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A mutant-based analysis of the establishment of Nod-independent symbiosis in the legume Aeschynomene evenia

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Abstract
Intensive research on nitrogen-fixing symbiosis in two model legumes has uncovered the molecular mechanisms, whereby rhizobial Nod factors activate a plant symbiotic signaling pathway that controls infection and nodule organogenesis. In contrast, the so-called Nod-independent symbiosis found between Aeschynomone evenia and photosynthetic bradyrhizobia, which does not involve Nod factor recognition nor infection thread formation, is less well known. To gain knowledge on how Nod-independent symbiosis is established, we conducted a phenotypic and molecular characterization of A. evenia lines carrying mutations in different nodulation genes. Besides investigating the effect of the mutations on rhizobial symbiosis, we examined their consequences on mycorrhizal symbiosis and in nonsymbiotic conditions. Analyzing allelic mutant series for AePOLLUX, Ca2+/calmodulin dependent kinase, AeCYCLOPS, nodulation signaling pathway 2 (AeNSP2), and nodule inception demonstrated that these genes intervene at several stages of intercellular infection and during bacterial accommodation. We provide evidence that AeNSP2 has an additional nitrogen-dependent regulatory function in the formation of axillary root hairs at lateral root bases, which are rhizobial-colonized infection sites. Our investigation of the recently discovered symbiotic actor cysteine-rich receptor-like kinase specified that it is not involved in mycorrhization; however, it is essential for both symbiotic signaling and early infection during nodulation. These findings provide important insights on the modus operandi of Nod-independent symbiosis and contribute to the general understanding of how rhizobial–legume symbioses are established by complementing the information acquired in model legumes.
**Introduction**

Plants have developed different strategies to cope with nutrient deprivation, notably by adapting their development and metabolism. Another ingenious solution is to establish symbiotic interactions with soil-borne microorganisms to enhance nutrient uptake. At least 80% of land plants are capable of forming a symbiosis with Glomeromycota fungi called arbuscular mycorrhiza. In this symbiosis, fungal hyphae penetrate plant roots and form arbuscules inside cortical cells. These symbiotic structures are the interface for the uptake of inorganic phosphorus (P) and other micronutrients used for plant nutrition. A limited number of flowering plants are also able to establish symbiosis with diazotrophic bacteria. Among these nitrogen-fixing symbioses, most members of the legume family (Fabaceae) interact with bacteria collectively known as rhizobia. These latter are hosted in a root-derived symbiotic organ, the nodule, where rhizobia reduce atmospheric nitrogen into ammonium to the plants’ benefit.

The rhizobium–legume symbiosis plays a prominent role in global biological nitrogen fixation, and legume crops are a major protein source for human and animal diets. Given their importance, intense research has been conducted on nodulation in legumes. Notably, forward genetic screens in two model legumes, barrel medic (Medicago truncatula) and Lotus japonicus, have identified numerous genes involved in the establishment of nodulation (Roy et al., 2020). It is now common knowledge that rhizobia produce lipochitooligosaccharidic Nod factors, which are perceived by plasma membrane-localized LysM-RLK receptors that act upstream of a Nod signaling pathway leading to the activation of a network of transcription factors in the cell nucleus. These latter coordinate the expression of genes involved in intracellular rhizobial infection via root hair infection threads, nodule organogenesis, and subsequent bacterial accommodation into nodules to fix nitrogen (Roy et al., 2020). These studies also provided visions on how nodulation could have evolved. First, several nodulation genes have also a nonsymbiotic function, suggesting that existing gene functions have been subsequently recruited for symbiosis (Liu et al., 2011). Second, many nodulation genes involved in signaling and infection appear to have been co-opted from roles in the more ancient mycorrhizal symbiosis (Gobbato, 2015; Radhakrishnan et al., 2020).

Research on M. truncatula and L. japonicus has boosted our understanding of nodulation mechanisms, but it is now also crystal clear that this knowledge is very model biased. The legume family is huge (approximately 20,000 species) and manifests a diversity of nodulation features (Masson-Bovin et al., 2009). In this respect, several Aeschynomene species are noticeable because they establish a symbiotic interaction with photosynthetic Bradyrhizobium strains without Nod factor recognition and infection thread formation (Giraud et al., 2007; Bonaldi et al., 2011). Whereas this so-called Nod-independent activation is singular in the legume family, rhizobial infection via intercellular penetration is observed in 25% of the genera (Ibáñez et al., 2017). In Nod-independent Aeschynomene species, bradyrhizobia first massively colonize axillary root hairs present at the base of lateral roots and then progress intercellularly in the root cortex. A few cortical cells are subsequently infected and divide repeatedly to give rise to the nodule (Bonaldi et al., 2011). This infection mode is proposed to be mechanistically simpler than the one involving infection thread formation.

Deciphering the molecular basis of the Nod-independent symbiosis is expected to provide critical insights into the evolution and diversity of nodulation (Quilbé and Arrighi, 2021). To identify genes important for this distinctive symbiosis, the genome of Aeschynomene evenia was recently sequenced and a forward genetic approach was conducted on this species (Quilbé et al., 2021). Approximately 70,000 ethyl methanesulfonate (EMS)-mutagenized seedlings were screened for defects in nodulation. Sequencing of nodulation mutant plants led to the identification of several symbiotic genes, AePOLLUX, Ca2+/calmodulin dependent kinase (AeCCaMK), AeCYCLOPS, nodulation signaling pathway 2 (AeNSP2), and nodule inception (AeNIN), coding for known components of the Nod signaling pathway in model legumes, that acts downstream of the Nod factor receptors. The isolation of these A. evenia mutants provide the opportunity to explore the roles of these conserved signaling genes in a symbiotic context that differs from the well-studied model legumes. This mutant-based approach also led to the discovery of a symbiotic gene, AeCRK, encoding a cysteine-rich receptor-like kinase (CRK) (Quilbé et al., 2021). Interestingly, AeCRK putative orthologs are ancestral in legumes but absent in model legumes. Although AeCRK is required to trigger nodulation in A. evenia, its precise role is not yet known.

