SHORT-ROOT paralogs mediate feedforward regulation of D-type cyclin to promote nodule formation in soybean

Chunhua Wang, Meng Li, Yang Zhao, Nengsong Liang, Haiyang Li, Pengxue Li, Liling Yang, Mengyuan Xu, Xinxin Bian, Mengxue Wang, Shasha Wu, Xufang Niu, Mengyao Wang, Xinxin Liu, Yi Sang, Wentao Dong, Ertao Wang, Kimberly L. Gallagher, and Shuang Wu

*College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou 350002, China; College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China; School of Life Sciences, Lanzhou University, Lanzhou 730000, China; Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China; and Department of Biology, University of Pennsylvania, Philadelphia, PA 19104

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Nitrogen fixation in soybean takes place in root nodules that arise from de novo cell divisions in the root cortex. Although several early nodulin genes have been identified, the mechanism behind the stimulation of cortical cell division during nodulation has not been fully resolved. Here we provide evidence that two paralogs of soybean SHORT-ROOT (GmSHR) play vital roles in soybean nodulation. Expression of GmSHR4 and GmSHR5 (GmSHR4/5) is induced in cortical cells at the beginning of nodulation, when the first cell divisions occur. The expression level of GmSHR4/5 is positively associated with cortical cell division and nodulation. Knockdown of GmSHR5 inhibits cell division in outer cortical layers during nodulation. Knockdown of both paralogs disrupts the cell division throughout the cortex, resulting in poorly organized nodule primordia with delayed vascular tissue formation. GmSHR4/5 function by enhancing cytokinin signaling and activating early nodulin genes. Interestingly, D-type cyclins act downstream of GmSHR4/5, and GmSHR4/5 form a feedforward loop regulating D-type cyclins. Overexpression of D-type cyclins in soybean roots also enhanced nodulation. Collectively, we conclude that the GmSHR4/5-mediated pathway represents a vital module that triggers cytokinin signaling and activates D-type cyclins during nodulation in soybean.

nodule primordia | GmSHR4/5 | cell division | cytokinin | GmCYCD6.1

Nitrogen fixation in soybean takes place in root nodules that arise from de novo cell divisions in the root cortex. Although several early nodulin genes have been identified, the mechanism behind the stimulation of cortical cell division during nodulation has not been fully resolved. Here we provide evidence that two paralogs of soybean SHORT-ROOT (GmSHR) play vital roles in soybean nodulation. Expression of GmSHR4 and GmSHR5 (GmSHR4/5) is induced in cortical cells at the beginning of nodulation, when the first cell divisions occur. The expression level of GmSHR4/5 is positively associated with cortical cell division and nodulation. Knockdown of GmSHR5 inhibits cell division in outer cortical layers during nodulation. Knockdown of both paralogs disrupts the cell division throughout the cortex, resulting in poorly organized nodule primordia with delayed vascular tissue formation. GmSHR4/5 function by enhancing cytokinin signaling and activating early nodulin genes. Interestingly, D-type cyclins act downstream of GmSHR4/5, and GmSHR4/5 form a feedforward loop regulating D-type cyclins. Overexpression of D-type cyclins in soybean roots also enhanced nodulation. Collectively, we conclude that the GmSHR4/5-mediated pathway represents a vital module that triggers cytokinin signaling and activates D-type cyclins during nodulation in soybean.

Nodulation in legumes is coordinated by two coordinated processes: rhizobial infection and nodule formation (1, 2). Studies over the past decade have identified key components of the Nod factor-activated signaling pathway in the early symbiotic stage (3–6). Nodule organogenesis starts with new cell divisions in root cortical tissues (4). The root cortex in soybean consists of multiple layers, and the Nod factor-induced cell division occurs first in the outer cortical cells followed by divisions in the inner cortical tissues and the pericycle (7). A number of regulators of Nod factor perception have been identified, including NODULE INCEPTION (NIN) (8), an ERF family protein (ERN), and two GRAS family proteins called Nodulation Signaling Pathway (NSP1 and NSP2) (4–6). Overexpression of NIN in the absence of rhizobia is sufficient to induce cortical cell divisions and leads to spontaneous nodule-like structures (9). ENOD40, a marker gene for nodule primordium initiation, is up-regulated at the onset of nodulation (10–12). These Nod factor-inducible genes can be directly activated by NSP1 (13). The soybean orthologs of all these components have been identified (14).

