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Summary
- Rhizobia and legumes establish symbiotic interactions leading to the production of root nodules, in which bacteria fix atmospheric nitrogen for the plant’s benefit. This symbiosis is efficient because of the high rhizobia population within nodules. Here, we investigated how legumes accommodate such bacterial colonization.
- We used a reverse genetic approach to identify a Medicago truncatula gene, SymCRK, which encodes a cysteine-rich receptor-like kinase that is required for rhizobia maintenance within the plant cells, and performed detailed phenotypic analyses of the corresponding mutant.
- The Medicago truncatula symCRK mutant developed nonfunctional and necrotic nodules. A nonarginine asparate (nonRD) motif, typical of receptors involved in innate immunity, is present in the SymCRK kinase domain. Similar to the dnf2 mutant, bacteroid differentiation defect, defense-like reactions and early senescence were observed in the symCRK nodules. However, the dnf2 and symCRK nodules differ by their degree of colonization, which is higher in symCRK. Furthermore, in contrast to dnf2, symCRK is not a conditional mutant.
- These results suggest that in M. truncatula at least two genes are involved in the symbiotic control of immunity. Furthermore, phenotype differences between the two mutants suggest that two distinct molecular mechanisms control suppression of plant immunity during nodulation.

Introduction
Legumes and rhizobia establish symbiotic interactions that result in the formation of root organs (the nodules) housing bacteria that fix atmospheric nitrogen for the benefit of the plant (Oldroyd et al., 2011; Udvardi & Poole, 2013). The symbiosis is efficient as a result of the chronic and massive bacterial invasion of the nodule cells. Despite the massive colonization of their tissues, plants do not develop defense reactions in response to the endosymbionts (bacteroids) (Berrabah et al., 2014). The relationship between immunity and symbiosis has been investigated before, but the majority of these studies focused on the early step of the symbiosis, that is, the infection process (Zamioudis & Pieterse, 2012). How plants tolerate massive intracellular infection in the symbiotic organ remains poorly documented (Zamioudis & Pieterse, 2012; Bourcy et al., 2013a). However, deciphering this mechanism is a necessity for the potential transfer of the nitrogen-fixing symbiotic capacity to nonlegume plants, which is currently a challenge (Charpentier & Oldroyd, 2010; Beaty & Good, 2011). In addition, understanding how eukaryotes can accommodate such massive intracellular infection by a foreign organism might also have impacts on other fundamental biological questions such as the evolutionary acquisition of endosymbionts, as well as the maintenance of intracellular pathogenic bacteria.

The recently released genome sequence (Young et al., 2011), the accumulation of transcriptomics data gathered in a gene expression atlas (Benedito et al., 2008; He et al., 2009) and the existence of large collections of tagged mutants (Tadege et al., 2008; Iantcheva et al., 2009; Cheng et al., 2011) with an increasing number of available flanking sequence tags (FSTs; http://bioinfo4.noble.org/mutant/database.php) make Medicago truncatula one of the favorite models to study rhizobium–legume interactions. In M. truncatula, nodules have a persistent meristem in the apical region, referred to as zone I, which is separated from zone III, in which bacteria fix nitrogen, by zone II, in which bacteria invade plant cells (Vasse et al., 1990). In zone III of M. truncatula nodules, the infected cells produce nodule-specific cysteine-rich (NCR) antimicrobial peptides that trigger the terminal differentiation of bacteroids (Van de Velde et al., 2010). As a consequence of the action of these peptides, the bacteroids are elongated and become polypliod (Kondorosi et al., 2013). In parallel, in zone III, the plant nuclei undergo endoreduplication, resulting in an endopolyploidy level up to 64C (Maunoury et al., 2010). Nodule aging causes the development of a fourth zone (IV) in which both bacteria and plant cells senesce (Vasse et al., 1990).
We recently described the DNF2 gene that is required for bacteroid differentiation and persistence in *M. truncatula* (Bourcy et al., 2013b). The nodules of the *dnf2* mutant display typical traits, suggesting defense-like reactions such as the induction of defense genes, the accumulation of phenolic compounds and the death of the symbiotic partner (Bourcy et al., 2013a,b; Berrabah et al., 2014). DNF2 encodes a nodule-specific phosphatidylinositol phospholipase C X domain containing protein, but the link between plant defense repression and the biochemical activity of DNF2 has not yet been described. To get deeper insight into symbiotic control of plant immunity, we searched for *DNF2* coexpressed genes and report here the discovery of a second gene required to suppress plant defense-like reactions in nodules.

