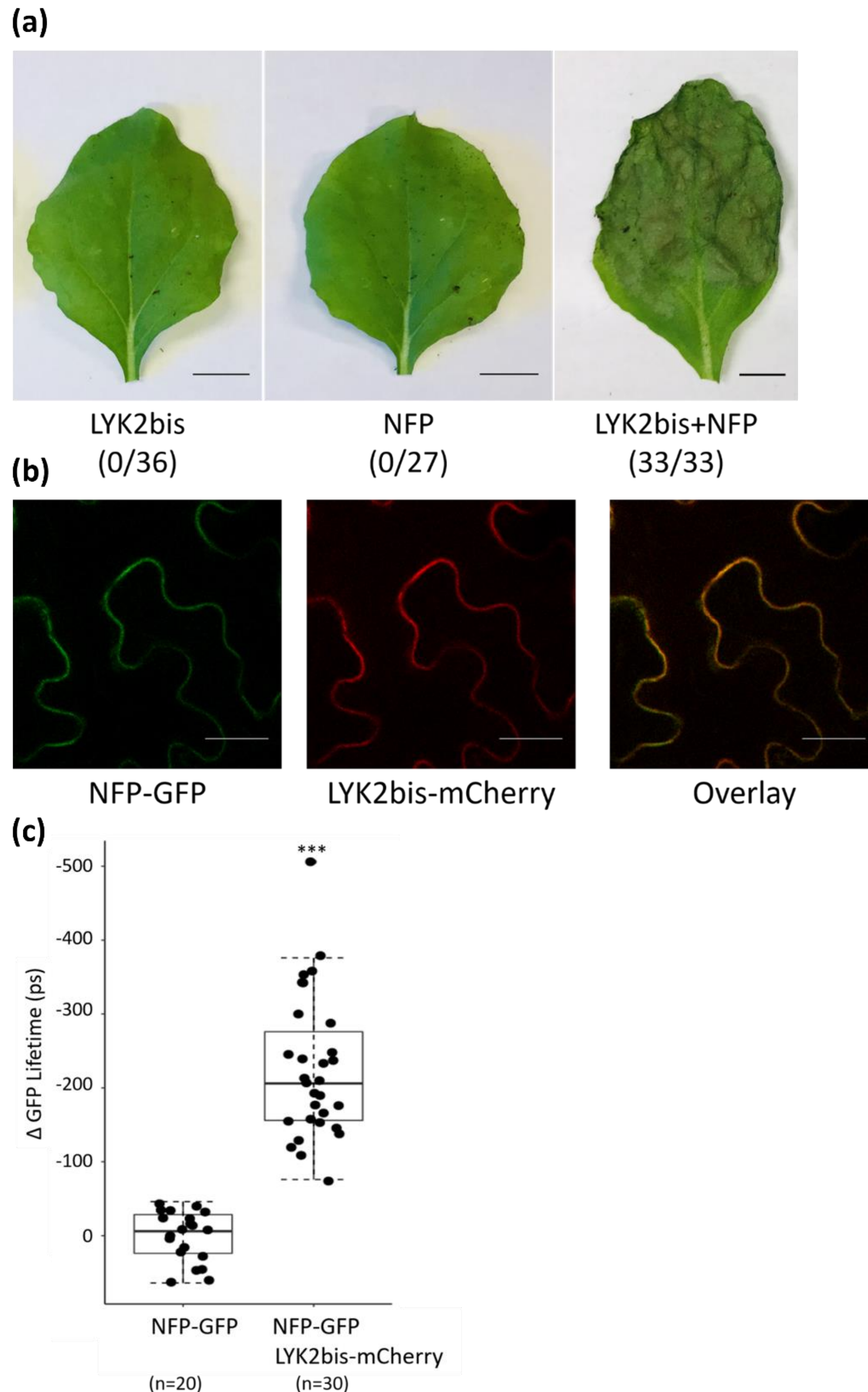

Fig. 8. LYK2bis physically interacts with NFP and this interaction can lead to cell-death at the plasma membrane of *Nicotiana benthamiana* leaves. (a) Co-expression of LYK2bis and NFP induces cell-death in *N. benthamiana* leaves at 3 dpi. Numbers in brackets indicate the number of leaves exhibiting cell death/total number of infiltrated leaves. Bars, 1 cm. (b) Co-localisation of NFP and LYK2bis at the plasma membrane of *N. benthamiana* leaves. Leaf discs were observed by confocal microscopy at 2 dpi. Bars, 25 µm. (c) LYK2bis physically interacts with NFP at the plasma membrane of *N. benthamiana* leaves by FRET FLIM technology. For each measurement, the difference to average GFP decay times (Δ) of the GFP-NFP in the absence of the LYK2bis-mCherry is given in ps; n: number of samples measured for each condition. Students' *t*-test was used for statistical analyses (***, $P < 0.001$).

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332 *LYK2bis possesses an active kinase and can transphosphorylate the pseudo kinase of NFP*

333 NFP has an inactive kinase domain (KD) which can be weakly trans-phosphorylated by the active
334 kinase domain of LYK3 (Arrighi *et al.*, 2006; Fliegmann *et al.*, 2016). To test whether LYK2bis
335 has an active kinase domain and can trans-phosphorylate NFP, we performed *in vitro*
336 phosphorylation assays on the intracellular regions of the two proteins (which includes the KD),