In this study, we aimed to better understand the roles of AePOLLUX, AeCCaMK, AeCYCLOPS, AeNSP2, AeNIN, and AeCRK in the establishment of the Nod-independent symbiosis. For this purpose, we undertook a detailed phenotypic and molecular characterization of allelic nodulation mutants altered in these genes in A. evenia. We found that the symbiotic signaling pathway intervenes at several steps of the establishment of the Nod-independent symbiosis and observed that, AeNSP2 has an additional nonsymbiotic function to control the formation of axillary root hairs. We also obtained indications on how AeCRK can mediate activation of nodulation and evidence that, unlike most genes of the Nod signaling pathway, this gene probably does not intervene in arbuscular mycorrhization.

**Results**

**Defining series of allelic mutants for conserved signaling genes and the AeCRK gene**

To uncover the molecular mechanisms underpinning the Nod-independent symbiosis, an EMS-mutant-based approach was recently conducted in A. evenia. It led to the identification, in isolated nodulation mutants, of causal
mutations for five genes of the Nod signaling pathway and the discovery of a symbiotic gene, AeCRK (Supplemental Table S1; Quilbé et al., 2021). To identify additional mutant alleles or genes, we conducted a mapping-by-sequencing approach on yet uncharacterized nodulation mutants. This approach applied to the mutant A21 by sequencing pooled DNAs from 140 F2 mutant plants, originating from a wild-type (WT) × A21 crossing, was successful in detecting a genetic linkage on chromosome Ae07 with a mutant allelic frequency reaching 98% at the AeNIN locus (Supplemental Figure S1). Another mutant, E26, was previously identified by a Targeted Sequence Capture as also being a candidate genotype, and the genotyping of 20 F2 backcrossed E26 mono- and recessive determinism for its symbiotic phenotype (Quilbé et al., 2021). We show that the mutant E26 has a genetic linkage on chromosome Ae07 with a mutant allelic type (WT).

All four nsp2 mutants were renamed according to the gene name and the allele can have different effects on a gene due to complete or partial loss of function. For clarity, the corresponding mutants were renamed according to the gene name and the allele number (Supplemental Table S1).

**nsp2 mutants lack nitrogen-controlled axillary root hairs normally colonized by bradyrhizobia**

While all the A. evenia mutants were primarily screened for defect in nodulation, an unexpected observation was that all four nsp2 mutants were completely devoid of axillary root hair crowns that normally surround lateral root bases (Figure 1A). These axillary root hairs are swollen structures covered with a thick mucilage and forming an orangecolored tuft. When present, bradyrhizobia intensively colonize the surface prior to inner root infection (Arrighi et al., 2012). In contrast to nsp2 mutants, lines altered in other genes of the Nod signaling pathway, AePOLLUX, AeCCaMK, AeCYCLOPS, AeNIN, as well as AeCRK, have a normal development of axillary root hairs (Figure 1A). A closer examination of calcofluor-stained sections of lateral root bases by confocal microscopy showed similar tufts of thick-walled axillary root hairs in the WT and the ccamk-3 mutant while in the nsp2-3 mutant the lateral root bases showed a continuous root epidermis and no bulge-like structures were visible (Figure 1B).

Since nitrogen is known to negatively regulate the rhizobial symbiosis and axillary root hairs constitute the initial infection sites, we analyzed the potential effect of nitrogen on their development by supplying different KNO3 concentrations to the plant growth medium. Observations at 10-day postgermination revealed that, for the WT line and the ccamk mutant, axillary root hair crowns were easily visible with a treatment of 0- or 0.5-mM KNO3. However, a clear reduction of axillary root hair development was apparent upon the addition of 5-mM KNO3 (Figure 1C). In that case, only a discrete orange coloration was observable at the base of lateral roots. In contrast, no nitrogen effect was observed on the nsp2 mutant, this latter is devoid of axillary root hairs (Figure 1C). We further explored the unusual phenotype observed in the nsp2 mutants by assessing root hair development and the root system architecture. The four nsp2 mutants produced root hairs with a similar tubular shape and with the same length as observed in WT plants and the ccamk-3 mutant globally (Figure 1D; Supplemental Figure S2A). In contrast, significant variations in the primary root length, lateral root length, and number were found relative to the WT line (Supplemental Figure S2, B–D). However, these variations were opposed among the four nsp2 mutants, suggesting that these variations are mutant-dependent rather than linked to AeNSP2. These observations reveal two features on the axillary root hairs present in A. evenia: (1) AeNSP2 is important for their development and (2) nitrate has a negative effect on their formation.

**nsp2 mutants show reduced expression of nitrogen-dependent genes**

The potential link between AeNSP2 and nitrogen homeostasis is supported by data from the A. evenia Gene Atlas that show that AeNSP2 is expressed in roots but that this expression is repressed by the addition of nitrogen and decreases during nodulation (Quilbé et al., 2021). To investigate this singular expression pattern, we searched for genes co-expressed with AeNSP2 and identified approximately 60 genes with a similar expression pattern. After an analysis of their putative biological functions, we retained a list of nine candidates with a potential role in symbiosis, root hair growth, or nitrogen homeostasis (Supplemental Table S4). In addition to AeNSP2, two other genes that we named as AeERN1 and AeERN3 encode predicted RERF required for nodulation (ERN) transcription factors, and they are the putative orthologs of the symbiotic genes MtERN1 and MtERN3 in M. truncatula (Andriankaja et al., 2007; Cerri et al., 2012). AeCDD7 encodes a putative carotenoid cleavage dioxygenase 7 and likely corresponds to MtCDD7 in M. truncatula, a gene previously shown to be involved in strigolactone biosynthesis and in rhizobium symbiosis (van Zeijl et al., 2015). AeAMT3 is predicted to encode an ammonium transporter, while AeHHP encodes a putative histidine phosphotransfer protein involved in cytokinin signaling. Two genes may be involved in cell wall modifications, AeEXP (expansin) and AePL (pectate lyase). Lastly, root hair defective 6-like 2 is predicted to encode a bHLH transcription factor that best matches with AtrSL2 and AtrSL4, both required for root hair growth in Arabidopsis thaliana.
To experimentally verify that the nonsymbiotic expression of these genes is regulated by nitrogen and to test their dependency on AeNSP2, we assessed their expression by reverse transcription–quantitative PCR (RT–qPCR) under different nitrogen conditions (0-, 0.5-, and 5-mM KNO₃) and in three genetic backgrounds (WT, ccamK-3, and nsp2-3 mutants). In WT roots, expression of all nine genes was maximal in the absence of nitrogen (0-mM KNO₃) and expression levels consistently decreased with increasing amounts of nitrogen. This negative relationship indicates that nitrogen has a repressive effect on the expression of these genes. A similar expression pattern was observed in the ccamK-3 mutant roots, indicating that AeCCaMK does not participate in the repression of these genes (Figure 2). In contrast, under the same nitrogen treatments, gene expressions in the nsp2-3 mutant roots of all tested genes were always lower than that in the WT line, and statistically significant for the 0- and 0.5-mM KNO₃ conditions (Figure 2). These results suggest that AeNSP2 is implicated, directly or indirectly, in the activation of expression of the nine identified genes.
The symbiotic mutants show blocks at several steps of nodule development