**Materials and Methods**

**Plant and bacterial cultures**

Except when explicitly mentioned, *Medicago truncatula* Gaertn ecotypes R108 (Hoffmann et al., 1997) and its corresponding mutants *dnf2*–4 (Pislaru et al., 2012; Bourcy et al., 2013b), and all mutants described in Supporting Information Table S1 were cultivated in vitro on buffered nodulation medium (BNM) (Ehrhardt et al., 1992) solidified with 2% bacto-agar. Phytagel (0.8%) was also used as an alternative solidifying agent. For flow cytometry material preparation, plants were cultivated in growth chamber on a mixture of perlite and sand (1/2, v/v). *Sinorhizobium meliloti* mutants described in Supporting Information Table S1 were cultivated in yeast extract broth (YEB) medium (Krall et al., 2001), strain 1021 *bacA* (Ferguson et al., 2002) and strain 2011 (Rosenberg et al., 1981), as well as *S. medicae* strain WSM419 (Howieson & Ewing, 1986), were cultivated in yeast extract broth (YEB) medium (Krall et al., 2002). The strain used for each experiment is indicated in the corresponding figure legend.

**Complementation experiment**

*SymCRK*, including its 4 kbp upstream region corresponding to the putative promoter region, was amplified using primers BG44 5′-CACCAGATTTGTGGCATCAGCTTATCC-3′ and MtRec3 5′-TATGACAACAACTTAAAAAACCTTGCA-3′, cloned into the pENTR/D-Topo (Invitrogen) and transferred to pK7WG. *Agrobacterium rhizogenes* strain Arqua1 was used to transform the root system of *symCRK* mutant (NF0737). Hairless root transformation was performed as described earlier (Boisson-Dernier et al., 2001). Briefly, after germination, plantlet root tips were removed using a scalpel and the cut roots were soaked in an *A. rhizogenes* suspension. Plants were then grown on BNM solid medium containing kanamycin.

**Identification of the PLA2 mutant**

Polymerase chain reaction screening was performed as described in (Tadege et al., 2008; Cheng et al., 2011, 2013) using PLA2-specific primers PLA2a 5′-CCACAAAAATGCTTC

CAGTACCTGT-3′ and PLA2b 5′-CCTTGTTAATTCCCTTGAAAATACCA-3′.

**Acetylene reduction assay**

Acetylene reduction assay (ARA) was conducted on individual plants with a protocol modified from Koch & Evans (1966). Briefly, 14 d after inoculation, individual whole plants were placed into 10 ml glass vials sealed with rubber septa. Acetylene (250 µl) was injected into each vial. Gas samples (200 µl) were withdrawn after at least 1 h incubation at room temperature and the ethylene that was produced was measured by GC. The assay was done in triplicate.

**SymCRK cDNA cloning**

*SymCRK* cDNA was cloned into PEntr/D-Topo after amplification from cDNAs prepared from R108 nodules using primers BG54 5′-caccATGGCTTACAATCTGAAACAAAACTG-3′ and MtRec3 5′-TATGACAACAACTTAAAAAACCTTGCA -3′ and sequenced.

**RT-qPCR**

RNA extraction, cDNA synthesis and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed as previously described (Bourcy et al., 2013b). The primers used in this study are listed in Table S2 and have been described previously (Gao et al., 2007; Samac et al., 2011; Nars et al., 2013).

**Microscopy**

Semithin sections (7 µm) of nodules were prepared as previously described (Van de Velde et al., 2006). For the live/dead staining procedure, nodule sections were stained, prepared and observed as previously described (Haag et al., 2011; Bourcy et al., 2013b). Phenolic compounds were stained using potassium permanganate and methylene blue (Vasse et al., 1993) and observed as previously described (Bourcy et al., 2013b).