337 purified after expression in *E. coli*. We also tested the ability of LYK2bis-KD to trans-
338 phosphorylate related proteins, LYR2-KD, LYR3-KD, LYR4-KD and LYK3-deadKD (Fliegmann
339 *et al.*, 2016), and the model kinase substrate myelin basic protein (MyBP). LYK2bis-KD had
340 strong autophosphorylation activity and could trans-phosphorylate GST fusions of NFP-KD,
341 LYR2-KD, LYR3-KD, LYR4-KD and LYK3-deadKD and MyBP in presence of [ɣ-^{32}P] ATP, but
342 not GST (Fig. **9**). These results show that LYK2bis has an active kinase domain and can trans-
343 phosphorylate NFP, some other kinase domains and the model kinase substrate, MyBP.

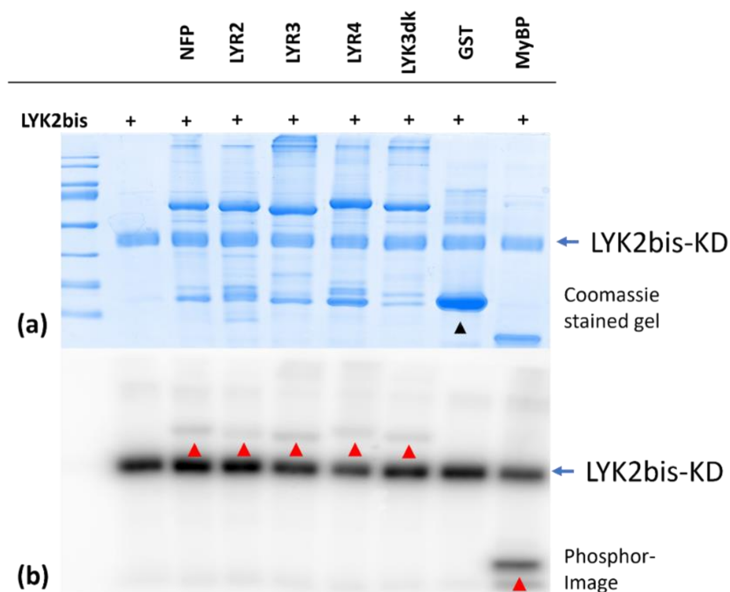

Fig. 9. LYK2bis has an active kinase and can transphosphorylate NFP-KD *in vitro*. The kinase domains (KD) of LYK2bis and NFP were expressed and purified from *E. coli* as fusions with GST. GST-cleaved LYK2bis-KD was incubated individually or co-incubated with purified GST/NFP-KD (NFP), GST/LYR2-KD (LYR2), GST/LYR3-KD (LYR3), GST/LYR4-KD (LYR4), GST/LYK3-deadKD (LYK3dk), GST or Myelin Basic Protein (MyBP) in the presence of radioactive [ɣ-^{32}P] ATP. Assays were analysed by SDS-PAGE, followed by coomassie staining (a) and phosphor imaging (b). Transphosphorylated proteins are marked by red arrowheads on the phosphor-image. The position of GST is marked on the coomassie gel (black arrowhead).

