miR393 and miR164 influence indeterminate but not determinate nodule development

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The roles of auxin in the regulation of symbiotic legume nodule formation are unclear. We recently showed that enhanced sensitivity to auxin resulting from overexpression of miR160 inhibits determinate nodule formation in soybean. We examined the roles of miR393 and miR164 in soybean (that forms determinate nodules) and Medicago truncatula (that forms indeterminate nodules). Our results together with previous studies suggest that indeterminate nodule formation requires a higher, but narrow window of auxin sensitivity and that miR164 regulation is not crucial for determinate nodule formation.

Symbiotic nodules are root lateral organs that result from interactions between leguminous plants and nitrogen-fixing rhizobia bacteria.1 There are 2 major types of nodules formed in legume roots: indeterminate and determinate.2,5 Indeterminate nodules are characterized by the presence of a persistent nodule meristem analogous to lateral roots. The types of initial cell divisions and meristem formation are also similar between lateral roots and indeterminate nodules. Indeterminate nodules are elongate due to the addition of new cells to the distal end of the nodule from the meristem. Examples of plants that form indeterminate nodules include temperate legumes viz pea, Medicago truncatula and clover. In contrast, determinate nodules are spherical and lack a persistent nodule meristem. There is no sustained cell division during determinate nodule development, and cell expansion rather than cell division results in nodule growth. Examples of plants producing determinate nodules include tropical legumes viz soybean, common bean, and Lotus japonicus. Expression of auxin-response markers at the sites of initiation of both these types of nodules indicates that auxin might play a role in their development.6,5 However, inhibition of polar auxin transport occurs at the site of indeterminate nodule initiation, but not determinate nodule initiation.6,7 Such inhibition of auxin transport is also crucial for indeterminate nodule development. Indeed, silencing of PIN auxin transporter gene expression results in reduced number of nodules in M. truncatula.8 The persistent nature of the meristem and requirement of auxin transport machinery are properties shared by lateral roots and indeterminate nodules. However, our knowledge on the role(s) of auxin in nodule development is minimal.

The majority of auxin responses are governed by the TIR1-like F-box family of auxin receptor proteins. Under low auxin conditions, auxin-responsive gene expression is repressed by Aux-IAA proteins that bind to and inhibit auxin response factor (ARF) transcription factors. Auxin perception by the auxin receptors causes degradation of Aux/IAA proteins resulting in auxin-inducible gene expression.9 A class of repressor ARF transcription factors that compete for promoter-binding with the activator ARFs above also regulate auxin-inducible gene expression. These, however, might act independent of TIR1.10 In addition, the auxin-dependent transcription factor NAC1 specifically plays a role in lateral root initiation.11 The auxin receptor gene family, several families of ARF transcription factors, and NAC1 family members are under the control of regulation by microRNAs (miRNAs).12 We recently overexpressed miRNAs that regulate auxin signaling components in soybean and demonstrated that hypersensitivity to auxin inhibits determinate nodule formation.13 We overexpressed miR393 to silence the auxin receptor gene family. This resulted in reduced sensitivity to auxin, but did not inhibit nodulation. On the other hand, suppression of ARF10-family of repressors (by overexpressing miR160) resulted in enhanced sensitivity to auxin and inhibition of nodule development apparently through a reduction in cytokinin sensitivity. These results suggested that determinate nodule development perhaps requires very low auxin sensitivity consistent with a lower level of auxin-inducible gene expression observed during determinate nodule initiation. Indeterminate nodules possess a persistent nodule meristem with a higher expression of auxin-inducible gene expression. This suggested that indeterminate nodules might require a higher level of auxin sensitivity for proper development. In this study, we tested this hypothesis by overexpressing miR393 in M. truncatula composite plants and examining nodule development. In addition, miR164 that plays

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a role in LR development also regulates indeterminate nodule formation. We tested its role in determinate nodule formation by overexpressing in soybean composite plants. Our results suggest that these miRNAs influence the development of indeterminate nodules, but not determinate nodules.