**Flow cytometry analysis**

Bacteroid material preparation and analysis were essentially conducted as described by Mergaert et al. (2006). Briefly, bacteroids were extracted from nodules 35 d after inoculation. Nodules were homogenized in a mortar and pestle with an ice-cold bacteroid extraction buffer (BEB; 125 mM KCl, 50 mM Na-succinate, 50 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt (TES) buffer, pH 7, 1% BSA). In order to eliminate debris, homogenates were centrifuged three times at 100 g at 18°C for 10 min and supernatants containing bacteroids were collected and centrifuged at 2000 g for 10 min. Bacteroid pellets were resuspended in 100 µl of BEB, and heated at 70°C for 10 min. Bacteroids were stained with 50 µg ml−1 4’,6’-diamidino-2-phenylindol (DAPI). Measurements were performed with a Beckman-Coulter (Danvers, MA, USA) ELITE ESP flow
cytometer. For nuclei preparation, 35-d-old nodules were treated as described by Mergaert et al. (2006).

Results

Identification of DNF2 coregulated genes as candidates for repression of plant immunity during the symbiotic process

In order to identify new genes potentially involved in the control of plant defenses during symbiosis, we searched for DNF2 coregulated genes, hypothesizing that some of them might be involved in plant immunity suppression. The M. truncatula gene expression atlas (MrGEA) was used for this purpose. Based on predicted function and on Pearson correlation coefficient calculated from expression data, three candidate genes were selected. These genes are annotated as a phospholipase A2 (PLA2), a sterol 3-β-glucosyltransferase and a cysteine-rich receptor-like kinase (CR RLK, Fig. 1) whose expression profile had the highest similarity with DNF2 (Fig. S1). Table S1 describes the Pearson correlation for the three candidate genes. Tnt1-tagged mutant lines with Tnt1 insertion in these genes were identified from the Noble Foundation M. truncatula mutant collection (http://bioinfo4.noble.org/mutant/database.php; Table S1, Fig. S1; Tadege et al., 2008). Mutant lines harboring insertions in CR RLK (NF0737) and in the sterol 3-β-glucosyltransferase gene (NF4831) were identified by searching the FST database, while the mutant with insertion in PLA2 (NF6259) was identified by PCR-based reverse screening as previously described (Cheng et al., 2013). In order to determine if the Tnt1 insertion in these genes alters nodule development and symbiosis, segregation analysis was performed on the T2 offspring of the mutant lines. Heterozygous, homozygous mutant or wild-type-like plants at the candidate loci were selected from the progenies of these lines (Table S3) and then tested for their capacity to establish symbiosis in vitro. Based on nodule pigmentation, only plants with insertion in the CR RLK locus and originating from the line NF0737 showed a defect in symbiosis and did not produce typical pink-colored functional nodules (Table S3, Fig 2b). Mutants with insertions in the other two genes, sterol 3-β-glucosyltransferase and PLA2, did not show symbiotic defects. Plants homozygous for insertion at the CR RLK locus had progenies with only white or necrotic nodules (Table S3). By contrast, progenies of a plant heterozygous for insertion at the CR RLK locus segregated as plants with wild-type nodules (19 plants) and plants with necrotic nodules (nine plants; Table S3), suggesting a strong link between the nodule phenotype and the mutation. In order to prove the

![Fig. 1](image1.png)

**Fig. 1** SymCRK is a member of the nonarginine aspartate (nonRD) kinase family. (a) The SymCRK gene structure; SymCRK has seven exons (black boxes) and six introns (2839 bp from ATG to Stop). The position of the Tnt1 insertion disrupting the third exon of SymCRK in the NF0737 line (1065 bp downstream the ATG) is indicated by an arrow. (b) The predicted protein domains. A predicted signal peptide is present on the N-terminal (SP), followed by two cysteine-rich domains of unknown function (DUF26), a transmembrane domain and a serine/threonine protein kinase domain. The activation loop of the protein kinase domain harbors a nonRD motif represented by a green C and a red D. This motif is typical of pattern recognition receptor such as At_EFR and At_FLS2 and is not present in most kinases, such as At_PEP1, At_THESEUS and At_WAK1, which harbor an RD motif represented by a blue R and a red D.

