Silencing of Putative Cytokinin Receptor Histidine Kinase1 Inhibits Both Inception and Differentiation of Root Nodules in *Arachis hypogaea*

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Rhizobia–legume interaction activates the SYM pathway that recruits cytokinin signaling for induction of nodule primordia in the cortex. In *Arachis hypogaea*, bradyrhizobia invade through natural cracks developed in the lateral root base and are directly endocytosed in the cortical cells to generate the nodule primordia. To unravel the role of cytokinin signaling in *A. hypogaea*, RNA-interference (RNAi) of cytokinin receptor histidine-kinase1 (*AhHK1*) was done. *AhHK1*-RNAi down-regulated the expression of type-A response regulators such as *AhRR5* and *AhRR3* along with several symbiotic genes, indicating that both cytokinin signaling and the SYM pathway were affected. Accordingly, there was a drastic downregulation of nodulation in *AhHK1*-RNAi roots and the nodules that developed were ineffective. These nodules were densely packed, with infected cells having a higher nucleo-cytoplasmic ratio and distinctively high mitotic index, where the rod-shaped rhizobia failed to differentiate into bacteroids within spherical symbiosomes. In accordance with the proliferating state, expression of a mitotic-cyclin *AhCycB2.1* was higher in *AhHK1*-RNAi nodules, whereas expression of a retinoblastoma-related (*AhRBR*) node that restrains proliferation was lower. Also, higher expression of the meristem maintenance factor *WUSCHEL-RELATED HOMEBOX5* correlated with the undifferentiated state of *AhHK1*-RNAi nodules. Our results suggest that *AhHK1*-mediated cytokinin signaling is important for both inception and differentiation during nodule development in *A. hypogaea*.

A compatible rhizobia–legume interaction activates the Nod factor-dependent SYM pathway that leads to a local accumulation of cytokinin as a trigger toward the induction of a de novo meristem in the root cortical cells (Frugier et al. 2008). Both exogenous cytokinin and rhizobia engineered to secrete *trans*-zeatin are able to initiate nodule primordium (Cooper and Long 1994; Heckmann et al. 2011; Podlesakova et al. 2013). Cytokinin signaling causes local auxin accumulation at the site of incipient nodule primordia by modulating the expression of auxin transporters (Ng et al. 2015; Plet et al. 2011). Together, these phytohormonal signals and the SYM pathway reprogram the cortical

cells and regulate their division, ultimately building a nodule primordium for the endocytic accommodation of the symbionts (Mathesius et al. 2000a; Suzuki et al. 2012).

Cytokinin is sensed by the extracellular cyclase/histidine kinase-associated sensing extracellular (CHASE) domain present in the cytokinin response1 (*MtCRE1*) and *Lotus* histidine kinase1 (LHK1) transmembrane receptors in *Medicago truncatula* and *Lotus japonicus*, respectively (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007). Loss-of-function mutations of LHK1 (*lhk1-1* allele, formerly known as *hit1*) and *MtCRE1* (*cre1-1/2*) lead to a strong reduction in nodulation (Murray et al. 2007; Plet et al. 2011), suggesting that cytokinin-dependent phosphorelay through these receptor histidine kinases (HK) is essential for nodule organogenesis. On the other hand, gain-of-function mutation of LHK1 (*snf2*) is sufficient to induce nodule primordia in the absence of rhizobia, demonstrating that cytokinin signaling is sufficient to initiate this developmental process (Tirichine et al. 2007). Cytokinin oxidase or dehydrogenase expressed during nodule development restricts the level of active cytokinin for balancing its positive role in nodule organogenesis with its negative effect on rhizobial infection (Reid et al. 2016). Downstream of cytokinin receptors, a histidyl-aspartyl multistep phosphorelay is initiated, leading to the activation of the type-B response regulators (RR) which, in turn, regulate the primary response genes such as the type-A RR (To et al. 2007). Several type-A RR such as *MtRR4*, *LjRR5*, and *MtRR9* are induced during nodulation and coordinate the downstream functional responses (Gonzalez-Rizzo et al. 2006; Op den Camp et al. 2011; Tirichine et al. 2007).

Several evidences suggest a hierarchical organization of the Nod factor-dependent activation of the SYM-Pathway and cytokinin signaling for the development of root nodule symbiosis (RNS). For example, spontaneous nodule formation is induced by constitutive activation of Nod factor signaling or cytokinin signaling (Gleason et al. 2006; Saha et al. 2014; Tirichine et al. 2006, 2007). Constitutive activation of cytokinin signaling (*snf2*) leads to spontaneous nodulation in the absence of a functional SYM pathway but constitutive activation of a SYM pathway (*snf1*) is unable to trigger the same in the absence of cytokinin signaling (Madsen et al. 2010). Recently, the Nod factors have been demonstrated to induce the accumulation of cytokinin, confirming its precedence to cytokinin signaling (Held et al. 2014; van Zeijl et al. 2015). Apart from the hierarchical organization, evidence also indicates a crosstalk between the SYM pathway and the cytokinin signaling. For example, cytokinin RRs are induced during the Nod factor-dependent symbiotic interactions (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Plet et al. 2011). In addition, cytokinin signaling activates several symbiosis-responsive transcription

Sequence data for *AhHK1* is available under accession number of KT153625.

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factors (TF) such as nodule inception (*NIN*) and *NODULATION SIGNALING PATHWAY 1* and 2 (*NSP1* and *NSP2*), ERF required for nodulation (*ERN1*), nuclear factor Y (NF-YA1, formerly known as *Hap2*), and early nodulation 40 (*ENOD40*) (Charon et al. 1997; Crespi et al. 1994; Gonzalez-Rizzo et al. 2006; Heckmann et al. 2011; Mathesius et al. 2000b; Murray et al. 2007; Op den Camp et al. 2011; Plet et al. 2011; Smit et al. 2005; Tirichine et al. 2007). Finally, apart from being the key endogenous signal for nodule development and differentiation (Plet et al. 2011), cytokinin also maintains the homeostasis of the symbiotic interaction by limiting the extent of additional infection at the root epidermis, indicating that the SYM pathway and cytokinin signaling are connected through feedback loops (Miri et al. 2016).

In *M. truncatula* or *L. japonicus*, the epidermal and the cortical processes are spatially and temporally coordinated to ensure rhizobial accommodation from the invading infection threads in the subtending nodule primordia in the cortex (Miri et al. 2016; Oldroyd 2013; Popp and Ott 2011; Saha et al. 2016). In both of these legumes, endoreduplication of host cells is found necessary for bacterial infection and differentiation of nodules whereas, in *Medicago* spp., the symbionts also endoreduplicate to mature into functional nitrogen-fixing symbiosomes (González-Sama et al. 2006; Mergaert et al. 2006; Truchet 1978). In approximately 25% of the legume species that are found mainly in tropical and warm temperate areas, rhizobial invasion does not involve the formation of infection threads (Sprent and James 2007). Examples are Aeschynomene legumes such as *Arachis hypogaea*, where rhizobia invade through natural annular cracks developed in the lateral root base and are directly endocytosed in the cortical cells to generate a nodule primordia (Bal et al. 1989; Chandler 1978). The rhizobia-infected cells within the nodule primordia divide repeatedly to form a typical aescynomenoid type of determinate nodule characterized by the absence of uninfected cells in the central infection zone (IZ) (Booger and Rossum 1997). After the host cells cease to divide in aescynomenoid nodules, the rod-shaped rhizobia differentiate into bacteroids within large, spherical nitrogen-fixing symbiosomes (Oono and Denison 2010). As seen with inverted repeat-lacking clade legumes such as *M. truncatula*, this differentiation is noted to be associated with endoreduplication (Czernic et al. 2015).