344 **Discussion**

345 The symbiotic interactions between legumes and rhizobia are highly specific and require proper
346 recognition of bacterial signals by plant receptors. NFs are key determinants of host specificity

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347 due to their species-specific chemical substitutions on both the reducing and non-reducing ends of
348 the NF, which are recognized by receptors on roots of their compatible hosts. In this study, we
349 demonstrate that two commonly used ecotypes of *M. truncatula*, A17 and R108, display a
350 contrasting nodulation specificity with the *S. meliloti nodF/nodL* mutant; while A17 has a
351 completely Nod$^-$ phenotype with this strain, R108 is able to form infected nodules with both the *S.*
352 *meliloti* WT and *nodF/nodL* strains (Fig. **1**). Using reverse genetics, we have identified *LYK2bis*
353 as the genetic determinant underlying this extension of nodulation specificity. Complementation
354 studies have clearly confirmed the essential role of *LYK2bis* in nodulation with the *nodF/nodL*
355 strain of R108 (Fig. **3**). Moreover, this exceptional characteristic can be transferred to another
356 genotype. As shown in Fig. **4**, the introduction of *LYK2bis* into A17 allows the plants to form
357 nodules with the *nodF/nodL* mutant strain. These results provide conclusive evidence for the role
358 of *LYK2bis* in nodulation with the *nodF/nodL* strain in R108.

359 *The evolution of LYK2bis*

360 *LYK2bis* is predicted to encode a *LysM-RLK* and is located between *LYK2* and *LYK3* on
361 chromosome 5 of the *M. truncatula* R108 genome (Fig. **2**). It is a chimeric gene with most of the
362 LysM domains of *LYK2* and the rest, including the kinase domain, of *LYK3* (Fig. **S2**). Such a gene
363 does not occur in other legumes such as *L. japonicus*, soybean and pea (De Mita *et al.*, 2014;
364 Sulima *et al.*, 2017). In *M. truncatula*, although all the 23 genotypes available for BLAST
365 screening in the Hapmap collection (https://medicagohapmap2.org/) contain sequences highly
366 related to *LYK2* and *LYK3* (Table **S3**), only R108 contains *LYK2bis*. R108 is the most distant of
367 the *M. truncatula* genomes (Zhou *et al.*, 2017), and the only one representing *M. truncatula* ssp.
368 *tricycla.* The most parsimonious explanation for the presence of *LYK2bis* in R108 is that it is a
369 newly evolved gene formed from the pre-existing *LYK2* and *LYK3* genes in this
370 genotype/subspecies, although we cannot exclude the possibility that it pre-existed in the most
371 recent common ancestor of *M. truncatula* and has been lost at an early stage of the divergence of
372 the two *M. truncatula* subspecies. In either case its phenotypic attributes suggest that it is a good
373 example of recent adaptive gene duplication (Kondrashov, 2012).