In addition to the transcriptional regulators, the phytohormone cytokinin (CK) also plays a key role in promoting cortical cell division in the early symbiotic stages (15–25). Depletion of the endogenous CK by overexpressing a CK oxidase/dehydrogenase (CKX) or by repressing expression of the cytokinin biosynthetic gene LONELY GUY 1 (LOG1) dramatically decreases nodulation in Lotus japonicus and Medicago truncatula (25, 26). The crucial role of cytokinin is also evidenced by the effects of overexpressing ARABIDOPSIS THALIANA RESPONSE REGULATOR 5 (ARR5), MtRR4 type-ARR, or Two-Component Signaling Sensor (TCS), all of which increase cortical cell divisions and nodulation (19, 27). Interestingly, gain-of-function mutants of both LOTUS HISTIDINE KINASE 1 (LHK1) (L. japonicus lotus, named snf2) and Cytokinin Response 1 (MtCRE1) result in “spontaneous nodule formation” in the absence of rhizobia (24). Genetic analyses indicate that the spontaneous nodule formation in snf2 requires NIN and NSP2 (24).

As nodules form on roots, it has been hypothesized that nodule organogenesis is derived from the lateral root developmental program (5, 28–31). Recent studies in M. truncatula showed that several transcription factors, including WUS-RELATED HOMEobox 5 (MiWOX5) and MiPLETHORA, are expressed during nodulation in soybean.
and function in the nodule meristem (32–34). In addition, RNA-seq of laser-microdissected nodules showed that close homologs for a series of key root meristem regulators are expressed in nodule primordia, including WOX3, ARABIDOPSIS CRINKLY 4 (ACR4), SHORT-ROOT (SHR), PLT, SCARECROW (SCR), and JACKDAW (JKD) (34). In Arabidopsis roots, SHR and SCR activate the expression of a D-type cyclin, CYCD6;1 to promote the periclinal division, which separates endodermis and cortex cell layers (35). Recently, it was revealed that MsSHR and MsSCR from M. truncatula form the module to drive the cell division in the cortex during nodule formation (36). These findings provided a hint that root developmental programs might be involved in nodulation. However, the function of important root meristem genes in nodule organogenesis has not been fully explored.

In this study, we obtained evidence that two distally evolved paralogs of the GRAS family protein SHR (GmSHR4 and GmSHR5) regulate cortical cell divisions and nodule organogenesis in soybean. After inoculation with rhizobia, GmSHR4/5 were activated mostly in outer cortical cells where the first cell divisions occur. Overexpression of GmSHR4/5 resulted in a substantial increase in nodule formation, whereas GmSHR4/5-microRNA roots formed significantly fewer nodules. The expression of GmSHR4/5 in the cortex enhanced cytokinin signaling and up-regulated the early nodulin genes. Downstream of this cascade were a set of D-type cyclins, which were regulated by a classic feedforward mechanism mediated by GmSHR paralogs. Therefore, our results revealed a mechanism regulating nodule primordia formation in soybean.

Results

GmSHR Paralogs Are Induced in Cortical Tissues at the Early Stage of Nodulation. In *Glycine max*, we identified six SHR homologs (named GmSHR1 to GmSHR6) by BLAST searches using AtSHR protein sequences as the query. The amino acid sequence homologs belong to the five conserved domains of typical GRAS proteins, including LHR1, VHIID, LHRII, PFYRE, and SAW (SI Appendix, Fig. S1). Phylogenetic analysis revealed that GmSHR1, GmSHR2, GmSHR3, and GmSHR6 are closely related to Arabidopsis SHR (AtSHR) and Medicago SHR (Medtr5g15490.1 and Medtr4g097080) (SI Appendix, Fig. S2A). Interestingly, two paralogs that arose during evolution of the soybean genome—GmSHR4 and GmSHR5—are only distally related to AtSHR (SI Appendix, Fig. S2A). These two GmSHR paralogs exhibited a tissue-specific expression pattern distinct from those of the other soybean SHRs (SI Appendix, Fig. S2B). In addition, the expression of GmSHR4 and GmSHR5 (GmSHR4/5 hereafter) in the roots was dramatically enhanced at 1 d after inoculation (dai) with rhizobia and then decreased with the progression of root nodulation (SI Appendix, Fig. S2C). By contrast, GmSHR1 did not appear to be up-regulated until 7 dai and its expression peaked in the mature nodule at 28 dai. Expression of GmSHR2 showed enhancement only in the mature nodule, while that of GmSHR3 and GmSHR6 changed very little during nodulation (SI Appendix, Fig. S2C). These results suggest that GmSHRs might participate in all different stages of nodule formation and GmSHR4/5 could be involved in the initiation stage.