To unravel the role of cytokinin signaling in *A. hypogaea*, RNA-interference (RNAi) of cytokinin receptor histidine-kinase1 (*AhHK1*) was done. We demonstrate that downregulation of *AhHK1* drastically reduced nodule formation, underscoring the importance of cytokinin signaling mediated through *AhHK1* during nodule initiation in *A. hypogaea*. The IZ of *AhHK1*-RNAi nodules contained mitotically active cells associated with high expression of a mitotic cyclin *AhCycB2.1*, indicating a prolonged proliferating state during nodule development in *A. hypogaea* in the absence of cytokinin signaling. These cells in the *AhHK1*-RNAi nodules contained a distinctly spherical nucleus with significant increase in nucleocytoplasmic index, suggesting that these cells retained some features of the dedifferentiated cells that continued to proliferate and failed to differentiate. Also, the endocytosed rod-shaped rhizobia failed to differentiate into bacteroids within spherical symbiosomes, indicating the endosymbionts to be in their undifferentiated state in the *AhHK1*-RNAi IZ cells. In accordance with the proliferating and undifferentiated state of the *AhHK1*-RNAi IZ cells, expression of a retinoblastoma-related protein (*AhRBR*) that restrains proliferation for favoring differentiation of plant cells was lower in *AhHK1*-RNAi nodules whereas expression of a meristem maintenance factor such as *WUSCHEL-RELATED HOMEBOX5* (*AhWOX5*) was significantly higher. Our results suggest that *AhHK1*-mediated cytokinin

signaling has a role in both nodule initiation and its subsequent differentiation during nodule development in *A. hypogaea*.

RESULTS

Cytokinin signaling is essential for inception and maturation of nodules in *A. hypogaea*.

To unravel the role of cytokinin signaling in *A. hypogaea* RNS, genes encoding the cytokinin receptor *AhHK1* (2,928 bp, KT153625) and its paralogues *AhHK2* (3,711 bp, KY368747) and *AhHK3* (3,102 bp, KY368748) were amplified from nodule cDNA by a degenerate priming approach. The predicted receptor proteins contain two transmembrane domains flanking the extracellular cytokinin perceiving CHASE domain in the N terminus and the HK domain followed by the receiver domain in the cytoplasmic C terminus (Supplementary Fig. S1). The steady-state level of *AhHK1* transcript was significantly higher than *AhHK2* and *AhHK3* in uninfected as well as nodulated roots and its level remained almost unchanged during the progress of RNS (Fig. 1A and B). In response to exogenous application of cytokinin, the expression of *AhHK1* alone increased rapidly in *A. hypogaea* roots, whereas that of *AhHK2* and *AhHK3* decreased significantly (Fig. 1C). In a phylogenetic tree of cytokinin receptors from diverse species, *AhHK1* clustered within the HK1/4 subgroup along with *MtCRE1* and *LHK1* (Fig. 1D), the HK that are demonstrated to be the most important for RNS (Gonzalez-Rizzo et al. 2006; Murray et al. 2007). Together, these results suggested that *AhHK1* is important for RNS in *A. hypogaea*. It may be noted that all the three *AhHK* (*AhHK1*, -2, and -3) from *A. hypogaea* are placed at the point of divergence of legumes from nonlegumes (Fig. 1D), which was also observed for *AhSYM* and *AhCCaMK* in their respective distance trees (Saha et al. 2014; Sinharoy and DasGupta (2009). Separation between the *A. hypogaea* genes and the other legume genes correlates with the rhizobial colonization by crack entry in Aeschynomene legumes versus rhizobial colonization through infection thread in other legumes such as *Cicer arietinum*, *Cajanus cajan*, *M. truncatula*, *Phaseolus vulgaris*, *L. japonicus*, and *Glycine max*.

To determine the role of *AhHK1*-mediated cytokinin signaling in *A. hypogaea* nodule development, the transcript encoding this receptor was downregulated by introducing an RNAi construct that targets the extracellular CHASE domain. There was approximately 80% reduction of *AhHK1* transcript in *AhHK1*-RNAi hairy roots without having any significant interference with *AhHK2* and *AhHK3* expression (Fig. 2A). Nodule development was monitored in vector-transformed and *AhHK1*-RNAi roots over a period of 5 weeks after infection (WAI) with *Bradyrhizobium* sp. SEMIA6144 (Fig. 2B). Bump-like protrusions in the lateral root base were noted in the vector-transformed hairy roots within 1 WAI and were identified as nodule primordia. *AhHK1*-RNAi roots at the same period did not show any sign of nodule development. At approximately 3 WAI, 83% of the vector-transformed plants ($n = 30$) developed functional nodules, as indicated by their ability to reduce acetylene (Fig. 2C and D). In *AhHK1*-RNAi roots, we noted bump-like protrusions by 3 to 4 WAI. Concomitant with the emergence of these nodule-like structures by 3 to 4 WAI, the composite plants with RNAi roots lost vigor and almost 50% of the plants died before nodulation was scored at 5 WAI. At approximately 5 WAI, 37% of the green fluorescent protein (GFP)-positive roots in the *AhHK1*-RNAi plants ($n = 40$) developed small white nodules at the lateral root bases (Fig. 2B and C). These nodules developed in *AhHK1*-RNAi roots at 5 WAI and were unable to reduce acetylene, indicating that they were nonfunctional (Fig. 2D). In comparison, nodules developed in vector-transformed roots were noted to be functional by 3 WAI. Overall, there was a considerable delay in nodule inception in the *AhHK1*-RNAi roots,

with approximately 80% reduction in nodule number, which could be attributed to approximately 80% reduction in *AhHK1* transcript level. A similar delay in nodulation was also observed in the RNAi lines of *MtCRE1* or *AeHK1* (Fabre et al. 2015; Gonzalez-Rizzo et al. 2006) or in the cytokinin receptor mutants such as *cre1-1* and *lhc1-1* (Murray et al. 2007; Plet et al. 2011). Because the transcript levels of *AhHK2* and *AhHK3* remained unaltered in the *AhHK1*-RNAi roots, it is possible that *AhHK2* and *AhHK3* partially compensated the loss of *AhHK1*, which enabled the residual nodulation in the *AhHK1*-RNAi lines (Fig. 2A). Moreover, the residual nodulation can also be attributed to the residual *AhHK1* expression in the *AhHK1*-RNAi lines.