![Fig. 2](image2.png)

**Fig. 2** SymCRK prevents nodule necrosis. (a–c) Nodules induced by Sinorhizobium meliloti Rm41 on Medicago truncatula wild-type (a), or on hairy roots of the NF0737 mutant line homozygous for SymCRK insertion (b, c), transformed with a control vector (b) or with a complementation vector (c). (d) The number of white (white bars), pink (pink bars) and necrotic (brownish) nodules produced per plant. Error bars, ± SE. A chi-squared test of homogeneity indicates that proportions of white and brownish nodules are different for dnf2–4 and symCRK (value of the test for one degree of freedom: 3.6 \( \times 10^{-16} \)). This experiment has been reproduced with similar results.

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requirement of CR RLK for the symbiotic process, the gene was cloned with its native promoter region (i.e. ~ 4 kbp upstream of the predicted start codon) and was expressed using hairy root transformation in NF0737 roots. In contrast to the NF0737 line that displayed white or necrotic nodules, the complemented root systems produced pink nodules similar to that of the wild-type R108 (Fig. 2b,c). Unambiguously, these results indicate that the CR RLK is required for the symbiotic process. For these reasons, we named this gene *M. truncatula* Symbiotic CR RLK (Mt_SymCRK).

**SymCRK encodes a nodule-specific receptor-like kinase of the nonarginine aspartate (nonRD) family**

The microarray data from the MtGEA suggested that the *SymCRK* gene is exclusively expressed in nodules but not in other plant tissues subjected to various physiological conditions included in the database (Fig. S1). However, the MtGEA dataset was mainly constructed with the *M. truncatula* ecotype Jemalong A17, while the NF0737 mutant is in the R108 ecotype. In order to determine whether the *SymCRK* gene is also specifically expressed in nodules of the *M. truncatula* R108 ecotype, RT-qPCR experiments were performed using RNA isolated from leaves, stems, petioles, roots, and nodules. In agreement with the data obtained with *M. truncatula* Jemalong, in the R108 background, the expression of *Mt_SymCRK* was detectable only in nodules (Fig. S2).

In order to determine the structure of the *SymCRK* (Medtr3g079850.1) gene in the R108 background, the corresponding cDNA was cloned and sequenced. The gene harbors six introns and seven exons (Fig. 1a) and corresponds to the Affymetrix Microarray probe sets, Mtr.39654.1.S1_at and Mtr.44507.1.S1_at (Table S1, Fig. S1). In the mutant line NF0737, the *Tnt1* insertion is located in the third exon (Fig. 1a). The gene encodes for a protein of 654 amino acid residues. Analysis of the primary sequence of the protein using the SMART server (http://smart.embl-heidelberg.de/; Schultz *et al.*, 1998; Letunic *et al.*, 2012) indicates that the predicted protein harbors a signal peptide, two extracellular cysteine-rich domains of unknown function 26 (DUF26), a transmembrane domain and a serine/threonine protein kinase domain (Fig. 1b), and thus belongs to the family of cysteine-rich kinases (CRKs; Chen, 2001). Interestingly, *Mt_SymCRK* contains a nonRD motif in the activation loop of the protein kinase domain (Figs 1b, S3) that is typical of pattern recognition receptors (PRRs) involved in plant and animal innate immunity (Dardick & Ronald, 2006; Dardick *et al.*, 2012). A careful analysis of the predicted protein database indicates that nonRD CRKs are only found in legume plants (one in *M. truncatula*, one in *Lotus japonicus* (chr6.CM0041.530.r2.a) and one in *Cicer arietinum* (XP_004502136.1)). By contrast, the 41 *Arabidopsis* CRKs are RD kinases (Fig. S3). Despite the presence of a nonRD motif, a phylogenetic analysis indicates that the kinase domains of these three nonRD CRKs of legumes are closer to RD CRK kinase domains than to nonRD kinase domains of PRRs such as AtFLS2 or AtEFR (Fig. S4). Interestingly, the occurrence of the nonRD motif in these three legume CRKs is correlated with an atypical motif in the N-terminal DUF26 domain (Fig. S3). Cysteine-rich RLK proteins contain two copies of C-X₆-C-X₂-C in the extracellular domains (DUF26; Chen, 2001). By contrast, *Mt_SymCRK* and its two legume homologs display a C-X₆-C-X₂-C motif in the N-terminal DUF26.