374 *The specificity of nodulation directed by LYK2bis*

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375 Comparison of nodulation of the *lyk2bis* mutant with different *nod* gene mutants (Fig. **5**) suggest
376 that it is the mutation in *nodL* (which is polar on *noeA, noeB*), and not in *nodF,* which is the major
377 cause of lack of nodulation of the *lyk2bis* mutant by the *nodF/nodL* strain. Although the roles of
378 *noeA* and *noeB* remain unknown, it is clear that NodL encodes an *O*-acetyl transferase and that the
379 only difference detected in the NFs produced by this *nodL* mutant is the complete lack of *O*-
380 acetylation of the non-reducing sugar (Ardourel et al., 1995). This evidence indicates a specific
381 role of *LYK2bis* in nodulation with strains producing non-*O*-acetylated NFs whereas other gene(s)
382 in R108 can enable nodulation with strains producing *O*-acetylated NFs, including ones without
383 the specific C16 acyl chain, specified by the *nodFE* mutant. A17 shows only a slightly reduced
384 nodulation with the *nodFE* mutant but a much reduced nodulation with a *nodL* mutant, and this
385 has been attributed to the *LYK3* gene (Smit *et al.*, 2007). LYK3-A17 may thus show a preference
386 for recognizing *O*-acetylated NFs but may tolerate C18 ones. It is relevant to note that in pea,
387 nodulation with C18:1 NFs produced by a *Rhizobium leguminosarum nodE* mutant is associated
388 with certain haplotypes of *PsSYM37,* which is orthologous and functionally equivalent to
389 *LYK3/NFR1* (Li *et al.*, 2011).

390 In pea, an interesting phenotype linked to *O*-acetylation of the reducing sugar of NFs has been
391 identified in cv Afghanistan: nodulation by *R. leguminosarum* requires this particular NF structural
392 modification, which is produced by strains containing the *nodX* gene (Firmin *et al.*, 1993). On the
393 plant side, *PsSym2* was identified as the determinant of this strain selectivity, and incidentally,
394 synteny with closely related *M. truncatula* led to the identification of the *LYK* cluster, containing
395 genes *LYK1* - *LYK7* (Limpens *et al.*, 2003). Recent studies have identified three *LysM-RLKs* in the
396 corresponding pea *LYK* cluster: *PsSYM37*, *PsK1* and *PsLYKX,* all of which play roles in nodulation
397 (Zhukov *et al.*, 2008; Li *et al.*, 2011; Kirienko *et al*., 2019; Sulima *et al.*, 2017). By analysing
398 different pea cultivars, it has been shown that nodulation with *nodX*$^{+}$ strains is correlated with
399 haplotypes of the *PsLYKX* gene, but not *PsSYM37* or *PsK1*, suggesting that *PsLYKX* corresponds
400 to *Sym2* (Sulima *et al.*, 2017, 2019).

401 *The importance of LYK2bis structure for NF recognition*

402 Recently, Bozsoki *et al.* (2020) have published the crystal structure of the extracellular domain of
403 LYK3-A17. By using a domain swapping and point mutation approach, the authors have identified

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404 the importance of two regions in the LysM1 domain of LYK3-A17 for the specificity of NF
405 signalling. As shown in Fig. **S1**, the extracellular domain of LYK2bis shows strong divergence to
406 LYK3-A17, especially in LysM1 (Table **S2,** Table **S5**). In particular, the two regions II and IV of
407 LysM1 that are essential in LYK3 for discrimination of NF and CO ligands and for specific
408 nodulation are very poorly conserved in LYK2bis. These regions could therefore be involved in
409 the discrimination of *nodF/nodL* and WT NFs. In pea, nodulation with *nodX*$^{+}$ strains is associated
410 with *LYKX* haplotypes containing specific amino acids in regions II and IV of LysM1, as described
411 above (Sulima *et al.*, 2019; Solovev *et al.*, 2021). Together, this evidence indicates the importance
412 of specific regions in the LysM1 of these highly-related LYK cluster proteins in the recognition of
413 specific NF decorations.