Next, we investigated the involvement of AePOLLUX, AeCCaMK, AeCYCLOPS, AeNSP2, AeNIN, and AeCRK in rhizobial symbiosis. For this purpose, we analyzed the nodulation kinetics of the six allelic mutant series after inoculation with Bradyrhizobium strain ORS278 over a time frame of 21 days. In all experiments, the WT line readily exhibited dark pink nodules upon inoculation and had well-developed green leaves. In contrast, all inoculated nodulation mutants showed symptoms of nitrogen starvation, having yellowish leaves, and a stunted growth habit at the end of the experiments (Supplemental Figures S3–S8). In the pollux series, pollux-2, pollux-3, and pollux-4 mutants did not form nodules after inoculation. However, pollux-6 produced a few bumps, and pollux-1 and pollux-5 formed nodules, albeit with consistently different frequencies (Figure 3A). For the four mutants of the ccamk series, the plants did not form nodule structures after inoculation while, in the cyclops series, the two mutants displayed different phenotypes, cyclops-2 being devoid of any nodule-like structure and cyclops-1 developing nodules at low frequency (Figure 3B).

In the nin series, most mutants were completely nonnodulating; however, nin-1 had a less severe phenotype since it was able to develop nodules (Figure 3C). In the nsp2 series, the phenotype was found to be homogeneous, with all mutants having noduleless roots (Figure 3D). Finally, in the crk series, the two mutants displayed an apparent null phenotype; however, microscopic examination at 21-day postinoculation (dpi) revealed the presence of a few very small bumps on their roots (Figure 3E). For nodule-producing mutants, the nodules tended to be smaller and paler than those of the WT plants, and acetylene reduction assays confirmed that the nodules had no (nin-1) or a weak (pollux-1, pollux-5, and cyclops-1) nitrogenase enzyme activity (Supplemental Figures S3, S5, and S7).

The obtained data show that three main symbiotic phenotypes can be distinguished within the isolated nodulation mutants: Nod⁺ (no nodule at all), Bump⁺ (limited nodule development), and Fix⁻ (ineffective nodules). Intriguingly, while the Nod⁻ phenotype was found in all allelic series for the conserved signaling genes, the two crk mutants have a
similar Bump+ phenotype. Therefore, we questioned the impact of these mutations on AeCRK function. First, to assess the predicted altered splicing in the \( \text{crk-1} \) mutant, we amplified AeCRK cDNAs in the WT and the \( \text{crk-1} \) mutant backgrounds (Supplemental Figure S9). While a single band of the expected size was readily obtained for the WT, three bands were observed in the \( \text{crk-1} \) mutant. This indicates that the \( \text{crk-1} \) mutation affects the splicing of AeCRK.

Figure 3 Nodulation kinetics of the nodulation mutants of \( A. \) evenia inoculated with \( Bradyrhizobium \) ORS278 and comparison with the WT line. A, \( \text{pollux} \) mutants. B, \( \text{ccamk} \) and \( \text{cyclops} \) mutants. C, \( \text{nin} \) mutants. D, \( \text{nsp2} \) mutants. E, \( \text{crk} \) mutants. Observations were performed at 4, 7, 10, and 14 dpi. Means and SD were derived from the phenotyping of 20 plants/line.
bands were recovered for crk-1. Their sequencing revealed variations that are predicted to lead to abnormal forms of AeCRK. Second, crk-2 has a G354E mutation falling in the first Gly of the highly conserved Gly-rich loop (GXGXXG), which is involved in ATP binding and essential for kinase activity (Quilbé et al., 2021). This observation prompted us to assay the kinase autophosphorylation activity, using anti-phospho-Thr antibodies, of the WT and mutated (G354E) forms of the AeCRK kinase domain produced in Escherichia coli and purified as GST-tagged proteins. In contrast to the two dead-kinase proteins used as control, GST-CRKinG354E phosphorylation was detectable, but was about 80-fold less than for the WT AeCRK kinase protein (Supplemental Figure S10). These results show that AeCRK has an active kinase domain and that the G354E mutated form present in the crk-2 mutant retains only residual kinase activity. Therefore, both crk-1 and crk-2 mutations can be considered as having a strong impact on AeCRK function.

The symbiotic mutants are differently altered in rhizobial infection

To analyze further the nodulation phenotypes evidenced with the Bradyrhizobium ORS278 strain, we focused within each allelic mutant series on mutants that had a clear difference in symbiotic phenotype. First, we determined whether these plant phenotypes were dependent on the bradyrhizobial strain used for inoculation. For this, the symbiotic phenotype of selected mutant lines at 21 dpi with Bradyrhizobium strain ORS285, which is also compatible but differs in its infectivity, was determined (Supplemental Figures S11–S16). Subsequently, we analyzed for these mutants the bacterial infection and nodule development using GUS-tagged versions of either ORS278 or ORS285.