RNAi of *AhHK1* affects both cytokinin signaling and the SYM pathway.

As a readout of cytokinin response, we monitored the level of three type-A RR transcripts in *AhHK1*-RNAi roots (namely *AhRR5*, *AhRR3*, and *AhRR9*) whose homologs are involved in the cytokinin response of symbiotic signaling (Fig. 3A). *AhRR5* is a homolog of *LjRR5/MtRR8* that is transcriptionally induced in the presence of rhizobia (Op den Camp et al. 2011; Saur et al. 2011; Tirichine et al. 2007). *AhRR3* is a homolog of *MtRR4* that functions downstream of *MtCRE1* in *M. truncatula* and is inducible by rhizobia (Gonzalez-Rizzo et al. 2006; Plet et al. 2011). *AhRR9* is a homolog of *MtRR9* that is transcriptionally induced in the presence of Nod factor (Op den Camp et al. 2011). There was a rapid increase in all three RR transcripts within 3 h of the application of 10^{-8} M 6-benzylaminopurine (BAP), with the highest expression being that of *AhRR3* (approximately sevenfold), indicating their cytokinin responsiveness in *A. hypogaea* roots (Fig. 3B). Also, transcripts for all three type-A RR were elevated in mature *A. hypogaea* nodules, with the highest expression being that of *AhRR5* (approximately 60-fold), suggesting their link with nodule development (Fig. 3C).

To clarify the role of *AhHK1* in determining the expression of these type-A RR, we monitored their expression in *AhHK1*-RNAi uninfected roots as well as in the ineffective nodules that developed at 5 WAI in these roots. As a control, we used the uninfected vector-transformed roots and the effective nodules that developed at 3 WAI in these control roots. The expression of *AhRR5* and *AhRR3* was significantly lower in both uninfected roots and infected *AhHK1*-RNAi nodules as compared with their counterparts in vector-transformed roots (Fig. 3C). However, expression of *AhRR9* was unaffected in both *AhHK1*-RNAi roots and nodules. Results indicate that *AhRR3* and *AhRR5* were transcriptionally upregulated by *AhHK1*-mediated cytokinin signaling whereas expression of *AhRR9* was apparently not affected by this pathway in *A. hypogaea* roots.

For the readout of the symbiotic signaling, we chose to monitor the expression of symbiotic genes such as *NIN*, *ENOD40*, *CYCLOPS*, *EFD*, and *SYMREM1*, whose homologs are significantly upregulated during progress of symbiosis in *Medicago* and *Lotus* spp., though their symbiotic functions remain to be established in *A. hypogaea* (Crespi et al. 1994; Lefebvre et al. 2010; Marsh et al. 2007; Toth et al. 2012; Vernie et al. 2008; Yano et al. 2008). *NIN* and *ENOD40*, the important markers for nodule organogenesis, are reported to be cytokinin induced in *Medicago* spp. (Gonzalez-Rizzo et al. 2006; Lee et al. 2007; Plet et al. 2011). In *A. hypogaea* roots, there was a rapid increase in transcripts encoding *AhNIN* and *AhENOD40* within 15 to 30 min of the application of 10^{-8} M BAP, indicating that their cytokinin responsiveness was conserved in *A. hypogaea* (Fig. 4A). Both *AhNIN* and *AhENOD40* were highly upregulated in wild-type (WT) nodules and, in *AhHK1*-RNAi nodules, their expression was significantly downregulated (Fig. 4B). This confirms the importance of cytokinin signaling in regulating their expression and suggests a conservation of their symbiotic function in *A. hypogaea*. Symbiotic genes such as *CYCLOPS/IPD3*, *SYMREM1*, and

Fig. 1. Expression and phylogeny of cyclase/histidine kinase-associated sensing extracellular domain containing putative cytokinin receptor histidine kinases (HK) in *Arachis hypogaea*. Quantitative reverse-transcription polymerase chain reaction analysis of *AhHK1*, *AhHK2*, and *AhHK3* in **A**, uninfected roots relative to *AhHK1* and **B**, infected roots at indicated time periods relative to uninfected roots (UI); 4DPI = infected roots at 4 days postinfection, 1/2 WAI = nodulated roots at 1 or 2 weeks after infection, and 3 WAI = nodules. **C**, Roots treated with 10^{-8} M 6-benzylaminopurine (BAP) for indicated time periods relative to untreated roots (Control). *AhACTIN* was used as reference. Histogram represents an average of three biological replicates, each having $n > 3$ plants, and error bar represents standard deviation. Wilcoxon matched-pairs signed rank test was used to assess significant differences, where ** and * indicate $P < 0.008$ and 0.05, respectively. **D**, Neighbor-joining distance tree (Saitou and Nei 1987) of HK using MEGA5 (Tamura et al. 2011) based on CLUSTALW alignment of 960 amino acids. Legumes are indicated by light branches and AhHK are indicated by closed circle.

EFD play essential roles in bacterial release and differentiation during nodule development in *M. truncatula* and *L. japonicus* (Lefebvre et al. 2010; Ovchinnikova et al. 2011; Toth et al. 2012; Vernie et al. 2008; Yano et al. 2008). Expression of *AhCYCLOPS*, *AhEFD1*, and *AhSYMREM1* was upregulated during symbiosis in *A. hypogaea* but, unlike *AhNIN* and *AhENOD40*, their expression was not induced in the presence of 10^{-8} M BAP (Fig. 4A and B). However, in *AhHK1-RNAi* nodules, expression of *AhCYCLOPS*, *AhSYMREM1*, and *AhEFD1* was significantly downregulated (Fig. 4B). It is possible that cytokinin signaling functions in concert with other signaling pathways in regulating the expression of these factors. Together, results in this section demonstrate the role of *AhHK1*-mediated cytokinin signaling in determining the expression of symbiotic genes and explains the drastic downregulation of nodulation in the *AhHK1-RNAi* roots.

Interference of *AhHK1*-mediated cytokinin signaling impedes the onset of differentiation in *A. hypogaea* nodules.