Within the 2-kb promoter regions upstream of GmSHR4/5, we identified many nodulin consensus sequence motifs (NODCON1GM and NODCON2GM) (SI Appendix, Fig. S2D). This implies that GmSHR4/5 expression could be triggered by the rhizobial infection. To understand the spatiotemporal expression pattern of GmSHR4/5 during nodulation, we first examined the nodule organogenesis in soybean roots. We performed histo-logical visualization and divided the early nodule development into six stages (I to VI). We numbered the cortical layers from the outermost to the innermost (C1 to C5). In stages I and II, anticlinal divisions were initiated in the C1 layer and then occurred in C2 (SI Appendix, Fig. S3 A, B, G, and H). In stage III, inner cortical cells started to divide (in C3 to C5), and more anticlinal divisions occurred in the C1 and C2 layers (SI Appendix, Fig. S3 C and F). In stage IV, divisions occurred in the endodermis and periclinal divisions started in the C1 layer (SI Appendix, Fig. S3 D and J). The pattern of cell divisions in the C1 layer also became more complex, leading to the formation of a primordial structure (SI Appendix, Fig. S3 D and J). In stage V, most of the cortical cells at the infection site had divided into smaller cells and cells in the pericycle started to divide (SI Appendix, Fig. S3 E and K). At stage VI, the young nodules distended (SI Appendix, Fig. S3 F and L). The newly formed nodules underwent steady growth and differentiation, in particular forming vascular bundles in stages VII through IX (SI Appendix, Fig. S3 M–O). To verify these histological observations, we performed in situ hybridization of the cell-cycle marker gene GmCyclB1.1. In stage V, the staining first became enriched mostly in the outer cortical cells (SI Appendix, Fig. S3P), followed by a high level of staining in the inner cortical cells, indicating a gradual expansion of mitotic activity during early nodulation (SI Appendix, Fig. S3Q). Consistent with the GmSHR4/5 expression pattern, GmCyclB1.1 expression gradually became undetectable in mature nodules (SI Appendix, Fig. S3 R and S). Further, we constructed promoter:GUS reporters of GmSHR4 and GmSHR5. In soybean roots, GmSHR4 had a weak expression and could occasionally be detected in the endodermal tissue, vascular tissues, and cortical cells in the mature zone (Fig. L4). GmSHR5 was expressed in the meristem (Fig. L1) and confined only in vascular tissues in the mature zone (Fig. L1 J and K). Upon inoculation with rhizobia, the expression of GmSHR4/5 was greatly enhanced (Fig. 1 B, L, and M). GmSHR4/5 expression was enriched in the outer cortical tissues during the early stage of nodulation (stages II to VI) (Fig. 1 C–E, M, and N) and also maintained a weak expression in the inner cortex (Fig. 1 D, E, M, and N). GmSHR4/5 expression gradually disappeared as the nodules matured (Fig. 1 F–H and O–Q). The spatiotemporal expression pattern of GmSHR4/5 shown by the promoter GUS reporters was validated by in situ hybridization (Fig. 1 R–Y), further suggesting the potential involvement of these two SHR paralogs in the early stage of the nodulation. Unlike GmSHR4/5, GmSHR1/2 were expressed only in the vascular tissues (SI Appendix, Fig. S2E).

GmSHR4/5 Promote Cortical Cell Division and Positively Regulate Root Nodulation. To examine GmSHR4/5 function, we manipulated their expression by overexpression (OX-GmSHR4/5 hereafter) and knockdown (GmSHR4-amiR, GmSHR5-amiR, and GmSHR4/5-amiR hereafter) in soybean roots (Fig. 2A and SI Appendix, Fig. S4 A–D). Interestingly, the expression of other GmSHRs appeared to be moderately reduced in the OX-GmSHR4/5 lines and increased in the GmSHR4/5-amiR roots, suggesting the existence of potential feedback inhibition among GmSHRs (SI Appendix, Fig. S4A).

In Arabidopsis, SHR regulates periclinal cell division in cortical initial cells (35, 37). Therefore, we examined the cortical cell layers that are derived from periclinal cell divisions in soybean roots. As expected, the OX-GmSHR4/OX-GmSHR5 roots showed three to six additional layers of cortical tissues, while the GmSHR4/5-amiR roots had fewer (Fig. 2 B–F). In addition, we transformed Arabidopsis with GmSHR4 and GmSHR5 and observed extra cell divisions in the cortical tissues of the transgenic plants (Fig. 2 G–J). These results suggest that GmSHR4/5 can induce cortical cell divisions.