The results show that plants from the mutant lines, pollux-2, ccamk-2, cyclops-2, nsp2-2, and nin-3, had also a Nod- phenotype when inoculated with the ORS285 strain. Macro and microscopic analysis of whole and sectioned roots evidenced again the absence of axillary root hairs in the nsp2-2 mutant, and no bacteria were detected on the root surface of this mutant after X-Gluc staining (Figure 4A). In contrast, the remaining mutants developed orange axillary root hair rosettes. They tended to be darker on roots from inoculated plants than on noninoculated roots, and X-Gluc staining revealed that they were colonized by bradyrhizobia (Figure 4A). A closer examination revealed that on pollux-2, ccamk-2, and nin-3 mutants, bacterial colonization was restricted to or in between axillary root hairs. The cyclops-2 mutant differed by displaying either a few inner infection spots or brownish areas, when inoculated with the strain ORS278-GUS or ORS285-GUS, respectively (Figure 4B). The mutant lines pollux-6 and crk-1, that developed bumps with the ORS278 strain, formed more discrete and infrequent bumps when inoculated with the ORS285 strain. Unlike the typical WT lobe-shaped bumps, these bumps appeared to be circular swellings, suggesting homogeneous but limited cell divisions all around the base of the lateral roots (Figure 4B). In sectioned mutant bumps, X-Gluc-stained ORS278 bacteria were contained in infection pockets, suggesting early aborted infection (Figure 4B). When the pollux-6 and crk-1 mutants were inoculated with the ORS285-GUS strain, the axillary root hair crowns were noticeably characterized by punctate darker zones, linked with underlying bacterial infection spots and brownish coloration that might correspond to defense reactions (Figure 4B). Thus, although ORS285 induced less cell divisions than ORS278, a similar premature aborted infection was observed.

Among the three mutants forming Fix- nodules with ORS278 (pollux-1, nin-1 and cyclops-1) only the cyclops-1 mutant was Nod- after inoculation with the strain ORS285 (Figure 5A). The pollux-1 mutant developed two types of nodules with the ORS285 strain. The majority of the nodules was small, round in shape, and contained a brownish spot while a small number of nodules were better developed and had a pink color. However, infection of the central tissue was noticeably patchy (Figure 5A). In contrast, whereas ORS278-induced nodules on the cyclops-1 mutant had a pink color, they were homogeneously infected, similarly to WT plants (Figure 5A). Finally, after inoculation with ORS285, the nin-1 mutant had the most severe phenotype, forming only white small rounded nodules. Sections of these nodules showed a limited bradyrhizobial infection and the presence of brown phenolic compounds (Figure 5A). To investigate the bacterial differentiation in nodules of the Fix- mutants, we used the nucleic acid-binding dye SYTO13 and observed the bacterial morphology by confocal microscopy (Figure 5B). Mature nodules of the WT plants contained typical spherical bacteroids and a similar morphology was observed for bacteria in the nodules of the pollux-1 mutant, suggesting that they are differentiated. In contrast, nodules of the cyclops-1 mutant contained bacteria with abnormal shape and size, showing that the process of bacteroid differentiation was altered. Finally, nodules of the nin-1 mutant contained only elongated bacteria, pointing to a failure of bacterial differentiation. These also showed a strong autofluorescence that is related to the brown pigmentation described above (Figure 5A). These confocal observations of bacterial infection and differentiation were in line correlated with the very low nitrogenase enzyme activity of pollux-1 and cyclops-1 nodules and its complete absence in nin-1 nodules with the ORS278 or ORS285 strain (Supplemental Figures S3, S5, S7, S11, S13, and S15).

The symbiotic genes are required for normal expression of early nodulin genes

Nodule organogenesis and bacterial infection are accompanied with the induction of numerous genes qualified as nodulins. To examine the effect of mutations in symbiotic genes on nodulin transcription, we selected a set of six genes whose expression is induced during nodulation, according to the A. evena Gene Atlas (Supplemental Table S4) (Quilbé et al., 2021). Four of them—AeNIN, symbiotic remorin (AeSymREM1), vapyrin (AeVPY), and AeENOD40—are
putative orthologs of genes with a well-described symbiotic function in model legumes. \textit{NIN} is a central regulator of nodulation (Liu et al., 2019). \textit{SymREM1} and \textit{VPY} are important for rhizobial infection (Lefebvre et al., 2010; Murray et al., 2011). \textit{Early nodulin 40} (\textit{ENOD40}) is required for the induction of cortical cell divisions (Charon et al., 1999). The two remaining nodulin genes, \textit{AeSBT} (subtilase) and \textit{AeCRK}, were selected based on transcriptomic and genetic studies on \textit{A. evenia} symbiosis (Quilbe et al., 2021; Gully et al., 2018).

We monitored the expression of these rhizobia-induced genes on mutants of \textit{AePOLLUX}, \textit{AeCCaMK}, \textit{AeCYCLOPS}, \textit{AeNSP2}, \textit{AeNIN}, and \textit{AeCRK}, and the WT line over a period of 7 days following inoculation with \textit{Bradyrhizobium} strain ORS278. RT–qPCR showed a clear induction of the analyzed nodulin genes at 2 or 4 dpi and still increasing at 7 dpi in the WT (Figure 6). In contrast, induction of expression of \textit{AeSymREM1}, \textit{AeVPY}, \textit{AeSBT}, and \textit{AeCRK} was totally abolished in all six tested mutant lines (Figure 6). For \textit{AeNIN}, induction of expression was severely reduced at 7 dpi in the \textit{nin-6} mutant background, and this induction was completely impaired in the other symbiotic mutants (Figure 6). For \textit{AeENOD40}, its level of expression remained unchanged after inoculation in the \textit{pollux-2}, \textit{ccamk-3}, \textit{nsp2-3}, and \textit{nin-6} mutants, whereas a low and transitory induction was visible for the \textit{cyclops-1} and \textit{crk-1} mutants. The latter is

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Bradyrhizobial infection in Nod\textsuperscript{−} and Bump\textsuperscript{+} mutants. A, Mutant lines showing only bacterial colonization of the axillary root hairs. B, Mutants showing inner root infection. Whole roots (upper) and 70-\textmu m-thick root sections before and/or after X-Gluc staining (middle and lower) of control WT (uninoculated) and mutant lines inoculated with either the ORS278-GUS or ORS285-GUS strain. Roots were harvested at 21 dpi. Arrows indicate inner infection spots. Bars represent 500 \textmu m (upper) or 50 \textmu m (middle and lower).}
\end{figure}
consistent with the absence of a null phenotype as observed for these mutants (Figure 6). The above results indicate that, as described in model legumes, nodulin gene expression is dependent on the symbiotic signaling pathway in *A. evenia* and that AeCRK is also required to assure normal symbiotic induction of the tested nodulin genes.