We next wanted to understand the specific stages of nodule development that were affected by the silencing of cytokinin signaling in addition to its requirement for cell proliferation during nodule initiation. For this, we monitored the ultrastructure of the primordia and nodules developed in *AhHK1-RNAi* roots. As indicated in Figure 2, nodule primordia developed within 1 WAI in vector-transformed roots. The longitudinal sections of these primordia revealed one or more centrally located pockets of rhizobia-infected cells that were surrounded by layers of uninfected cells (Fig. 5A). These rhizobia-containing cells were distinct by having reduced calcofluor-binding ability, indicating that they are thin walled (Fig. 5B and C). Such alteration in the cell wall structure of the rhizobia-containing cells in early stages

Fig. 2. RNA interference (RNAi) of *AhHK1* affects nodulation in *Arachis hypogaea*. **A**, Quantitative reverse-transcription polymerase chain reaction analysis of *AhHK1*, *AhHK2*, and *AhHK3* in nodules formed in vector-transformed roots (Control) at 3 and 5 weeks after infection (WAI) and *AhHK1-RNAi* roots at 3 WAI (primordia) and 5 WAI relative to their expression in nodules formed on control roots at 3 WAI. *AhACTIN* was used as a reference gene. Histogram represents an average of three biological replicates, each having $n > 4$ root systems, and error bar represents standard deviation. Wilcoxon matched-pairs signed rank test was used to assess significant differences, where * indicates $P < 0.05$. **B**, Nodulation events in *AhHK1-RNAi* roots as compared with control roots at 1 to 5 WAI. Open box indicate primordia and shaded box indicate nodules. Error bar represents standard deviation ($n = 30$ for control plants and $n = 40$ for *AhHK1-RNAi* plants). **C**, Stereomicrographs of indicated nodules shown as bright-field and green fluorescent protein. Arrow = nodule and double arrowhead = primordia. Scale bar: 500 μ m. **D**, Acetylene reduction assay in control and *AhHK1-RNAi* nodules at indicated WAI. Histogram represents an average of three biological replicates, each having $n > 2$ root systems, and error bar represents standard deviation.

of infection of *A. hypogaea* was also reported by Chandler et al. (1978). In the primordia, cells within the infection pocket and in the surrounding uninfected zone (SUZ) showed an enlarged nucleus with bigger and denser nucleolus in comparison with root cells (Supplementary Fig. S2). A magnified view revealed the intracellularized rhizobia within the infection pocket to be undifferentiated and rod shaped (Fig. 5D). These infection

pockets act as IZ founder cells and it is their uniform division and differentiation that give rise to the distinct aescynomenoid-type IZ in mature nodules. There has not been a single case where an uninfected primordia was noted ($n = 20$), which is in accordance with the proposition of infection preceding development of aescynomenoid nodules (Fabre et al. 2015). In *AhHK1-RNAi* roots, the primordia were noted only 3 WAI (Fig. 5E). Similar to

Fig. 3. Phylogeny and expression analysis of *AhRR3*, *AhRR5*, and *AhRR9*. **A**, Neighbor-joining distance tree (Saitou and Nei 1987) of indicated type-A response regulators (RR) from *Arachis hypogaea*, *Medicago truncatula*, and *Lotus japonicus* was generated using MEGA5 (Tamura et al. 2011) based on CLUSTALW alignment of 225 nucleotides. *AhRR* are indicated by a closed circle. Quantitative reverse-transcription polymerase chain reaction analysis of *AhRR3*, *AhRR5*, and *AhRR9* in **B**, roots treated with 10^{-8} M 6-benzylaminopurine (BAP) for the indicated time periods relative to untreated (Control) roots and **C**, uninfected vector-transformed and *AhHK1-RNAi* roots and in nodules developed in vector-transformed (3 weeks after infection [WAI]) and *AhHK1-RNAi* (5 WAI) roots relative to Control uninfected roots (UI). *AhACTIN* was used as a reference gene. Histogram represents an average of three biological replicates, each having $n > 4$ plants, and error bar represents standard deviation. Wilcoxon matched-pairs signed rank test was used to assess significant differences, where ** and * indicate $P < 0.008$ and 0.05, respectively.

the primordia in vector-transformed roots, *AhHK1-RNAi* primordia had centrally located, rod-shaped, rhizobia-containing cells surrounded by uninfected cells (Fig. 5E to H). As such, apart from a delay and a reduction in the number which unambiguously indicates a role of *AhHK1* in nodule initiation and the establishment of nodule primordia, no further differences were apparent in the anatomy of the early primordia stage in the *AhHK1-RNAi* and control roots.

The ultrastructure of ineffective nodules developed by 5 WAI in *AhHK1-RNAi* roots was compared with the functional nodules that developed in vector-transformed roots by 3 WAI. The vector-transformed roots developed characteristic aescynomenoid nodules by 3 WAI, with no uninfected cells in the IZ, and the rod-shaped rhizobia differentiated into bacteroids within characteristic spherical symbiosomes (Fig. 5I to K). In the *AhHK1-RNAi* nodules, the organization of the aescynomenoid IZ was unaffected (Fig. 5L) and the IZ/SUZ ratios were similar to those of the WT nodules (Supplementary Fig. S3). The load of the symbionts also appeared to be uniform in all IZ cells of the *AhHK1-RNAi* nodules, indicating that proper synchronization of cell division between the host and the symbionts was not perturbed by *AhHK1* downregulation (Fig. 5M). However, there were stark differences between the control nodules and the *AhHK1-RNAi* nodules in the later stages of development, the primary distinctions being (i) the IZ of the *AhHK1-RNAi* nodules comprised actively dividing cells with mitotic index > 6, as

opposed to the WT mature nodules that were devoid of any dividing cells (Fig. 5I to K and L to R; Supplementary Fig. S4; Table 1); and (ii) the IZ cells of the *AhHK1-RNAi* nodules had a twofold higher density of infected cells as compared with the IZ of the vector-transformed nodules. However, the total number of infected cells in the IZ of *AhHK1-RNAi* nodules was always higher as compared with a similar-sized WT nodule (Supplementary Table S1). The proliferative state can be explained by a delay in the process of nodule development but a simple delay doesn't explain the increase in the number of total cells in *AhHK1-RNAi* nodules. Finally, unlike WT nodules where the rhizobia mature into bacteroids within a spherical symbiosome, in the *AhHK1-RNAi* nodules, rhizobia maintained their native rod form, indicating that cytokinin signaling is also important for the differentiation of the symbionts (Fig. 5L to R). Taken together, these results indicate that cytokinin signaling is important for the proliferation or differentiation of both host cells and the symbionts during the development of aescynomenoid nodules in *A. hypogaea*.

Characteristics of the undifferentiated *AhHK1-RNAi* nodules.

Apart from being characterized by their smaller size and higher mitotic index, both the IZ and SUZ cells in the *AhHK1-RNAi* nodules showed a significant increase in nucleo-cytoplasmic index compared with the control vector-transformed nodules (Fig. 6A).

Fig. 4. RNA interference (RNAi) of *AhHK1* affects the SYM pathway. Quantitative reverse-transcription polymerase chain reaction analysis of indicated genes in **A**, roots treated with 10⁻⁸ M 6-benzylaminopurine (BAP) for the indicated time periods relative to untreated (Control) roots and **B**, uninfected vector-transformed and *AhHK1-RNAi* roots and nodules developed in vector-transformed (3 weeks after infection [WAI]) and *AhHK1-RNAi* (5 WAI) roots relative to Control uninfected roots (UI) roots. *AhACTIN* was used as a reference gene. Histogram represents an average of three biological replicates, each having each $n > 4$ plants, and error bar represents standard deviation. Wilcoxon matched-pairs signed rank test was used to assess significant differences, where * indicates $P < 0.05$.