Next, we evaluated the nodulation of soybean roots with altered GmSHR4/5 expression at 28 dai with rhizobia. The total number and dry weight of nodules were significantly higher on OX-GmSHR4/5 roots and substantially lower on GmSHR4/5-amiR roots (Fig. 2J and K and SI Appendix, Fig. S4E), indicating that GmSHR4/5 functioned as the positive regulators of soybean.
nodulation. Interestingly, unlike GmSHR5-amiR, GmSHR4-amiR did not change the nodulation (Fig. 2L). This finding suggests that GmSHR5 could be more involved than GmSHR4 in the initiation of nodule primordia.

To rule out the potential artifacts that can arise from overexpression, we specifically expressed GmSHR4/5 in nodule primordia under the promoter of GmENOD40. Both pENOD40-GmSHR4 and pENOD40-GmSHR5 lines resulted in a higher number of nodules (Fig. 2M–P). The total dry weight of nodules of the pENOD40-GmSHR4 and pENOD40-GmSHR5 lines was 2.72 and 2.19 times that of the control, respectively (Fig. 2Q). These data further support that GmSHR4/5 function in nodule formation.

**GmSHR4/5 Function in Cortical Tissues during the Nodule Initiation.**

The spatial expression pattern of GmSHR genes during nodulation in soybean. (A–H) GmSHR4pro::GUS expression pattern during nodulation. The cross-sections of the roots without rhizobial infection (A) and the roots at 10 dai with rhizobia (B–G) and 20 dai (H) are shown. (I–Q) GmSHR5pro::GUS expression pattern during nodulation. The cross-sections of the roots at 0 dai (I–K), 10 dai (L–P), and 20 dai (Q) are shown. (R–U) In situ hybridization of GmSHR4/5 in roots at 10 dai. (V–Y) The areas in the yellow wireframe are the enlarged views of the boxed regions in R–U. IZ, infection zone. White arrows indicate the nodule parenchyma. Yellow and black arrows indicate the division of the outer cortical cells and inner cortical cells, respectively. White asterisks point to the division of the pericycle cells. Yellow asterisks point to the outer ground tissues. (Scale bars, 100 μm.)

![Fig. 1. Spatial expression pattern of GmSHR genes during nodulation in soybean. (A–H) GmSHR4pro::GUS expression pattern during nodulation. The cross-sections of the roots without rhizobial infection (A) and the roots at 10 dai with rhizobia (B–G) and 20 dai (H) are shown. (I–Q) GmSHR5pro::GUS expression pattern during nodulation. The cross-sections of the roots at 0 dai (I–K), 10 dai (L–P), and 20 dai (Q) are shown. (R–U) In situ hybridization of GmSHR4/5 in roots at 10 dai. (V–Y) The areas in the yellow wireframe are the enlarged views of the boxed regions in R–U. IZ, infection zone. White arrows indicate the nodule parenchyma. Yellow and black arrows indicate the division of the outer cortical cells and inner cortical cells, respectively. White asterisks point to the division of the pericycle cells. Yellow asterisks point to the outer ground tissues. (Scale bars, 100 μm.)](https://doi.org/10.1073/pnas.2108641119)
nodule structure. In addition, divisions throughout the cortex contribute to the formation of nodules.

**GmSHR4/5 Promote Nodule Formation by Activating Cytokinin Signaling.** It is known that cytokinin promotes cortical cell divisions during nodulation (15–24). To dissect the relationship between cytokinin and GmSHRs, we examined the expression of cytokinin-related genes in GmSHR4/5 transgenic systems. It was previously reported that CRE1/HK1 is the major cytokinin receptor involved in the nodulation of *Medicago sativa* and *L. japonicus* (17, 19). In soybean, we identified three HK1 homologs (named *GmHK1-1*, *GmHK1-2*, and *GmHK1-3*). Our qRT-PCR results showed that GmSHR4/5 had different activation targets, with GmSHR4 regulating *GmHK1-1* and GmSHR5 targeting *GmHK1-3*.
Effect on the expression of 6-Benzylaminopurine (6-BA) treatment seemed to have little cytokinin oxidase/dehydrogenase gene that negatively regulates of and GmSHR4/5 amiR (Fig. 4 A–D). When GmSHR4/5 amiR roots were treated with 10 nM 6-BA, their nodulation phenotype was fully rescued (Fig. 4G and SI Appendix, Fig. S6E–H).

To further examine the function of HK1s, we knocked down their expression (GmHK1-1,1-2,1-3-amiR hereafter) in soybean roots (SI Appendix, Fig. S6I). As expected, the total number of nodules was significantly lower on GmHK1-1,1-2,1-3-amiR roots (Fig. 4 H–J and W).