**Certain nodulation mutants are defective for arbuscular mycorrhizal symbiosis**

In model legumes, many genes of the Nod signaling pathway or involved in rhizobial infection have been shown to play a role in arbuscular mycorrhizal symbiosis. However, in the view of the semi-aquatic habit of *A. evenia*, an environment...
Expression of early nodulation genes in the WT and the mutants pollux, ccamk, cyclops, nsp2, nin, and crk in A. evenia. Expression values were normalized to AeEF1a and Ubiquitin levels. Means and SD were derived from three biological replicates.

unfavorable for mycorrhizal symbiosis, and the absence of literature data, it is not known if this species is able to establish such a fungal symbiosis. Therefore, we first assessed the ability of A. evenia WT to interact symbiotically with *Rhizophagus irregularis* under aeroponic conditions. Eight weeks after inoculation, typical fungal structures could be observed on the roots (Figure 7A). The presence of both extra- and intra-cellular hyphae was indicative of effective root infection. In addition, inner cortical cells with well-developed arbuscules and vesicles suggested that this symbiosis is fully developed. Next, we questioned whether AePOLLUX, AeCCaMK, AeCYCLOPS, AeNSP2, AeNIN, or AeCRK are important for mycorrhization in *A. evenia*, by evaluating the mycorrhizal phenotype of one strong allele mutant for each signaling gene and the two allelic mutants available for AeCRK. Microscopic analysis of the fungi-inoculated roots revealed that the *nsp2-3*, *nin-6*, *crk-1*, and *crk-2* mutants are mycorrhized, whereas the *pollux-2*, *ccamk-3*, and *cyclops-2* mutants showed almost no mycorrhizal colonization (Figure 7B). When fungal colonization was quantified as the mycorrhization frequency, there was no difference between the *nsp2-3*, *nin-6*, and *crk-2* mutants and the WT line (Figure 7C). Although the mycorrhization intensity was more variable between the *nsp2-3*, *nin-6*, *crk-1*, and *crk-2* mutants and the WT line, these variations were not significantly different (Figure 7D). Thus, these phenotypic observations support a major role of AePOLLUX, AeCCaMK, and AeCYCLOPS for the mycorrhizal symbiosis in *A. evenia*.

To further characterize the mycorrhizal symbiosis in *A. evenia*, we developed seven plant mycorrhization markers by identifying putative orthologs of mycorrhiza-induced genes described in *M. truncatula* (Supplemental Table S5) (Takeda et al., 2011; Park et al., 2015; Gibelin-Viala et al., 2019). Among them, reduced arbuscular mycorrhizal 1 (*AeRAM1*) and required for arbuscule development (*AeRAD1*) are involved in mycorrhizal signaling. AeVPY, exocyst 70I (*AeEXO70I*), stunted arbuscule (*AeSTR*), and arbuscular mycorrhiza-induced subtilase 1 (*AeSBT1*) are markers for mycorrhizal infection and arbuscule development, while phosphate transporter 4 (*AePT4*) is involved in arbuscule functioning. In addition to plant markers, two mycorrhiza-specific genes, 25S-large ribosomal subunit (*RiLSU*) and glyceraldehyde 3-phosphate dehydrogenase (*RiGADPH*), were selected (Xue et al., 2015; Buendia et al., 2016). All seven plant genes were checked to be effectively induced during mycorrhization and the two mycorrhiza-specific genes to enable quantification of the fungal colonization of the roots (Supplemental Figure S17). Next, we measured the expression levels of these selected mycorrhiza marker genes by RT–qPCR in WT and mutant plant roots. Although variability could be found between the WT line and the mycorrhized *nsp2-3*, *nin-6*, *crk-1*, and *crk-2* mutants, in most cases, these differences were statistically not significant (Figure 8). In contrast, consistent with the observed absence of fungal colonization in the roots of the *pollux-2*, *ccamk-3*, and *cyclops-2* mutants, fungal genes were hardly detected, and plant marker genes were not induced or showed a very much reduced expression level (Figure 8).

Exploiting the observation that both AeCRK and AeNIN genes are induced during nodulation, we investigated if such upregulation of expression also occurs during mycorrhization, as is the case for AeVPY (Figures 6 and 8). For this purpose, a
comparative analysis of WT plant roots, inoculated with *Bradyrhizobium* ORS278 (nodulation) or *R. irregularis* (mycorrhization), was conducted by RT–qPCR. Concordant with other transcriptomic data, an increase in *AeCRK* and *AeNIN* expression was observed in nodulated roots relative to control roots (Figure 9A). In contrast, no significant difference in expression was detected between mycorrhized roots and noninoculated roots (Figure 9A). We also compared *AeCRK* and *AeNIN* expression during the two symbioses in the WT line and the completely symbiosis-deficient *ccamk*-3 mutant. While in rhizobial symbiosis their expression levels were consistently higher in WT roots as compared to *ccamk*-3 roots, no such differences were observed during mycorrhizal symbiosis (Figure 9B). Thus, the absence of both a mycorrhizal phenotype and the induction of symbiotic gene expression during symbiosis with *R. irregularis* suggest that *AeCRK* and *AeNIN* are not involved in mycorrhization in *A. evenia.*

**Figure 7** Mycorrhizal phenotypes of the WT and the mutants *pollux, ccamk, cyclops, nsp2, nin,* and *crk* in *A. evenia.* Plant roots of the different lines cultivated for 8 weeks and inoculated with *R. irregularis* were stained with ink for microscopy analysis and quantification of fungal colonization according to the Mycocalc method. A. Typical fungal structures observed in the WT roots with hyphae (h), intraradical hyphae (ih), arbuscules (a), and vesicles (v). B. Representative fungal colonization patterns observed in the different mutant lines. C and D. Box plots represent the mycorrhization frequency and intensity, respectively, both expressed in percentage in the WT and mutant lines. The central rectangle spans the first quartile to the third quartile; the bold segment inside the rectangle shows the median; and the whiskers above and below the box show the locations of the maximum and minimum value, respectively. Outliers are represented by dots. Data are from three biological repeats, each with five plants/line. Statistical analyses were performed using Student’s *t* test (*P* < 0.05, ***P* < 0.001, ****P < 0.0001) by comparing the mutant lines to the WT. Bars = 100 μm (A) and 50 μm (B).