414 *Mechanism of action of LYK2bis*

415 MtNFP, and its orthologs in *L. japonicus* (NFR1)*, P. sativum* (SYM10) and other legumes, is the
416 key LysM-RLK involved in NF perception and signalling (Krönauer & Radutoiu, 2021). Many
417 studies have shown the involvement of this protein and one or two LYK cluster proteins in
418 perception of NFs and signalling for nodulation (Radutoiu *et al.*, 2003; Kirienko *et al.*, 2019). In
419 this study, we observed that the co-expression of LYK2bis and NFP in *N. benthamiana* leaves
420 leads to defence-like responses, which were also observed in co-expression of LYK3-A17 and
421 NFP (Pietraszewska-Bogiel *et al.*, 2013), indicating a similar mechanism of action between these
422 two protein pairs. In addition, we have shown that LYK2bis physically interacts with NFP in *N.*
423 *benthamiana* leaves suggesting the formation of a heteromer *in planta,* which is possibly
424 responsible for recognition of non-*O*-acetylated NFs. This hypothesis is supported by the work on
425 the interaction between PsLYKX and PsSYM10 receptor complex with NFs (Solovev *et al.*, 2021).
426 In this study, using molecular modelling and ligand docking, the authors have shown the potential
427 for stable heterodimers between PsLYKX and PsSYM10, which may interact with NFs at the
428 heterodimer interface. Indeed in LYK3, region III of LysM1 is envisaged to interact with NFP
429 (Bozsoki *et al.*, 2020), and is quite highly conserved between LYK2bis and LYK3 (7/12 residues
430 – Fig. **S2**). Moreover, the LYK2bis intracellular domain is almost identical to that of LYK3-A17
431 (three amino acids in difference) and both possess an active kinase, which *in vitro* can trans-
432 phosphorylate NFP-KD, albeit non-specifically (Fig. **8** and Fliegmann *et al.*, 2016). This similarity
433 suggests a mechanism by which LYK2bis could integrate into the LYK3 signal transduction

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434 pathway. This may be through substitution for LYK3, rather than interaction with this protein, as
435 we have shown that the LYK2bis-dependent gain of A17 nodulation by the *nodF/nodL* strain can
436 occur in a *lyk3* mutant.

437 Finally, our work has shown that *LYK2bis* is very important for nodulation of R108 by many
438 natural strains of *S. meliloti* and *S. medicae*. However, some strains of each species do not require
439 this gene for R108 nodulation (Table **S4** and Fig. **6**). This difference in *LYK2bis* dependence could
440 be explained by the strains differing in the proportion of non-*O*-acetylated/*O*-acetylated NFs that
441 they produce: the dependent strains producing a greater proportion than the *LYK2bis*-independent
442 strains. Analysis of the *nodL* gene from the available sequences of these strains did not reveal any
443 differences that correlated with the two classes (B. Gourion, personal communication). However,
444 recent preliminary analysis of the NFs from *S. medicae* WSM419 supports this hypothesis as *O*-
445 acetylated NFs were not detected among the most abundant detected NFs (V. Puech-Pagès, F.
446 Maillet, B. Gourion, P. Ratet, personal communication), whereas the *LYK2bis* independent strain
447 *S. meliloti* 2011 produces a majority of *O*-acetylated NFs (Roche et al., 1991). R108 was collected
448 from Israel (Garmier *et al.*, 2017) but no correlation was observed between *LYK2bis*-dependence
449 and strains from the Middle East region, suggesting that *LYK2bis* has not evolved to adapt to a
450 particular strain, specific to this geographical region. Genomic studies on *S. meliloti* and *S.*
451 *medicae* suggest a complex history of horizontal gene transfer between these two species, which
452 is restricted almost exclusively to plasmid genes, including the *nod* genes (Epstein *et al.*, 2012).
453 Thus, the ability to produce non-*O*-acetylated NFs may be widespread but patchy in *Sinorhizobium*
454 and the evolution of *LYK2bis* may thus present an adaptive advantage to allow nodulation by a
455 greater variety of strains.

456 Based on data obtained in this study, we thus propose a model for the nodulation of R108 in which
457 rhizobia may produce different proportions of *O*-acetylated/non-*O*-acetylated NFs. LYK2bis
458 forms a receptor complex with NFP that perceives non-*O*-acetylated NFs and activates the kinase
459 of LYK2bis, which then trans-phosphorylates NFP leading to downstream signalling and finally
460 to nodule formation (Fig. **10**). Other *LYK* genes such as *LYK3*, possibly redundant with *LYK2bis*,
461 may be involved in the perception of O-acetylated NFs.