Distinct expression profiles of miR393 and miR164 in soybean and *Medicago truncatula* during nodule development

We examined the expression profiles of miR393 and miR164 in soybean and *M. truncatula* roots along a time-course of rhizobium inoculation. Whole roots were harvested from plants inoculated with the appropriate rhizobia (*B. japonicum* for soybean and *S. meliloti* for *M. truncatula*) at different time-points and miRNA expression assayed by stem-loop qPCR. Expression levels were normalized to U6 and the 0 h time point. We also included mock-inoculated controls for each time point. Comparing fold change of miRNA expression in inoculated roots vs. control roots at each time point indicated that miR393 levels showed only a moderate change in expression levels along the time course in soybean (Fig. 1A). In contrast, in *M. truncatula* roots there was a clear (2–7 fold), but transient increase in miR393 levels at 6 h post inoculation (hpi). Subsequently, miR393 levels showed an increase at 7 d post inoculation (dpi; Fig. 1B). The levels of miR164 showed a clear but small decrease early during the time-course in soybean (at 1 and 3 hpi) before returning to basal levels by 6 hpi (Fig. 1C). In *M. truncatula* roots, miR164 levels showed 2 transient increases at 6 and 24 hpi (Fig. 1D). We observed considerable variation in miRNA expression between replicate experiments at certain time points likely due to the use of whole roots rather than nodule development zones for expression assays.

Nevertheless, these data suggested that auxin sensitivity and action are more dynamically regulated during nodule development in *M. truncatula* roots by these miRNAs.

Overexpression of miR393 and miR164 in soybean and *M. truncatula*

The distinct expression profiles of these miRNAs prompted us to examine their roles in determinate vs. indeterminate nodule development. We reported a reduced sensitivity to auxin in soybean roots overexpressing miR393, but this did not influence determinate nodule development. We overexpressed miR393 in *M. truncatula* composite plant roots using *A. rhizogenes*-mediated transformation. miR393 precursor was driven by the constitutive Cassava Vein Mosaic Virus CVP2 promoter (CsVMV). These roots had ~4–5 fold higher levels of mature miR393 (data not shown). We examined LR density and root growth as a measure of auxin sensitivity. As expected, miR393-overexpressing *M. truncatula* roots had slightly longer roots (Fig. 2A) and significantly reduced LR density (Fig. 2B) suggesting that these roots were indeed hyposensitive to auxin. We inoculated these plants with *S. meliloti* and counted the number of nodules at 14 dpi. In contrast to results from soybean, miR393 overexpression resulted in a significant reduction in nodule numbers in *M. truncatula* (Fig. 2C). This suggested that auxin perception by TIR1/AFBs is crucial for indeterminate nodule formation. We conclude that the level of auxin sensitivity required for indeterminate nodule formation is higher than that required for determinate nodule formation.

Overexpression of miR164 reduced nodule formation in *M. truncatula*. We overexpressed miR164 in soybean composite plant roots to examine its role in determinate nodule development.
Overexpression of miR164 precursor resulted in a 15–20 fold increase in mature miR164 levels in these roots (data not shown). As reported previously in Arabidopsis and *M. truncatula*, overexpression of miR164 had no effect on primary root length (Fig. 3A), but resulted in reduced LR density (Fig. 3B). We inoculated these plants with *B. japonicum* and examined nodule formation 14 dpi. In contrast to results from *M. truncatula*, overexpression of miR164 did not significantly affect nodulation in soybean (Fig. 3C). This suggested that NAC-proteins regulated by miR164 do not play a crucial role in determinate nodule formation, but do regulate indeterminate nodule formation. We cannot rule out the possibility that other NAC1 homologs that are not under miR164 regulation might play a role in soybean nodule development.

In summary, comparison of results from miR393ox experiments indicate that the basal level of auxin sensitivity required for the formation of indeterminate nodules is higher than that of determinate nodules. Interestingly, it appears that enhanced sensitivity to auxin inhibits the formation of both these types of symbiotic legume nodules. Enhanced sensitivity to auxin resulting from overexpression of miR160 inhibited determinate nodule formation. Overexpression of miR160 also inhibited nodule formation in *M. truncatula* that forms indeterminate nodules. The results suggest that a relatively narrow window of high auxin concentration/sensitivity is required for proper development of indeterminate nodules. In contrast, determinate nodules can develop over a larger window.
of relatively low auxin concentration/sensitivity. Examination of spatio-temporal auxin signaling and activity domains of specific miRNAs will reveal how miRNA regulation achieves precise auxin activity required for proper nodule development.

Author Contributions

M. truncatula experiments were performed by Mao G and Yu O. Soybean experiments and qPCR assays were performed by Turner M and Subramanian S. The study was conceived and coordinated by Yu O and Subramanian S.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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