Nucleus/cell and nucleolus/cell ratios both were higher in the *AhHK1-RNAi* nodules whereas the nucleus/nucleolus ratio was lower (Fig. 6B to D). Additionally, the IZ cells in the *AhHK1-RNAi* nodules had a distinctly spherical nucleus and dense cytoplasm (Fig. 6E and F to H; Supplementary Fig. S5). The IZ cells were occasionally binucleated and sometimes a single nucleus had two nucleoli (Fig. 6G and H). These are some of the

characteristic features of pluripotent stem cells in plants (Verdeil et al. 2007) and, therefore, it is possible that the infected cells in the *AhHK1-RNAi* nodules have retained some features of the dedifferentiated cells that continued to proliferate and failed to differentiate in the absence of cytokinin signaling.

We then used molecular markers to justify the proliferating and undifferentiated state of the IZ cells in *AhHK1-RNAi*

Fig. 5. Ultrastructure analysis of nodules developed in *AhHK1-RNAi* roots. Sections of nodule primordia from **A** to **D**, control vector-transformed roots at 1 week after infection [WAI] and **E** to **H**, *AhHK1-RNAi* roots at 3 WAI (**E-H**). **A** and **E**, Bright field and propidium iodide (PI) merged. **B** to **D** and **F** to **H** are magnified images of **A** and **E**, respectively. **B** and **F**, Calcofluor stained; **C**, **D**, **G**, and **H**, calcofluor and PI merged. Dashed line encircles the infection zone (IZ). SUZ = surrounding uninfected zone, arrow = rod-shaped rhizobia, and asterisk indicates identical position in **A** to **D** and **E** to **H**. Scale bar: 100 µm (**A** to **C** and **E** to **G**) or 10 µm (**D** and **H**). Sections of nodules from **I** to **K**, control vector-transformed roots at 3 WAI and **L** to **R**, *AhHK1-RNAi* roots at 5 WAI. **J** to **K** and **M** to **N** are magnified images of IZ shown in **I** and **L**, respectively. Inset in **K** shows magnified IZ cells. Magnified images of IZ cells in **N** showing **O**, early prophase; **P**, metaphase; **Q**, anaphase; and **R**, telophase. Arrows indicate spherical symbiosome (**J**) and rod-shaped rhizobia (**M**); wedged arrowhead indicates dividing cells (**N**). **I**, **L**, and **O** to **R**, Calcofluor and PI merged; **J**, **K**, **M**, and **N**, calcofluor, PI, and Syto9 merged. Scale bar: 100 µm (**I** and **L**) and 10 µm (**J**, **K**, and **M** to **R**). Inset scale bar = 10 µm.

nodules. As a molecular marker for proliferation, we monitored the level of expression of a type-B cyclin, *AhCycB2.1*. It is a homolog of *GmCycB2.1* that has been implicated to have a role in regulating host cell division during nodule organogenesis in *G. max* (Mortier et al. 2011). In vector-transformed nodules, there was upregulation of *AhCycB2.1* at 1 WAI, after which its expression drastically decreased in the mature nodules. In *AhHK1-RNAi* roots, there was a steady increase in the level of expression of *AhCycB2.1* from 2 WAI onward and the expression was approximately threefold more in the *AhHK1-RNAi* nodules (Fig. 6I). This high expression of *AhCycB2.1* is in accordance with the proliferating state of the IZ cells in the *AhHK1-RNAi* nodules. As a marker for the proliferative state of the IZ cells in the *AhHK1-RNAi* nodules, we also monitored the level of *AhRBR*, whose downregulation causes overproliferation and delayed differentiation in the root apical meristem (RAM) of *Arabidopsis* (Borghi et al. 2010). We wanted to check whether the level of *AhRBR* expression was correlated with the proliferating state of *AhHK1-RNAi* nodules. Our results reveal that, in vector-transformed roots, there was a significant increase (20-fold) of *AhRBR* expression during nodule development by 2 WAI, after which it decreased (Fig. 6J). In *AhHK1-RNAi* nodules, *AhRBR* level was maximal at 3 WAI, with only fivefold increase in expression. It is possible that the role of RBR in restraining cell division is functionally conserved during nodule development because its downregulation in *AhHK1-RNAi* nodules correlates with the proliferative and undifferentiated state of the infected host cells.

The homeobox protein WOX5 is a marker for meristem maintenance in RAM of *Arabidopsis thaliana* and *MtWOX5* is found to be expressed within nodule meristems in *M. truncatula* (Franssen et al. 2015; Osipova et al. 2012; Sarkar et al. 2007). Therefore, we chose to monitor the expression of *AhWOX5* with the progress of symbiosis in *AhHK1-RNAi* roots. In vector-transformed roots, *AhWOX5* expression was approximately 50-fold higher in developing nodules at 2 WAI as compared with the uninfected roots. The level of *AhWOX5* almost reduced to its basal level in the mature nodules at 3 WAI (Fig. 6K). This transient expression pattern of *AhWOX5* was similar to what was observed for *MtWOX5* in *M. truncatula* and *PsWOX5* in *Pisum sativum* (Osipova et al. 2012). In *AhHK1-RNAi* roots, the *AhWOX5* level gradually increased up to 85-fold by 5 WAI in the ineffective nodules as opposed to only 7-fold higher expression in the effective and mature nodules that developed in the vector-transformed roots by 3 WAI (Fig. 6K). Thus, unlike the differentiation-promoting factors such as *AhRBR*, whose expression decreased, the expression of a meristem maintenance factor such as *AhWOX5* increased in *AhHK1-RNAi* nodules. In situ hybridization was done to determine the spatial pattern of *AhWOX5* expression during nodule development (Fig. 6L to O). In the vector-transformed roots at 1 WAI, *AhWOX5* transcript was detected primarily in the IZ of the nodule primordia, with weak expression in the vascular bundles (Fig. 6L). In the mature nodules at 3 WAI, *AhWOX5* was absent from the IZ but was detected in the vascular bundles (Fig. 6M). In the *AhHK1-RNAi* nodule primordia

at 3 WAI, *AhWOX5* expression was restricted to the IZ and vascular bundles, which is similar to what was observed in the control nodule primordia (Fig. 6N). However, in the *AhHK1-RNAi* nodules at 5 WAI, *AhWOX5* expression was still detectable in both the IZ and the vascular bundles (Fig. 6O). These results demonstrate that both the level and the site of *AhWOX5* expression changes in the *AhHK1-RNAi* nodules. Because the WUSCHEL HOMEBOX (WOX) TF are central regulators of stem cell specification in diverse meristems, the elevated level of *AhWOX5* transcript in the *AhHK1-RNAi* nodules is in accordance with the undifferentiated state of infected host cells in the absence of *AhHK1*-mediated cytokinin signaling.