**Mt_SymCRK is required for nitrogen fixation**

Like the *dnf2* mutant, the *symCRK* mutant produces a mixture of white and necrotic nodules when grown on an agar-based substrate (Fig. 2d, Table S3). However, the proportion of necrotic nodules to white nodules was significantly higher in the *symCRK* than in the *dnf2* mutant (Fig. 2d). We previously observed that DNF2 is facultative for symbiosis *in vitro* when Phytagel was used instead of agar to solidify the medium (Berrabah *et al.*, 2014). In order to determine if *symCRK* also displays this conditional requirement, we grew and inoculated *M. truncatula* wild-type, *dnf2* and *symCRK* plants with *Sinorhizobium medicae*, *in vitro*, on medium solidified with agar or Phytagel, and evaluated the symbiotic capacity of the plants using the ARA. In contrast to *dnf2*, which displayed the conditional phenotype (i.e. fix⁺ on Phytagel and fix⁻ on agar), *symCRK*-nodulated plants were unable to reduce acetylene on both substrates tested (Fig. 3). Together, our data support an essential role for *SymCRK* in nitrogen fixation.

**SymCRK is required for normal nodule zonation**

In order to get further insight into the role of *SymCRK* during symbiosis, histological analyses of the mutant nodules were performed. The initial steps of the symbiotic process were not altered in the *symCRK* mutant. Indeed, the mutant nodules displayed the typical elongated shape of indeterminate nodules, and symbiotic cells seemed to be correctly infected (Fig. 4). However, in *symCRK* nodules, zone III was drastically reduced compared...
with the wild-type nodules and a senescent zone with plant cells devoid of bacteria was observed. Comparison with dnf2 nodule sections revealed that the modification of the nodule zonation is less severe in the symCRK mutant than in the dnf2 mutant in which zone III is smaller and zone IV is larger than in wild-type plants. This observation was further confirmed by comparisons of the endoreduplication indexes (EI) determined using flow cytometry analysis of the nodule nuclei (Fig. S5, Table S4). The EI was higher in the symCRK than in dnf2 (Fig. S5, Table S4). In addition, in the symCRK nodules, degradation of material was observed in the vacuoles of infected cells (Fig. 4f).

SymCRK is required for bacteroids differentiation

Bacteroid differentiation in symCRK was evaluated by flow cytometry analysis (Fig. 5A). For this experiment, the Sinorhizobium meliloti strain Sm1021 was used together with its derivative mutant bacA, which is unable to undergo terminal bacteroid differentiation (Glazebrook et al., 1993). A bacA mutant does not exist in another rhizobium strain that can efficiently nodulate M. truncatula R108. The DNA content of the bacteroids extracted from dnf2 and symCRK nodules induced by wild-type S. meliloti (Sm1021) were similar to bacteroids prepared from wild-type M. truncatula R108 plants infected by the S. meliloti bacA mutant (Fig. 5A). In agreement with this observation, analysis of confocal microscopy images revealed that the bacteroids in symCRK and dnf2 mutants were smaller than those in the wild-type M. truncatula plants (Fig. 5B). Wild-type, dnf2 and symCRK bacteroids had average sizes of 7, 3 and 1.5 μm, respectively. Bacteroids of the bacA mutant on wild-type plants had the same size as symCRK bacteroids (Fig. 5B).

As mentioned earlier, in M. truncatula, terminal differentiation of bacteroid is triggered by NCR peptides. In order to determine if the lack of bacteroid differentiation observed in symCRK is correlated with reduced NCR production, expression of six genes encoding NCR peptides was investigated by RT-qPCR in nodules of M. truncatula (R108) wild-type, symCRK, dnf2 and in bacA-induced nodules (Fig. 5C). Expression of the early NCR