To examine the genetic interactions between SHR and HKI, we combined GmHK1-1,1-2,1-3-amiR with both OX-SHR5 and SHR5/5 amiR roots in a transgenic hair root system (SI Appendix, Fig. S6 J and K). Compared with OX-SHR5 roots, OX-SHR5/ GmHK1-1,1-2,1-3-amiR roots exhibited significantly decreased nodulation, showing the similar phenotypes to GmHK1-1,1-2,1-3-amiR roots (Fig. 4 H–V). Quantification of nodule numbers confirmed the similarity between OX-SHR5/GmHK1-1,1-2,1-3-amiR and GmHK1-1,1-2,1-3-amiR roots, suggesting the potential epistasis of HK1 over SHR5 (Fig. 4X). In addition, no further reduction of nodules was observed in GmSHR4/5-amiR/GmHK1-1,1-2,1-3-amiR lines, suggesting they presumably act in the same pathway.

**Early Nodulin Genes Act Downstream of GmSHR4/5.** Cytokinin interacts with Nod factor signaling to control the expression of nodulin genes during nodulation. Therefore, we next examined how GmSHR4/5 interplay with these symbiotic marker genes (11, 27, 38, 39). We first verified that cytokinin indeed activated the early nodulin genes (SI Appendix, Fig. S4B). Most of the early nodulin genes, including GmNIN, GmNSP1, and GmENOD40, were up-regulated in OX-GmSHR4/5 roots while down-regulated in GmSHR4/5-amiR roots (SI Appendix, Fig. S7A). In situ hybridization showed that GmNIN was mostly expressed in the pericycle cells (SI Appendix, Fig. S7B), along with weak expression in the epidermis in soybean roots (SI Appendix, Fig. S7B). During nodulation, GmNIN was mainly expressed in the dividing cortical cells (SI Appendix, Fig. S7C). Notably, GmNIN expression was noticeably decreased in GmSHR4/5-amiR roots (SI Appendix, Fig. S7 E–G). Conversely, we also tested whether GmSHR4/5 expression was affected by the early nodulin genes. In the soybean roots overexpressing GmENOD40 or GmNSP1, we detected no dramatic change in the expression of GmSHR4 (SI Appendix, Fig. S7H). However, GmSHR5 was surprisingly down-regulated in the presence of high levels of GmENOD40 or GmNSP1 (SI Appendix, Fig. S7H). These findings suggest that GmSHR4/5 likely function upstream of early nodulin genes but that GmSHR5 expression may be subject to feedback inhibition by these genes, which could partially explain why GmSHR5 transcript levels were dramatically reduced during the later stages of the nodulation. Furthermore, our Y1H results showed that GmSHR4 directly targeted the GmNSP1 promoter for activation (SI Appendix, Fig. S7F). Therefore, we concluded that these early nodulin genes are downstream components of the GmSHR4/5 and cytokinin signaling pathway.

**GmCYCD6;1 Homologs Are Functional Components of GmSHR4/5-Mediated Nodulation.** In *Arabidopsis*, SHR regulates pericel cell divisions by promoting the specific expression of a D-type cyclin, CYCD6;1 (35). Therefore, we asked whether this conserved module is part of the developmental program for nodulation in soybean. In the *G. max* genome, there are six GmCYCD6;1 homologs (named GmCYCD6;1-1 to GmCYCD6;1-6). Phylogenetic analysis
revealed that GmCYCD6;1-1 to GmCYCD6;1-4 are closely related to Arabidopsis AtCYCD6;1 but GmCYCD6;1-5 and GmCYCD6;1-6 are distantly related (SI Appendix, Fig. S8A). Transcriptional analysis showed that all GmCYCD6;1s were substantially up-regulated in OX-GmSHR4/5 roots and significantly down-regulated in GmSHR4/5-amiR roots (SI Appendix, Fig. S8B). We further examined the spatial expression patterns of GmCYCD6;1 using in situ hybridization. The results showed that GmCYCD6;1-1, GmCYCD6;1-2, GmCYCD6;1-3, GmCYCD6;1-4, and GmCYCD6;1-5 were all expressed in the vascular tissues (SI Appendix, Fig. S8 C–Q). In contrast, both in situ hybridization and the promoter:GUS reporter showed that GmCYCD6;1-6 expression was mostly enriched in the dividing cortical cells (SI Appendix, Fig. S8 S, U, and V). In mature nodules, GmCYCD6;1-6 was expressed in nodule parenchyma (SI Appendix, Fig. S8W).
Next, we performed a Y1H assay to determine whether the regulation of *GmCYCD6;1-6* by GmSHR4/5 is direct. We tested four regions of the *GmCYCD6;1-6* promoter (P1, B1, B2, and P3), as shown in Fig. 5A. We found that GmSHR4 and GmSHR5 directly bound to the B1 and P1 regions of the *GmCYCD6;1-6* promoter (Fig. 5B). To validate this result, we performed ChIP-qPCR using transgenic hairy roots expressing 35S:*GmSHR4/5-GFP. To detect immunoprecipitated DNA fragments bound by GmSHR4/5-GFP, we designed primers targeting the *GmCYCD6;1-6* promoter (regions A1, A2, and B1) (Fig. 5A). We observed a 2.05-fold enrichment of GmSHR4 in region B1 (Fig. 5C) and 2.48- and 5.15-fold enrichments of GmSHR5 in regions A1 and A2, respectively (Fig. 5D). Despite these results, we failed to detect the activation of *GmCYCD6;1-6* by GmSHR4/5 in the LUC activation reporter system (Fig. 5E).