DISCUSSION

In *A. hypogaea*, rhizobia are directly endocytosed in cortical cells to generate the founder cells that divide repeatedly to develop the typical aescynomenoid nodule (Fig. 5). *AhHK1-RNAi* resulted in a decrease of nodule numbers, indicating that cytokinin signaling mediated through this receptor is important for inception of nodule primordia in *A. hypogaea* (Figs. 2, 3, 4, and 5). In the later stages, *AhHK1-RNAi* IZ cells were noted to be in a proliferative state, suggesting that *AhHK1* has a role in differentiation of these cells for the development of mature and effective nodules (Figs. 5 and 6). The endocytosed rhizobia in the *AhHK1-RNAi* nodules remained in their native rod-shaped form without being differentiated into bacteroids within the spherical symbiosomes (Fig. 5), which is directly linked to nitrogen-fixing efficiency (Oono and Denison 2010). This explains the lack of nitrogen-fixing capability of *AhHK1-RNAi* nodules and indicates that *AhHK1* signaling is important for the differentiation of both host and symbionts during the development of aescynomenoid nodules (Figs. 2D and 5). It may be noted that, in both *Medicago* and *Lotus* spp., the redundancy of cytokinin signaling mediated through *MtHK2/LHK2* and *MtHK3/LHK3* led to formation of proper nitrogen-fixing nodules after a considerable delay (Boivin et al. 2016; Held et al. 2014). It is possible that the observed undifferentiated state of host and the symbionts in *AhHK1-RNAi* nodules was due to a lag in the onset of differentiation and functional nodules could develop after such considerable delay. However, a simple delay does not explain why there would be an increase in infected cell number in the IZ of *AhHK1-RNAi* nodules. Therefore, it is also possible that the presence of proliferating IZ cells in *AhHK1-RNAi* nodules is due to lack of a specific signal for differentiation in the absence of *AhHK1*-mediated cytokinin signaling.

Earlier reports have indicated that the expression of SYM pathway effectors and type-A RR decrease in *MtCRE1-RNAi* and *cre1/hit1* mutants (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Plet et al. 2011). Similarly, under *AhHK1-RNAi* conditions, we find drastic downregulation of expression of type-A RR such as *AhRR3* and *AhRR5*, whose homologs were previously been reported to play a significant role during nodulation downstream of cytokinin signaling (Murray et al.

Table 1. Mitotic index (MI) of infection zone in *AhHK1-RNAi* nodules^a

Set	Number of cells	Interphase	Prophase	Metaphase	Anaphase	Telophase	MI ± SD (%) ^b
1	219	204	11	3	1	0	7 ± 1.67
2	234	222	8	2	1	1	5 ± 1.15
3	186	170	10	3	2	1	9 ± 0.41
4	230	216	10	3	1	0	6 ± 1.8
5	212	201	9	1	0	1	5 ± 2.46

^a Nodules were harvested from *AhHK1-RNAi* roots 5 weeks after infection. Three different areas (272 by 272 µm each) in a nodule section were analyzed for dividing cells. Each row represents observations from a single nodule.

^b MI is calculated using the formula $MI = [(P + M + A + T)/N] \times 100\%/N$, where P = prophase, M = metaphase, A = anaphase, T = telophase, and N = total number of cells. SD = standard deviation.

2007; Op den Camp et al. 2011; Saur et al. 2011). In addition, several SYM pathway effectors were significantly down-regulated upon silencing of *AhHK1* in *A. hypogaea* (Figs. 3 and 4). *NIN* and *ENOD40* are cytokinin-inducible effectors of the SYM pathway, where *MnIN* is required to initiate nodule formation in the cortex and *MtENOD40* is a long noncoding RNA that is essential for nodule organogenesis (Crespi et al. 1994; Lee et al. 2007; Marsh et al. 2007). Although both these factors are yet to be characterized in *A. hypogaea*, inducibility of *AhNIN* and *AhENOD40* by cytokinin and their high expression in *A. hypogaea* nodules suggests their conserved role in nodule development (Fig. 4). Finally, the significant down-regulation of their expression in *AhHK1-RNAi* nodules confirms their cytokinin responsiveness during *A. hypogaea* nodule development. CYCLOPS transactivates *NIN* and functions downstream of CCaMK in the SYM pathway, and SYMREM1 is a symbiotic remorin that interacts with an upstream receptor-like kinase responsible for bacterial recognition (Singh et al. 2014; Toth et al. 2012). Both CYCLOPS and SYMREM1 are essential for bacterial release from infection threads in *M. truncatula* and *L. japonicus* but their role in aescynomenoid nodule development is unknown (Lefebvre et al. 2010; Ovchinnikova et al. 2011; Toth et al. 2012; Yano et al. 2008). Silencing of *AhHK1* led to decreased expression of *AhCYCLOPS* and *AhSYMREM1*, which possibly explains the drastic downregulation of nodulation because nodule organogenesis is preceded by bacterial endocytosis in *A. hypogaea* (Booger and Rossum 1997). EFD is an ethylene-dependent TF required for nodule differentiation in *M. truncatula* (Vernie et al. 2008). Considering the role of EFD in nodule differentiation, the immature and undifferentiated state of the *AhHK1-RNAi* nodules might be attributed to a low level of *AhEFD1* (Fig. 4). Because *AhCYCLOPS*, *AhSYMREM1*, and *AhEFD1* were not induced by exogenous application of cytokinin, their low expression in *AhHK1-RNAi* roots indicates that cytokinin signaling might be necessary in concert with other pathways for regulating their expression. It may be noted that *MtEFD* negatively affects nodule number in *M. truncatula* by inducing type-A RR (*MtRR4*) (Vernie et al. 2008). This causes feedback inhibition of *MtCRE1* and thereby explains why the *Mtefd1* mutant shows higher nodulation. In uninfected *AhHK1-RNAi* roots, there was a significant upregulation of *AhEFD1* expression, which is in accordance with our present understanding about the negative role of EFD on nodule number. However, elevated expression of *AhEFD1* does not explain the reduced expression of *AhRR3* and *AhRR5* in *AhHK1-RNAi* roots and, in turn, low expression of type-A RR does not explain the low nodule number under the same conditions (Figs. 3C and 4). This paradox was noted earlier, when RNAi of type-A RR led to a reduction in nodule numbers, which should have increased as per our present understanding on cytokinin signaling (Op den Camp et al. 2011).

The importance of *MtCRE1/LHK1* in the inception of nodulation has been widely demonstrated in model legumes such as *Medicago* spp. (Gonzalez-Rizzo et al. 2006; Plet et al. 2011) or *Lotus* spp. (Murray et al. 2007). It may be noted that the additional role of *HK1* in regulating the transition between meristematic and cell differentiation during RNS was first noted in the *Medicago cre1* mutants (Plet et al. 2011). However, RNAi of *MtCRE1* did not reveal any such sign of incomplete differentiation and, like *L. japonicus hit1* mutants, could only demonstrate the role of this HK in the early stages of inception (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Plet et al. 2011). In *A. hypogaea*, the IZ in *AhHK1-RNAi* nodules were densely packed with infected cells with distinctively high mitotic index (Table 1). This was in accordance with significantly high expression of a mitotic cyclin *AhCycB2.1* in *AhHK1-RNAi* nodules (Fig. 6I). This is similar to the observations in

Arabidopsis RAM, where an inhibitory role of cytokinin is demonstrated to control the number of cell divisions that occur prior to differentiation (Takatsuka and Umeda 2014). Also, reductions in expression of the cytokinin receptor in *Arabidopsis* cause higher numbers of dividing cells in RAM (Dello Ioio et al. 2008). Cytokinin signaling is also known to activate retinoblastoma-related proteins that restrain the cell cycle in RAM of *Arabidopsis* for promoting differentiation (Perilli et al. 2013) but its role in nodule meristem is unknown. The proliferative state in the *AhHK1-RNAi* nodules that was associated with low expression of *AhRBR* suggests that the function of RBR proteins in restraining cell division could be conserved during nodule development, or low expression of *AhRBR* could also be a consequence of the aberrant morphology of the RNAi nodules (Fig. 6J).