Fig. 4 SymCRK is required to prevent early senescence. Sections of Medicago truncatula wild-type (WT; a, d, g), dnf2 (b, e, h), and symCRK (c, f, i) nodules 38 d after inoculation with Sinorhizobium meliloti strain Rm41. Meristematic (I), infection (II), fixation (III or III*) and senescent (IV) zones are delimited in panels (a)–(c) by dashed lines. (d–i) These represent enlargement of (a)–(c) in the indicated zones (roman numbers on the pictures). Nodule sections were stained using toluidine blue. Arrows in (f) indicate the degraded material in the vacuole. The material deposited between cells and staining darker in (h) and (i) may represent phenolics. Bars: (a–c) 200 μm; (d–i) 50 μm.
Fig. 5 SymCRK is required for bacteroid differentiation. (A) Flow cytometry analysis of 4′,6′-diamidino-2-phenylindol (DAPI)-stained bacteroids extracted from Medicago truncatula wild-type (WT; a, d), dnf2 (b) and symCRK plants (c) 35 d after inoculation with Sinorhizobium meliloti Sm1021 (a–c) or with the Sm1021 bacA mutant (d). Results obtained with isolated bacteroids and free-living bacteria are represented in green and in blue, respectively. On these diagrams, the y-axis represents the numbers of counted objects, and the x-axis represents DAPI fluorescence measurements on a log scale (reflecting the DNA contents). (B) Bacteroid size determined by image analysis: wild-type, dnf2 and symCRK bacteroids are an average of 7.3 and 1.5 μm long, respectively. symCRK bacteroids display the same size as the bacA mutant and free-living bacteria (1.5 μm). Error bars, ± SD from 60 measurements. A parametrical one-way ANOVA test (P-value < 2.2e-16) and a post hoc Tukey-Kramer test (95%) were performed, and statistically identical values are attributed with identical letters (n = 60). (C) NCR gene expression was evaluated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in the indicated nodules 14 d after inoculation with Sm1021 or Sm1021 bacA. Fold change vs WT is presented after normalization with an Actin gene as an internal control. Error bars, ± SE from two technical replicates from two independent experiments. Expression of late nodule-specific cysteine-rich (NCR) peptides is altered in the defective nodules.

SYMCRK is required to repress defense-like reactions in nodules

In a previous study, DNF2 was shown to be required to prevent phenolics accumulation and nodule necrosis and to repress defense-related gene expression (Bourcy et al., 2013b; Berrabah et al., 2014). In order to determine if SymCRK also prevents defense-like reactions in the nodules, accumulation of phenolic compounds was investigated in the nodules formed by the symCRK mutant by staining nodule sections with KMnO4/methylene blue (Nasse et al., 1993). Fig. 6(A) represents the nodules sections. Phenols are revealed by blue pigmentation in panels (b), (d) and (f). The brown pigmentation observed in images (b), (d), and (f) correspond to KMnO4 residues. Similar to dnf2 nodules, symCRK displayed accumulation of phenolics at the base of the nodules induced by both S. medicae and S. meliloti (Figs 6A (c)–(f), S6). In necrotic nodules, accumulation of phenolics was localized with necrosis. In order to investigate the expression of defense gene markers, RT-qPCR experiments were performed using RNAs extracted from wild-type, dnf2 and symCRK nodules induced by both S. medicae and S. meliloti. The expression of six defense gene markers, that is, the PR10 gene (Samac et al., 2011), chitinase (Nars et al., 2013), vegetative storage protein (VSP), polygalacturonase (PAL), β-endoglucanase (BGL; Gao et al., 2007) and nondisease resistance 1 (NDR1; Century et al., 1997), was evaluated. In the nodules of both dnf2 and symCRK, expressions of all the six defense genes tested were stronger than in the wild-type inoculated with S. medicae or S. meliloti (Figs 6B and S6, respectively). The viability of the bacteroids in the wild-type, dnf2 and symCRK nodules was also evaluated with the life-dead staining procedure that stains either green or red for living or dead bacteria, respectively (Haag et al., 2011). In agreement with the defense-like reactions observed in nodules of the two mutants, bacteroid viability was altered as compared with the wild-type. However, in contrast to dnf2 in which all bacteria were dead in zone III, symCRK zone III displayed a mixture of dead and live bacteroids (Fig. 6C).