In *Arabidopsis*, AtSHR was reported to form a complex with AtSCR to activate CYCD6;1 (35). To see whether this mechanism is also conserved in soybean, we next examined the effect of GmSCRs on the expression of *GmCYCD6;1-6*. There are six GmSCR genes in the soybean genome, clustering into four groups in our phylogenetic analysis (SI Appendix, Fig. S9A). Using Y2H assays, we detected physical interactions between multiple GmSHR-GmSCR pairs (GmSHR4 and GmSCR3, GmSHR5 and GmSCR5, and GmSCR4 and GmSCR5) (SI Appendix, Fig. S9B). We next examined whether the various GmSCRs can help activate *GmCYCD6;1-6*. In a LUC reporter system, we indeed detected activation of *GmCYCD6;1-6* when both GmSHR and GmSCR were added (Fig. 5E).

**GmCYCD6;1s Function Downstream of Early Nodulin Genes.** In *OX-GmENOD40*, *OX-GmNSP1*, and *OX-GmNSP2* roots, the expression of *GmCYCD6;1-3* to *GmCYCD6;1-6* was markedly increased, while the expression of *GmCYCD6;1-1* and *GmCYCD6;1-2* seemed to be increased only in *OX-GmENOD40* and *OX-GmNSP2* roots (Fig. 6A). Although the transcriptional analysis supported that *GmCYCD6;1s* function downstream of early nodulin genes, *GmCYCD6;1* seemed to have a feedback effect on nodulin gene expression. When *GmCYCD6;1-2* was overexpressed in soybean roots (Fig. 6B), the expression of *GmNSP1* and *GmNSP2* was up-regulated (Fig. 6B). Interestingly, the spatial expression pattern of p*GmENOD40-GUS* was similar to that of p*GmCYCD6;1-6-GUS* (SI Appendix, Figs. S8 R–W and S9 C–F).

Cytokinin is a key regulator of root nodulation and the nodulin genes (23, 26). In soybean roots, cytokinin also markedly activated the expression of *GmCYCD6;1-3* (SI Appendix, Fig. SSC). We next examined the role of the *GmCYCD6;1* genes in soybean root nodulation. We manipulated the expression of all three of the *GmCYCD6;1* genes by overexpression and microRNA-mediated knockdown (SI Appendix, Fig. S10 A and B). As shown in SI Appendix, Figs. S7 C–R and S9 C–J, overexpression of *GmCYCD6;1-2*, *GmCYCD6;1-3*, and *GmCYCD6;1-6* all significantly increased nodulation, as evidenced by increased nodule number, total nodule fresh weight, and nodule size. By contrast, knockdown of any of these three *GmCYCD6;1* genes resulted in inhibition of nodulation. These results indicate that the *GmCYCD6;1* genes function as positive regulators of soybean nodulation downstream of the early nodulation genes.
Spatially Separated Cortical Cell Divisions Account for the Formation of Nodule Primordia and Vascular Founder Cells. The two distinct locations of cortical cell divisions in the early stage of nodulation prompted us to hypothesize that the cell divisions in the outer cortical layers are mainly responsible for the formation of nodule primordia and the divisions in the inner cortex produce the founder cells for the vascular tissues of the nodules. To test this hypothesis, we first examined the expression of GmSHRI