Ultrastructure analysis of *AhHK1-RNAi* nodules revealed the IZ cells to have some characteristic features of pluripotent stem cells such as high nucleocytoplasmic ratio, spherical nucleus, and multiple nucleoli (Verdeil et al. 2007). Additionally, we noted binucleated IZ cells in the *AhHK1-RNAi* nodules, which was previously reported for *A. hypogaea* in *AhCCamK-RNAi* nodules and for *P. sativum* in the early primordia stage (Sinhroy and DasGupta 2009; Torrey and Barrios 1969). Therefore, it is possible that these cells have retained some features of the dedifferentiated cells that continued to proliferate due to either a delay in the process of nodule development or a lack of signal for differentiation. The observed undifferentiated state of *AhHK1-RNAi* nodules could be correlated with *AhWOX5*, because its expression was significantly higher in the ineffective *AhHK1-RNAi* nodules as compared with the nodules developed in vector-transformed roots that were fully mature and effective (Fig. 6K). WOX TF are central regulators of stem cell maintenance in different meristem types and are also expressed in nodule meristems of *M. truncatula* (Franssen et al. 2015). In *Arabidopsis* RAM, *AtWOX5* expression decreases with the onset of differentiation and, conversely, its ectopic expression induces a stem-cell-like program in differentiated cells (Sarkar et al. 2007). It may be noted that, in RAM, cytokinin is known to negatively regulate *AtWOX5* expression (Zhang et al. 2013). Because higher expression of *AhWOX5* is associated with silencing of cytokinin signaling in *AhHK1-RNAi* nodules, cytokinin-regulated repression of *AhWOX5* might be conserved in nodule meristems.

Overall, our observations indicate that *AhHK1*-mediated cytokinin signaling is essential for both inception of nodules and their transition from proliferating to a differentiated cell fate during aescynomenoid nodule development. Interference of *AhHK1* was associated with lower expression of factors such as *AhRBR* that restrain proliferation to promote differentiation of plant cells (Perilli et al. 2013). Under the same conditions, expression of *AhWOX5* was significantly elevated, which is consistent with the undifferentiated state of *AhHK1-RNAi* nodules. A role of cytokinin in nodule differentiation was first hinted at in *M. truncatula* (Plet et al. 2011) but whether factors such as RBR and WOX5 are functionally conserved in root nodule meristems is a subject for future investigation.

MATERIALS AND METHODS

Plant and rhizobial strains.

A. hypogaea JL24 strain seed is from ICRISAT, India; *Agrobacterium rhizogenes* R1000 from C. Somerville, Stanford University; binary vector pK7GWIWG2D(II) from Ghent University, Belgium; and *Bradyrhizobium* sp. SEMIA6144 from Adriana Fabra, Universidad Nacional de Rio Cuarto, Cordoba, Argentina.

Cloning of *A. hypogaea AhHK1, AhHK2, and AhHK3*.

A. hypogaea HK were amplified from cDNA prepared from nodulated roots of *A. hypogaea* and cloned following

standard procedures (Sinharoy and DasGupta 2009). *AhHK1* was isolated by amplification of nodule cDNA using a de-generate priming approach based on legume *HK1* sequences. Primers 5'ATGGBTYTKGGGTTTSARGATG3' and 5'CAA CVKCRTRKTANAGATTYTSTTC 3' were used. Similarly, *AhHK2* and *AhHK3* were amplified using primers 5'ATGTY TGTSAAATAWAGNAWTTTCAGG3' and 5'-CSCTTCBTCAA GCNTAGTTCATAG-3' for *AhHK2* and 5'ATGSGTTTGWTC CATNTTGNTGGG-3' and 5'CGGTTWTCCSABCWTCNT GA-3' for *AhHK3*.

Phylogenetic analysis.

A neighbor-joining tree was generated by MEGA 5.2 (Tamura et al. 2011), with protein sequences of *AhHK1*, *AhHK2*, and *AhHK3* along with their orthologs available in the online database based on CLUSTALW sequence alignment. For the RR, the neighbor-joining tree was generated with nucleotide sequences of *AhRR3*, *AhRR5*, and *AhRR9* along with their orthologs from *M. truncatula* and *L. japonicus* based on CLUSTALW sequence alignment. The sequences used for generating the phylogenetic tree are listed in Supplementary Table S2.

Constructs.

The *AhHK1-RNAi* construct was generated by amplification from *AhHK1* cDNA template with primers 5'-CACCAACCA TGTCCATGCCCTTGCC-3' and 5'-GCAAGTTGACCAAGTAA ATTCTCCAC-3' complementary to CHASE domain, 522 bp in length. Amplified fragments were cloned into pENTR/D-TOPO (Life Technologies) and then into binary vectors pK7GWIG2D (II) (Karimi et al. 2002) by gateway technology through the LR Clonase reaction kit (Life Technologies). Constructs are then transformed in *Agrobacterium rhizogenes* strain R1000 for further experiments.

Transformation with *A. rhizogenes* and nodulation of transgenic hairy roots.

Transformation was done according to (Sinharoy et al. 2009). Transformed and untransformed roots in the composite transgenics were differentiated from each other by the expression of the visible marker enhanced GFP by a Leica M205FA stereofluorescence microscope equipped with a Leica DFC310FX digital camera (Leica Microsystems) and are referred to in the article as GFP positive. The selected composite transgenic plants were transplanted either in agar plates containing half-strength Murashige and Skoog (1/2 MS; Hi-media) or vermiculite pots and the plants were maintained in a growth chamber at 26°C on a schedule of 16 h of light and 8-h of darkness under 60 mmol/m²/s. Nodulation was scored 1, 2, 3, 4, and 5 WAI for RNAi and control plants. Nitrogen fixation of control and RNAi-transformed roots bearing a similar number of nodules was assayed using nitroge-nase acetylene reduction Sinharoy and DasGupta (2009).

Phenotypic analysis and confocal microscopy.

Images of whole-mount nodulated roots were captured using a Leica M205FA stereofluorescence microscope equipped with a Leica DFC310FX digital camera (Leica Microsystems). Detached nodules were embedded in Shandon cryomatrix (Thermo Scientific) and sliced into 30-µm-thick sections with a CM1850 rotary cryomicrotome (Leica Microsystems). For confocal microscopy, sample preparation was done according to Haynes and associates (2004) and Sinharoy and DasGupta (2009). Sections were stained with Calcofluor (Life Technologies), propidium iodide (Life Technologies) and Syto9 (Life Technologies). Images were acquired with a Leica TCS SP5 II AOBs confocal laser-scanning microscope (Leica Microsystems). All digital micrographs were processed using Adobe Photoshop CS5.