Thus, taken together, the accumulation of defense-like phenolic compounds, the induction of defense marker genes and the
SymCRK is required to prevent defense-like reactions in nodules. (A) Wild-type (WT; a, b), dnf2 (c, d), and symCRK (e, f) Medicago truncatula nodule sections with (b, d, f) or without (a, c, e) KMNQ/methylene blue staining reveal the presence of phenolic compounds in dnf2 (d) and symCRK (f) nodules observed 21 d after inoculation (dai) with Sinorhizobium medicae WSM419 strain. Bars, 500 μm. (B) Expression of defense-related genes is induced in dnf2 (black bars) and symCRK (white bars) nodules as compared with the wild-type. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on RNAs extracted from nodules 21 dai with S. medicae WSM419. Fold change vs WT is presented after normalization with an Actin gene as an internal control. Error bars, ± SE from two independent experiments with two technical replicates for each experiment. (C) The bacteroid viability is altered in nodules of symCRK and dnf2 mutants. Bacterial viability was evaluated in WT (a, b), dnf2 (c, d) and symCRK (e, f) nodules 21 dai with S. meliloti 1021 using live/dead staining; live and dead bacteria are stained green and red, respectively. Bars: (a, c, e) 200 μm; (b, d, f) 20 μm.

A few functional studies support a role for the CRK members in plant immunity: in Arabidopsis, overexpression of either CRK5 or CRK13 enhanced resistance against P. syringae pv. tomato strain DC3000 (Chen et al., 2003; Acharya et al., 2007). Such a positive effect of CRK on plants challenged by a pathogenic organism is not a general feature of CRK as the loss of function of CRK20 in Arabidopsis also results in increased resistance to P. syringae pv. tomato strain DC3000 (Ederli et al., 2011). Similar to CRK20, transient gene silencing of HvCRK1 in barley (Hordeum vulgare) triggers an enhanced resistance against the biotrophic fungus Blumeria graminis (the causal agent of powdery mildew; Rayaparam et al., 2012). The authors suggested that HvCRK1 is involved in the negative regulation of basal resistance. In nodules, the negative effect of SymCRK on plant defense-like reactions resembles the phenotypes of HvCRK1 silencing and Arabidopsis crk20 mutant. However, sequence relationships between these CRK members do not correlate with the suggested functions. For example, At_CRK20 and At_CRK5 cluster in the same group (Figs S4, S7).

Interestingly, the DUF26 domain is not restricted to CRKs and is also present in nonkinase proteins. Amongst these is the ginkobilin -2, a protein accumulated in Ginkgo biloba seeds that displays antifungal activity (Sawano et al., 2007). The structure of ginkobilin-2 has been resolved (Miyakawa et al., 2007), but the molecular basis of its antifungal activity remains unknown. Proteins containing DUF26 domains were also associated with other plant–fungus interactions. Upon infection with the phytopathogenic fungus Magnaporthe oryzae, secreted DUF26 containing proteins are more abundant in intercellular washing fluids of partial mortality of the bacteroids suggest that, similar to DNF2, the SymCRK gene is required to repress defense-like reactions during the symbiotic process.

**Discussion**

By using genomics resources available for M. truncatula (Benedito et al., 2008; He et al., 2009; Cheng et al., 2011), we identified a gene required for nitrogen fixation, bacteroid differentiation, preventing nodule early senescence, and preventing defense-like reactions in nodules. This gene is expressed specifically during the symbiotic process and encodes a receptor-like kinase of the cystein rich superfamily. Interestingly, a close homolog of SymCRK is present in the L. japonicus genome and is also specifically expressed in nodules (Verdier et al., 2013), suggesting a common function for these genes.

Cysteine-rich kinase family members generally harbor two extracellular cystein-rich motifs (Chen, 2001) of unknown function (the DUF26 domains). Little is known about this superfamily, but in Arabidopsis, some members were identified as being up-regulated upon treatment with the defense-related phytohormone salicylic acid (SA) or after inoculation with the phytopathogenic bacteria Pseudomonas syringae pv. tomato strain DC3000 (Du & Chen, 2000). The transcriptional behavior of the complete superfamily of CRK has been studied in Arabidopsis and transcription of some members is induced upon treatment with H2O2, the plant immunity activator peptide flg22, SA, and O3 (Wrzaczek et al., 2010), thus suggesting a role for these receptors in plant immunity.