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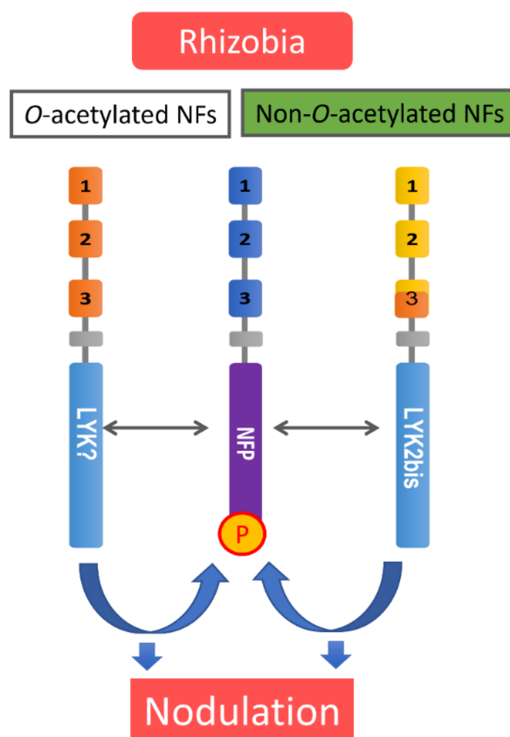

Fig. 10. Model of NF recognition and nodulation in *Medicago truncatula* R108. Different rhizobia may secrete different proportions of *O*-acetylated and non-*O*-acetylated NFs. The nodulation of R108 with strains producing non-*O*-acetylated NFs is dependent on LYK2bis and possibly involves NFP while the *O*-acetylated NFs may involve other LYK(s). The active LYK kinases can transphosphorylate (P) NFP, which may be important for signal transduction.

462 In conclusion, we have identified a newly-evolved gene in R108, *LYK2bis*, which is required for
463 efficient nodulation by many but not all *Sinorhizobium meliloti and medicae* strains and is
464 responsible for extending the nodulation specificity of R108 to include a *nodF/nodL* mutant.
465 *LYK2bis* appears to have a specific role in recognising NFs which are non-*O*-acetylated on the
466 terminal non-reducing sugar. *LYK2bis* is a chimera formed from the *LYK2* and *LYK3* genes and is
467 located between them in the *LYK* cluster on chromosome 5. Studies on the diversity of this locus
468 and the expansion of the *tricycla* subspecies, coupled with analysis of rhizobial symbionts, would
469 establish whether the evolution of this gene in *M. truncatula* has led to host-range expansion.

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491 **Author contribution**

492 TBL, NP and JC designed and performed experiments, interpreted the data and wrote the
493 manuscript. AO contributed to experiments on natural rhizobium strains and performed the
494 mycorrhization test. CP performed and analysed the FRET-FLIM experiments.

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635 **Supporting Information**

636 Additional supporting information may be found in the online version of this article.

637 **Fig. S1** *Medicago truncatula* R108 is able to form fixing nodules with both *Sinorhizobium meliloti*
638 2011 WT and its *nodF/nodL* mutant.

639 **Fig. S2** Amino acid alignment of LYK3-A17, LYK3-R108, LYK2bis, LYK2-A17 and LYK2-
640 R108

641 **Table S1** *Sinorhizobium meliloti* 2011 strains used in this study

642 **Table S2** Percentage of identity/similarity of LYK2, LYK3 in A17 and LYK2bis and LYK3 in
643 R108

644 **Table S3** Presence of *LYK2bis* in *Medicago truncatula* genomes

645 **Table S4** Characteristics of natural *Sinorhizobium* strains and their ability to nodulate R108 and
646 the *lyk2bis-1R* mutant.

647 **Table S5** Number of identical amino acids/total amino acids in each LysM between LYK2bis and
648 LYK3-A17 and LYK2-A17