Fig. 6. D-type cyclins act downstream of early nodulin genes. (A) The relative expression of GmCYCD6;1s in EV and the OX-GmNSP1, OX-GmNSP2, and OX-GmENOD40 lines. The RNA was extracted from the noninoculated roots. GmEF1α (accession no. X56856) was used as the internal control. Significant differences are observed between transgenic plants and EV plants (**P < 0.01, *P < 0.05; Student's t-test). Error bars represent SD. D6, CYCD6 (n = 3). (B) The relative expression of early nodulin genes in EV and the OX-GmCYCD6;1-2, OX-Gm CYCD6;1-3, and OX-CYCD6;1-6 lines. GmEF1α (accession no. X56856) was used as the internal control. Significant differences are observed between transgenic plants and EV plants (**P < 0.01, *P < 0.05; Student's t-test). Error bars represent SD (n = 3). (C–G) Nodulation in OX-GmCYCD6;1-6 roots. D6, CYCD6. (D and F) Close-up view of the boxed areas in C. (Scale bars: C, 2 cm; D and F, 5 mm.) D6, GmCYCD6. (H-L) Nodulation in CYCD6;1-2/3/6-amiR roots. (I and K) Close-up view of the boxed areas in H. (Scale bars: H, 2 cm; I and K, 5 mm.) D6, GmCYCD6. (M–R) The quantification of the nodulation in OX-CYCD6;1-6 roots and CYCD6-1;2/3/6-amiR. Shown are nodule number (M and P), nodule fresh weight (N and Q), and nodule size (O and R) (n ≥ 18). Significant differences are observed between transgenic plants and EV plants (**P < 0.01, *P < 0.05; Student's t-test). Error bars represent SD. n represents the number of independent biological samples. All experiments were repeated three times.
in nodule, a close homolog of AtSHR, which is expressed when the vascular founder cells are initiated in the globular stage of embryo development (39). Similar to AtSHR, GmSHR1 had a specific expression in the stele (SI Appendix, Figs. S2E and S1A). The GmSHR1 was not induced by the rhizobia inoculation, but showed patchy expression underlying the newly formed nodule primordia (SI Appendix, Fig. S11B–D). This expression persisted and gradually became restricted to the newly formed vascular tissues of the nodule (SI Appendix, Fig. S11E and F). In line with this hypothesis, knockdown of GmNSP4/5 considerably disrupted the cell divisions in inner cortex and affected the formation of nodule vascular tissues (Fig. 3J–L).

In the outer cortex, repeated cell divisions drove the emergence of primordia-like structures from the soybean roots (SI Appendix, Fig. S3 E, K, and P). This bulging tissue was similar in morphology to the lateral root primordia. We therefore tested whether the primordia-like structures have stem cells by examining the expression of the marker gene GmWOX5. In situ hybridization showed that expression of GmWOX5 was initiated in the early stage of nodulation, when the bulging structures were formed (SI Appendix, Fig. S11G). The spatial expression of GmWOX5 in these structures was very similar to that in lateral roots, with enrichment in a few cells located at the top of the bulging tissue (SI Appendix, Fig. S11H). In the mature nodule, GmWOX5 expression was maintained, but was mostly restricted to the nodule parenchyma (SI Appendix, Fig. S11J).

Discussion
Several lines of evidence have shown that nodule organogenesis is evolutionarily related to root development (5, 28, 29). Genes important for regulating root stem cells, including WOX5 and PLETHORA (PLT1-4), play vital roles in the establishment of root nodule primordia (32–34). Nodule-specific knockdown of MtPLETHORA genes results in a reduced number of nodule primordia and generates nodules with inactive meristems. Similar to lateral root development, nodule organogenesis starts from oriented cell divisions in root cortical tissues. However, we have very limited understanding of the mechanism behind this cortical cell division during nodulation. In Arabidopsis roots, SHRSCR-CYC6D1 forms a regulatory module that spatiotemporally promotes periclinal cell divisions in cortex/endodermis initials (35). There is also evidence that AtSHR and rice SHR homologs can induce periclinal cell divisions in cortical tissues in root meristem (40, 41). The first cell division of a determinate nodule (such as those of soybean) occurs subepidermally in the outer cortex (42, 43). In this study, we provide evidence demonstrating that two SHR paralogs in soybean regulate nodulation by promoting cortical cell divisions in the cortex.