Scanning electron microscopy.

Scanning electron microscopy (SEM) was performed as described by Sinharoy and associates (2009), with some modifications. Whole nodulated roots harvested 3 WAI (control) or 5 WAI (RNAi) were vacuum infiltrated with 2.5% (vol/vol) glutaraldehyde (Sigma-Aldrich) in 0.1 M PIPES (pH 7.2) buffer

(Sigma-Aldrich) and incubated for 1 h at room temperature. They were rinsed twice (30 min each) in 0.1 M PIPES (pH 7.2). The harvested nodules were dehydrated through a series of ethanol gradients (20, 40, 60, 75, 80, 90, and 100%) for 1 h each at room temperature. Nodules were then transferred to 100% chloroform through a gradient of ethanol/chloroform (3:1, 1:1, and 1:3 [vol/vol]) mixtures. Paraplast (Sigma-Aldrich) was dissolved in chloroform (Sigma-Aldrich) for embedding the tissue and then incubated at 37 and 60°C for 2 h each. Sections (20 µm) were prepared using rotary microtome RM2235 (Leica Microsystems) and placed on poly-L-lysine-coated slides (Sigma-Aldrich). Paraplast was removed with xylene. SEM was done using a Zeiss EVO 18 Special Edition (Carl Zeiss).

RNA extraction, cDNA synthesis, and quantitative reverse-transcription polymerase chain reaction.

Frozen root samples and detached nodules and primordia were ground in liquid nitrogen and total RNA extraction was performed with a NucleoSpin RNA (Macherey-Nagel) plant kit. RNA quality and quantity were analyzed with a NanoDrop spectrophotometer (Thermo Scientific). Total RNA (500 ng) was reverse-transcribed by using Super-ScriptIII RT (Life Technologies) and oligo (dT)₁₇. RNA quantity from each sample in each biological replicate was standardized prior to first-strand cDNA synthesis. Quantitative reverse-transcription polymerase chain reaction (PCR) was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems), with primers designed using Oligoanalyzer software (Integrated DNA Technology) (Supplementary Table S3). Calculations were done using the $\Delta\Delta$ cycle threshold method, with *AhActin* as the endogenous control, whose constitutive expression was verified against another housekeeping gene *AhUbiquitin* (*AhUBI2*) (Supplementary Fig. S6). The reactions were run in the Applied Biosystems 7500 Fast HT platform as follows: 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 5 min; 40 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s; followed by melt curve analysis at 1 cycle at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. A negative control without cDNA template was checked for each primer combination. Nucleotide sequence data for the genes used in this article can be found in the GenBank data library under the following accession numbers: *AhARR3* (KT820784), *AhARR5* (KT820785), *AhARR9* (KT820786), *AhNIN* (KT820787), *AhWOX5* (KT820790), *AhENOD40* (KY401676), *AhCYCLOPS* (JQ839282.1), *AhREMORIN* (SYMREM1) (KT988038), *AhEFD1* (KT962993), *AhHK1* (KT153625), *AhActin* (EU982407), *AhHK2* (KY368747), *AhHK3* (KY368748), *AhRBR* (KT820789), *AhCycB2.1* (MF784810), and *AhUBI2* (MF784809).

Cytokinin treatments.

Ten embryonic axes were grown on 1/2 MS (Hi-media) plates for 10 days at 26°C on a schedule of 16 h of light and 8-h of darkness at 60 mmol/m²/s. After 10 days, rooted embryonic axes were treated with 10⁻⁸ M BAP (Sigma-Aldrich) for various

Fig. 6. Characteristics of the undifferentiated *AhHK1-RNAi* nodules. Analysis of nodules collected from control vector-transformed roots at 3 weeks after infection [WAI] and *AhHK1-RNAi* roots at 5 WAI. Ratio of diameters of **A**, nucleus/cytoplasm; **B**, nucleus/cell; **C**, nucleolus/cell; and **D**, nucleus/nucleolus. Data scored from 200 infection zone (IZ) cells and 50 surrounding uninfected zone (SUZ) cells from 15 nodules. Error bars represent mean $\pm$ standard error of the mean. A two-way analysis of variance followed by Bonferroni posttests were used to assess significant differences, where *** and ** indicate $P < 0.001$ and 0.01, respectively. Scanning electron microscopy image of an infected cell from the IZ of **E**, control and **F** to **H**, *AhHK1-RNAi* nodules. Arrow indicates spherical symbiosome (E) or rod-shaped rhizobia (F to H); n = nucleus and nu = nucleolus. Scale bar = 2 µm (E to H). Quantitative reverse-transcription polymerase chain reaction analysis of **I**, *AhCycB2.1*; **J**, *AhRBR*; and **K**, *AhWOX5* with progress of nodulation in control vector-transformed roots (1 to 3 WAI) and *AhHK1-RNAi* roots (1 to 5 WAI). For control roots, UI = uninfected roots; 1 to 2 WAI = nodulated roots, and 3 WAI = Control nodules. For *AhHK1-RNAi* roots, UI = uninfected roots, 1 to 2 WAI = infected roots, 3 to 4 WAI = primordia containing roots, and 5 WAI = RNA interference (RNAi) nodules. *AhACTIN* was used as a reference gene. Histogram represents an average of three biological replicates, each having $n > 4$ plants, and error bar represents standard deviation. Wilcoxon matched-pairs signed rank test was used to assess significant differences, where * indicates $P < 0.05$. *AhWOX5* was localized by in situ hybridization using antisense probes in indicated tissue sections. Nodule primordia: **L**, control at 1 WAI and **N**, *AhHK-RNAi* at 3 WAI. Nodules: **M**, control at 3 WAI and **O**, *AhHK-RNAi* at 5 WAI. Signal corresponds to precipitate marking the activity of the alkaline phosphatase detection system; v = vascular bundle, iz = infection zone, and suz = surrounding uninfected zone. Scale bar = 250 µm (L to O).

incubation times and maintained under the same growth conditions. Roots were collected at the indicated time points and immediately frozen in liquid nitrogen for RNA extraction.

In situ hybridization.

The in situ hybridization probe for *AhWOX5* was created by first amplifying 648-bp fragments by PCR from a template cDNA prepared from total RNA from inoculated roots. The primer set 5'-ATGCAGACGGTCCGAGATCTGTC-3' and 5'-TTACATGAACTTAAGCGAAGG-3' was used for the amplification. The fragments were inserted into the pGEM-T easy vector by TA cloning (Promega Corp.). Digoxigenin (DIG)-labeled probes were generated with the SP6/T7 DIG-RNA labeling kit (Roche). Probe synthesis, preparation of sections, and in situ hybridization were performed as described previously (Javelle et al. 2011). In situ hybridization signals were analyzed by light microscopy using a Leica M205FA stereofluorescence microscope equipped with a Leica DFC310FX digital camera (Leica Microsystems).

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