**Discussion**

In this study, we performed a phenotypic and molecular analysis on a series of allelic mutants for the genes *AePOLLUX, AeCCaMK, AeCYCLOPS, AeNIN,* and *AeCRK.* This allowed us to specify the modalities of the establishment of the Nod-independent symbiosis in *A. evenia* and it provided insights into the biological functions that these genes fulfill (Figure 10). Here, we discuss three main features of these gene involvements in *A. evenia* and how this information contributes to a better understanding of the mechanisms underpinning the establishment of symbioses in legumes.

**Involvement of the symbiotic signaling pathway in intercellular rhizobial infection and mycorrhization**

Knowledge acquired on rhizobial and mycorrhizal symbioses in model legumes facilitates research in other legume species
that have alternative symbiotic processes. So far, variations to the major studied nodulation mechanisms have been best investigated in two robinoid legumes, *Sesbania rostrata* and *L. japonicus*, which exhibit dual rhizobial infection pathways (partially intercellular infection and infection thread formation) (Capoen et al., 2010; Montiel et al., 2021). The Nod signaling pathway was recently shown to be equally important for both infection routes in *L. japonicus* (Montiel et al., 2021). 

*Arachis hypogaea* (peanut) and *A. evenia*, belonging to the Dalbergioid clade, are two examples of legumes that only use an intercellular infection process. Genetic studies showed that several genes of the Nod signaling pathway are conserved and important for symbiosis in both these legumes (Fabre et al., 2015; Sharma et al. 2020; Quilbé et al., 2021; Peng et al., 2021). Here, we specified the involvement of symbiotic signaling genes of *A. evenia* in intercellular infection. We showed that strong mutants for the *AePOLLUX*, *AeCCaMK*, *AeNSP2*, and *AeNIN* genes were characterized by a Nod\(^-\) phenotype, where the bradyrhizobia remain at the surface of axillary root hairs. Lack of bacterial penetration resembles the phenotype repeatedly described for mutants of the Nod signaling pathway in model legumes and indicates that this pathway also intervenes in the initial step of the symbiotic interaction in *A. evenia*. Besides a complete Nod\(^-\) phenotype, we evidenced among the allelic series of mutants of *AePOLLUX*, *AeCYCLOPS*, and *AeNIN* genes a spectrum of nodulation phenotypes. The broadest gradient was obtained for *AePOLLUX*, with the *pollux-1* and *pollux-6* mutants that are classified as Bump\(^+\) or Fix\(^-\). In these mutants, infection was impaired with abortive infection pockets in mutant bumps and scattered infection present in mutant nodules. A somewhat similar picture was obtained for *AeNIN*, for which the *nin-1* mutant is able to develop nodules but with a severely impaired bacterial infection and differentiation. This phenotyped the few weak *nin* phenotypes observed in *M. truncatula* and supported the recent evidence that NIN is important not only for nodule inception but also for transition to nitrogen fixation (Liu et al., 2021; Feng et al., 2021). Interestingly, both mutants for *AeCYCLOPS* were completely Nod\(^-\) with *Bradyrhizobium*
strain ORS285, while two distinct phenotypes were observed with Bradyrhizobium strain ORS278. The strong phenotype, found in cyclops-2, equated to infection pockets that were not associated with bump formation and the weaker phenotype, observed in cyclops-1, corresponded to a few nodules that contained bacteria altered in their accommodation. This situation is reminiscent of the ones described for the ipd3-2/ipd3l double mutant and the ipd3-2 mutant of M. truncatula, respectively (Horváth et al., 2011; Jin et al., 2018). All these observations highlighted, despite the important differences between intercellular and intracellular infections, a similar involvement of the Nod signaling pathway all throughout the process of rhizobial invasion and symbiosome formation (Figure 10). Aeschynomene evenia also provided a good opportunity to assess an involvement in mycorrhiza formation of symbiotic signaling genes. Mutant phenotyping indicated that mutations in AePOLLUX, AeCCaMK, and AeCYCLOPS drastically affected root colonization by R. irregularis, in contrast to AeNSP2 and AeNIN. In M. truncatula, NSP2 was found to facilitate mycorrhizal root colonization, although it is not essential to trigger mycorrhizal symbiosis (Maitillet et al., 2011). In contrast, MtNIN was demonstrated to be not required and not induced during mycorrhization (Kumar et al., 2021). We found that AeNIN was not induced either during mycorrhization. Therefore, conserved signaling genes in A. evenia appear to be similarly involved or not in mycorrhization as in model legumes (Figure 10) (Gobbato, 2015; Kumar et al., 2021).

**AeNSP2 has functions in symbiotic signaling and the control of rhizobia-colonized axillary root hair development**

In the Nod signaling pathway, NSP2 is a key transcriptional regulator that regulates the expression of symbiotic genes (Quilbé and Arrighi, 2021). In A. evenia, phenotypic and molecular data obtained for the nsp2 mutants suggest that this function is conserved (Figure 10). However, AeNSP2 has a singular expression profile, being repressed in roots by nitrogen and during nodulation (Quilbé et al., 2021). This negative regulation was not restricted to AeNSP2 and actually involved a small set of genes. We showed for nine of these genes that their expression was not only dependent on nitrogen but also on AeNSP2, suggesting that they are directly or indirectly activated by AeNSP2. A nonsymbiotic function for NSP2 was previously reported in M. truncatula by Liu et al. (2011). In this legume, NSP2 is important for the expression of the DWARF27 gene that intervenes in the carotenoid and strigolactone biosynthetic pathway. Here we show that in A. evenia, another gene of this biosynthetic pathway, CCD7, is expressed in roots in an NSP2-dependent manner. Therefore, it is likely no coincidence that the DWARF27 and CCD7 genes appear to be co-expressed during nodulation in M. truncatula, suggesting that they have been conjointly co-opted in rhizobium symbiosis (van Zeijl et al., 2015). Additionally, we show that NSP2 is required for the expression of two genes of the symbiotic signaling pathway, AeERN1 and AeERN3. In M. truncatula, ERN1 and ERN2