We identified six SHR homologs in soybean, which were differentially expressed in roots, stems, leaves, and nodules, implying that GmSHR could participate in a wide range of developmental processes in soybean. Four of the soybean SHR homologs are closely related to AtSHR and MsSHR1 (Medtr5g015490) (36) and exhibited a similar vascular expression pattern, while two distal paralogs (GmSHR4 and GmSHR5) had a distinct expression pattern. This implies that subfunctionalization of SHR homologs may have occurred during soybean evolution. Interestingly, GmSHR4 and GmSHR5 exhibited high levels of expression in the initial stage of nodule development, when nodule vascular tissues had not yet formed. In contrast, GmSHR1 and GmSHR2 were also induced during nodulation but were specifically expressed in the vascular tissues of the mature nodules. These results suggest that GmSHR family members may differentially regulate different stages of soybean nodulation. Upon the Rhizobium infection, the expression of GmSHR4/5 was activated in outer cortical cells, where nodule primordia were initiated. In the outer cortical cells, GmSHR5 seemed to play a more dominant role as the inhibition of GmNSP4/5 affected the formation of the nodule primordia. However, GmNSP4 and GmNSP5 could also be functionally redundant during nodulation. The overexpression of either of these two GmSHR genes substantially improved nodulation, suggesting that they could functionally replace each other.

Previous studies revealed that cytokinin signaling interacts with Nod factors to promote cortical cell division during nodulation (24). Although we know that a number of early nodulin genes are involved, the detailed mechanism of this stimulation of cortical cell division remains a mystery and it is not clear how the upstream signaling cascade is transduced to regulate cell division. Our results provide evidence that soybean GmSHR4/5 are a signaling component downstream of Nod factor perception but upstream of cytokinin as well as early nodulin genes, including GmNSP1, GmNIN, and GmENOD40. Interestingly, this regulation seems to involve a complex network rather than a linear cascade. A recent report showed increased MtSHR1 expression after 6-BA treatment in Medicago, and 6-BA effect seemed to be abolished in MtSHR1-SRDX hairy roots (36). However, in soybean roots, 6-BA treatment was insufficient to promote GmSHR4/5 expression and genetic analysis showed that GmHK1 presumably functions downstream of GmSHR4/5 during the nodulation in soybean. This difference might reflect the differential mechanism between indeterminate nodules and determinate nodules.

The expression of GmSHR5 could be suppressed when GmNSP1 and GmENOD40 were overexpressed. This potential feedback loop is also evidenced by the differential ability of GmSHR4 and GmSHR5 to induce nodules. Compared to the substantially increased nodule number induced by overexpression of GmSHR4, overexpression of GmSHR5 only moderately enhanced nodulation, which may reflect the feedback inhibition of GmNSP1 on GmSHR5. Both GmSHR4 and GmSHR5 seemed to play roles only in the early stage of nodulation, as their expression was hard to detect in mature nodules. Therefore, GmSHR4/5 could represent a key regulatory module of NFR-mediated early symbiotic events.

ENOD40 was previously shown to promote cortical cell division (44). However, the link between ENOD40 and downstream mitosis is not known. Here we showed that GmENOD40 induced the expression of D-type cyclins. Considering the role of the Arabidopsis D-type cyclin homolog AtCYCD6;1 in promoting periclinal cell division, it is likely GmENOD40 promotes cortical cell division during nodulation by inducing GmCYCD6;1 expression. In line with this, GmCYCD6;1 expression in soybean roots was also enhanced by the application of cytokinin, which could induce spontaneous nodules without rhizobial infection.

In Arabidopsis, it was discovered that AtSHR regulates the development of vascular tissues by repressing cytokinin biosynthesis (45). Interestingly, in poplar, PhSHR1 seemed to increase cytokinin biosynthesis to promote both primary (height) and secondary (girth) growth (46). Soybean has six SHR homologs that can be clustered into two groups. GmSHR4/5 not only are distally related to one another but also have a number of early nodulin genes phylogenetically, but also exhibited expression patterns distinct from the other GmSHR genes. This suggests that the GmSHR genes have likely undergone subfunctionalization during their evolution. Through multiple assays, we showed that soybean SHR4s promote nodulation by enhancing both cytokinin biosynthesis and signaling. In addition, GmSHR4/5 seemed to promote GmCYCD6;1 expression by directly binding to its promoter. This direct activation mechanism appears to be evolutionarily conserved as a similar mechanism also exists in Arabidopsis roots. Finally, GmSHR4/5 can also activate the expression of D-type cyclins via the early nodulins GmNIN and GmENOD40. The two SHR-mediated cascades that activate D-type cyclins form a classic feedforward loop to ensure the progression of nodule organogenesis (SI Appendix, Fig. S12).
Additional materials and methods are described at length in SI Appendix, SI Materials and Methods.

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