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rice (Shenton et al., 2012). All these data suggest a functional link between DUF26-containing proteins and phytopathogenic interactions. However, the role of DUF26 might not be restricted to biotic stress responses. Indeed, reduction of CRK36 expression by RNAi in Arabidopsis triggers a higher sensitivity to the stress hormone ABA and to osmotic stress during the post-germination growth phase (Tanaka et al., 2012). This suggests that the CRK family might be involved in response to both biotic and abiotic stresses.

An intriguing feature of SymCRK is the presence of a nonRD motif in the activation loop of the protein kinase domain. Association of a nonRD motif to this class of receptor kinases is specific to legumes. In plants and animals, the nonRD motif is associated with PRR (Dardick & Ronald, 2006; Dardick et al., 2012; Schwessinger & Ronald, 2012) perceiving microbial invaders and triggering pattern-triggered immunity (PTI) (Zipfel, 2008; Boller & Felix, 2009; Segonzac & Zipfel, 2011). In this context, it is interesting to note the presence of a signal peptide and the expression of SymCRK in zones II and III (Roux et al., 2014) of the nodules. These together suggest that SymCRK could be localized to the symbosome membrane so that the extracellular DUF26 domains might be in close vicinity with the bacteroids. Considering the induction of defense-like reactions in nodules of the symCRK mutant, it is thus tempting to speculate that during evolution legumes coopted defense perception elements to build a molecular mechanism preventing the elimination of the symbiont.

Influence of innate immunity during rhizobia/legume symbioses has been studied previously, notably in Lotus and Medicago. These studies indicated that both SA, the defense-related phytohormone (Stacey et al., 2006), and Flg22, a PTI elicitor (Lopez-Gomez et al., 2012), have a negative impact on the early steps of the symbiotic process. In addition, transcriptome analysis indicates that defenses can be elicited during the first step of the symbiosis, but that bacterial exopoly saccharides repress the development of the plant defenses (Jones et al., 2008). Nodulation factors, produced by rhizobia, are also able to suppress immunity triggered by microbial-associated molecular pattern on soybean as well as on nonlegume plants, such as corn, tomato and Arabidopsis (Li et al., 2013). These data, as well as other recent studies (Scheid et al., 2005; Yang et al., 2010; Okazaki et al., 2013; Rey et al., 2013), suggest a major role for immunity modulation in the successful establishment of the symbiotic process. However, these studies did not address the role of immunity in the later steps of the symbiosis (chronic infection), which is reported in this manuscript.

What triggers defense-like reactions in dnf2 and symCRK nodules remains to be elucidated. Despite similarities between the two mutants, symCRK does not have the conditional phenotype observed for dnf2. Furthermore, in the dnf2 nodules, the infected zone is smaller and the symbiotic cells are less well differentiated than in the symCRK mutant, and in addition bacteroid loss of viability is higher in the dnf2 nodules. All these differences suggest that two similar but distinct pathways can activate plant defense-like reactions in the nodule and the action of only one, repressed by DNF2, depends on the environmental conditions. The second one is repressed by SymCRK, and whether the two pathways are independent or converge should be the focus of future studies and will require the analyses of the dnf2 symCRK double mutant.

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References


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

Fig. S1 Expression profile of candidate genes.

Fig. S2 SymCRK expression is nodule-specific.

Fig. S3 SymCRK harbors atypical motifs in the DUF26 and kinase domains.

Fig. S4 SymCRK kinase domains are closer to RD-CRK kinases than to nonRD kinases.

Fig. S5 The symCRK endoreduplication index is higher than dnf2.

Fig. S6 Defense-like reactions in dnf2 and symCRK nodules induced by strain Sm2011.

Fig. S7 Phylogenetic tree of DUF26.

Table S1 Candidate genes and corresponding mutant lines

Table S2 Primers used to evaluate genes expression by RT-qPCR

Table S3 Phenotype segregation analysis of the candidate mutant lines

Table S4 Endoreduplication levels of wild-type, dnf2 and symCRK nodule cells

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