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**Figure 9** Comparative expression of AeCRK and AeNIN during nodulation and mycorrhization. A, Gene expression levels in the WT line either noninoculated (NI) or inoculated (I) with the symbiont. B, Gene expression in the WT and the ccamk mutant inoculated with the symbiont. B, Gene expression in the WT and the ccamk mutant inoculated with the symbiont. Expression levels were determined at 7 dpi with Bradyrhizobium ORS278 and at 8-week postinoculation with R. irregularis by RT-qPCR analysis. Expression values were normalized to AeEF1a and Ubiquitin levels. Means and SD were derived from four (A) and seven (B) biological replicates. Asterisks indicate significant differences ( \( P < 0.05 \), Student’s t test) between the control and the other samples.
AeCRK is important for symbiotic signaling and early rhizobial infection but not mycorrhization

A distinct component used by A. evenia to trigger nodulation is the AeCRK gene that belongs to the large plant CRK gene family in which many members have been associated with reactive oxygen species (ROS) sensing (Quilbé et al., 2021; Bourdais et al., 2015). To assess AeCRK roles, we investigated its involvement in mycorrhiza formation. Mutant phenotyping indicated that mutations in AeCRK do not affect root colonization by R. irregularis. Furthermore, AeCRK was shown to be strongly induced during nodulation but not during mycorrhization. This analysis showed that, similar to AeNIN, AeCRK symbiotic function seems to be specific to nodulation (Figure 10) (Kumar et al., 2021). When inoculated with Bradyrhizobium strain ORS278, crk mutants developed swellings that circled lateral roots and which often contained an infection pocket. These circular bumps were reminiscent of those spontaneously induced in A. evenia lines expressing deregulated forms of AeCCaMK (Fabre et al., 2015). This suggests that, in crk mutants, cortical cell divisions occur to form a bump, but that a positional information of the bacteria is missing to form a nodule. The infection pockets located in the outer cortical cell layers indicated an early block in the infection process. Interestingly, in WT A. evenia, the first infected cortical cells have been described to collapse, while underneath infected cells remain intact and divide to give rise to the nodule primordium (Bonaldi et al., 2011; Arrighi et al., 2012). Similarly, in S. rostrata, intercellular colonization at lateral root base is associated with local cortical cell death and ROS production (D’Haese et al., 2003). These data provide directions to further investigate the potential link between AeCRK, ROS, and early infection. However, at present, it is not known whether the phenotype observed in crk mutants reflects the first stage where AeCRK intervenes in the symbiotic pathway. Indeed, it cannot be excluded that the phenotype is due to mutations in the ck-1 and ck-2 mutants that do not completely inhibit CRK function or is caused by a functional...
redundancy with another member of the CRK cluster to which AeCRK belongs (Quilbé et al., 2021). The latter has for instance been observed for the symbiotic receptor gene LYK3 within the LYK cluster in M. truncatula (Limpens et al., 2003). In addition, the expression of AeCRK throughout nodulation suggests that this gene is also involved in later stages. A similar involvement throughout nodulation has been shown for the symbiotic receptor genes, NFP, LYK3, and DMI2, in M. truncatula (Limpens et al., 2003, 2005; Arrighi et al., 2006). We showed that AeCRK induction following inoculation required the conserved Nod signaling pathway. Since, downstream of this signaling pathway, NIN is known to regulate many rhizobial-induced genes, it is possible that AeCRK is part of the NIN regulon (Liu et al., 2019). However, rhizobial induction of nodulin genes, including AeNIN, was completely abolished in the crk mutant, indicating that AeCRK is required for the symbiotic signaling process. A reconciling hypothesis is that AeCRK expression depends on AeNIN and that AeCRK activity both increases signaling to enhance symbiotic responses and promote rhizobial infection (Figure 10). Such dual commitment has already been proposed for the symbiotic receptors LjEPR3 and LjRINRK in L. japonicus, highlighting the tight link between symbiotic signaling and rhizobial infection (Li et al., 2019; Kawaharada et al., 2015). Continuing research on this receptor-kinase will likely reveal new and exciting features of rhizobial symbiosis in A. evenia and legumes in general.

Materials and methods

Plant material and mutant analysis

All A. evenia nodulation mutants characterized in this study were obtained from the phenotypic screen of an EMS-mutagenized population derivated from the reference CIAT22838 line (Quilbé et al., 2021). Mutant characteristics are detailed in Supplemental Table S1. For the A21 and E26 mutants, genetic analyses (genetic determinism and allelism tests) and sequencing approaches (genotyping and mapping-by-sequencing) were performed as indicated in Quilbé et al. (2021).

For seed germination, seeds were scarified for 40 min with sulfuric acid (96% v/v) and rinsed with distilled water; germination was induced over night with 0.01% (v/v) ethrel (BAYER) as described earlier (Chaintreuil et al., 2016). For root hair lengths, measurements were taken with a binocular loupe at 0, 7, 14, and 21 dpi and the number of root length or density performed with the Optimas version 6.1 software (Media Cybernitics, Silverspring, MD, USA). For the nodulation kinetics, roots were observed with a binocular loupe at 0, 7, 14, and 21 dpi and the number of bumbs, white and pink nodules were recorded. Nontreated, nodulated, and mycorrhized roots were observed using a stereo-macroscope (Nikon AZ100, Champigny-sur-Marne, France) and pictures taken with the Nikon Advanced software. For root hair lengths, measurements were taken with ImageJ version 1.53e software (http://imagej.nih.gov/ij). Bacterial infection of plant tissue was analyzed using 70-µm-thick vibratome (Leica VT1000S) sections of fresh material. In case of infection with strain ORS278-GUS, sections were stained with X-gluc (Fabre et al., 2015) and observed using a Nikon microscope. To analyze the infection process, 70-µm root sections of plants inoculated with ORS278 or ORS285 were stained with Syto13 and Calcofluor White and visualized by confocal microscopy with the following excitation laser/emission cutoffs: 405/410–500 nm (Calcofluor White: intensity 5%–11%; gain: 500–800), 488/493–525 nm (Syto-13:
intensity 5%–11%; gain: 500–750), 555/560–630 nm (auto-fluorescence: 6%–11%; gain: 500–800). Analysis and photos were taken using a confocal laser-scanning microscope (Carl Zeiss LSM 700; Jen, Germany), and obtained images were processed using the Carl Zeiss Confocal Microscope software.

**In silico gene expression and co-expression analysis**

Expression patterns for genes of interest were obtained using the A. evenia gene atlas available at the AeschynomeneBase (http://aeschynomenebase.fr/content/gene-expression). To identify genes co-expressed with AeNSP2, a hierarchical clustering analysis using Pearson correlation was performed. Raw RNAseq data available for A. evenia were converted into fragments per kilobase of exon per million mapped fragments. After filtering data to remove genes with low expression levels, a hclust hierarchical clustering was performed on a distance matrix calculated with the Pearson method included in the AMAP package (version 0.8.17). The clustering tree was initially split into 12 and then 100 clusters. The one containing AeNSP2 was analyzed and co-expressed genes subsequently validated by RT–qPCR are listed in Supplemental Table S4. Homology and orthology were inferred for A. evenia with previously ORTHOFINDER-delineated groups of orthologous (Quilbé et al., 2021), and with A. thaliana genes using the Blast tool on the TAIR web portal (https://www.arabidopsis.org).

**RNA extraction and expression studies**

All shown experiments contained at least three biological replicates. To study the gene regulation by nitrogen and AeNSP2 at 10-day postgermination, we took for each mutant line and nitrogen concentration (0, 0.5, and 5 mM KNO₃) a pool of three plant roots for analysis and the experiment was repeated 3 times. For the nodulation kinetics, was done and the experiment was repeated 3 times. For the nodulation kinetics, we took for each mutant, and the four allelic nsp2 mutants of A. evenia, which had been modified to include a glutathione-S-transferase (GST) sequence after the encoded 6 × His residues. The G354E mutation (corresponding to the crk-2 allele) was created by site-directed mutagenesis using primers AeCRKmutj42F (5′-GAAACCAAGCTTAGAAGAAGATTAAAAGG-3′) and AeCRKmutj42R (5′-GAAGCCACCTTCCAAGCCTTGGTTC3′). The resulting plasmids, pCDFDuet-6hisGST-CRKkin and pCDFDuet-6hisGST-CRKkin-G354E, were checked by sequencing and then transformed into E. coli Rosetta/DE3 (Novagen). The fusion proteins were expressed and purified on Glutathione-Sepharose4B (Amersham Biosciences Amersham, UK) as described (Klaus-Heisen et al., 2011). Kinase activity assays used the same conditions except that no radioactivity was used and the concentration of ATP was increased to 20 μM. Autophosphorylation was detected by western blotting using anti-phospho-Thr antibodies as described (Klaus-Heisen et al., 2011), except that a dilution of 1/5,000 was used. Duplicate gels were stained with Coomassie blue protein stain. The protein purifications and kinase assays were repeated at least twice. Two dead kinase proteins from M. truncatula, GST-NFPkin (Arrighi et al., 2006) and GST-LYK3kin-G334E (Klaus-Heisen et al., 2011) and GST were used as controls. Quantification of the bands used the volume tools of Image Lab version 6.0 (BioRad Laboratories, Hercules, CA, USA).

**Statistical analysis**

All measurements and gene expression levels were compared between the WT and mutant lines or between the control condition and tested conditions using a Kruskal–Wallis test or Student’s t test with R package.

**Accession numbers**

The mapping-by-sequencing data generated for the nin-1 mutant in this study were deposited in the NCBI database under BioProject ID: PRJNA727694. The A. evenia gene identifiers are shown in Supplemental Tables S1, S4, and S5.

**Supplemental data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Identification of a nin mutant allele by mapping-by-sequencing.

**Supplemental Figure S2.** Comparison of root hair development and root system architecture in the WT line, the ccamk-3 mutant, and the four allelic nsp2 mutants of A. evenia.
Supplemental Figure S3. Nodulation properties of the \textit{pollux} mutants with \textit{Bradyrhizobium} strain ORS278.

Supplemental Figure S4. Nodulation properties of the \textit{ccamk} mutants with \textit{Bradyrhizobium} strain ORS278.

Supplemental Figure S5. Nodulation properties of the \textit{cyclops} mutants with \textit{Bradyrhizobium} strain ORS278.

Supplemental Figure S6. Nodulation properties of the \textit{nsp2} mutants with \textit{Bradyrhizobium} strain ORS278.

Supplemental Figure S7. Nodulation properties of the \textit{nin} mutants with \textit{Bradyrhizobium} strain ORS278.

Supplemental Figure S8. Nodulation properties of the \textit{crk} mutants with \textit{Bradyrhizobium} strain ORS278.

Supplemental Figure S9. Abnormal splicing of \textit{AeCRK} transcripts in the \textit{crk-1} mutant.

Supplemental Figure S10. Analysis of the kinase activity of \textit{AeCRK} and the G354 mutant protein, corresponding to the mutation in \textit{crk-2}.

Supplemental Figure S11. Nodulation properties of \textit{pollux} mutants with \textit{Bradyrhizobium} strain ORS285.

Supplemental Figure S12. Nodulation properties of a \textit{ccamk} mutant with \textit{Bradyrhizobium} strain ORS285.

Supplemental Figure S13. Nodulation properties of \textit{cyclops} mutant with \textit{Bradyrhizobium} strain ORS285.

Supplemental Figure S14. Nodulation properties of a \textit{nsp2-2} mutant with \textit{Bradyrhizobium} strain ORS285.

Supplemental Figure S15. Nodulation properties of \textit{nin} mutants with \textit{Bradyrhizobium} strain ORS285.

Supplemental Figure S16. Nodulation properties of \textit{crk} mutants with \textit{Bradyrhizobium} strain ORS285.

Supplemental Figure S17. Expression of mycorrhization-related genes in the \textit{A. evenia} WT line.

Supplemental Table S1. Molecular and phenotypic properties of \textit{A. evenia} nodulation mutants.

Supplemental Table S2. Genetic determinism of \textit{nin} mutants.

Supplemental Table S3. Allelism tests performed on \textit{nin} mutants.

Supplemental Table S4. Expression data of \textit{A. evenia} genes induced or repressed during nodulation.

Supplemental Table S5. List of genes with the primers used for gene expression analysis.

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