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The *M. truncatula SUNN* gene is expressed in vascular tissue, similarly to *RDN1*, consistent with the role of these nodulation regulation genes in long distance signaling

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Encoding a conserved protein of unknown function, the *Medicago truncatula RDN1* gene is involved in autoregulation of nodulation through signaling in the root. In contrast, the SUNN kinase in *M. truncatula* has been shown by grafting of mutant scions to control nodule number in the root by communication of a signal from the shoot to the root. GUS staining patterns resulting from expression of the *SUNN* promoter fused to *uidA* showed expression of *SUNN* in most parts of plant including the root, but confined to the vascular tissue, a pattern that overlaps with that published for *RDN1*. Real Time qRT-PCR analysis showed levels of both *SUNN* RNA and *RDN1* RNA did not change significantly during early nodulation signaling (0–72 h after inoculation). The similarity in expression in cell types strongly suggests vascular signaling for nodule number regulation, while the lack of changes over early nodule development suggest post transcriptional mechanisms such as protein association or phosphorylation transmit the signal.

The symbiosis between legumes and rhizobia involves signaling, response and regulation by both the bacterial and plant partners. The regulation of nodule number by the plant, termed "autoregulation," includes a long distance signal transduction pathway involving both the shoot and the root. Because supporting active nodules has an energy cost to the plant in the range of 12–17 g of carbon per gram of nitrogen obtained,[1] regulation of nodule number by the plant presumably balances the need for fixed nitrogen with the cost of supporting the bacteria. Isolation of plant mutants unable to regulate the number of nodules that form on the roots identified multiple genes encoding proteins that control nodule number from different parts of the plant (for review see[2,3]). Grafting experiments in which mutant shoots were grafted onto wild type roots and the reciprocal experiments of wild type shoots grafted onto mutant roots revealed that for some genes, the genotype of the shoot determined the number of nodules that formed on the root, while for other genes, it was the genotype of the root which determined the number of nodules that formed.[2,3] We recently identified a gene, *RDN1* (*ROOT DETERMINED NODULATION 1*), that controls nodule number from the root but is expressed in both shoots and roots.[4] A fusion of the *RDN1* promoter to the *uidA* (*GUS*) gene gave staining in the vasculature of transgenic roots.[4]

The *SUNN* gene in *M. truncatula* encodes a leucine-rich repeat receptor kinase that controls nodule number from the shoot but like *RDN1* is expressed in both shoots and roots.[5] To assess tissue-level expression of *SUNN*, we constructed a transgenic *M. truncatula* plant containing 1360 bp of sequence upstream of *SUNN* driving expression of an *mGFP-GUS* gene fusion. This construct includes the entire region of 5' DNA between the start of the *SUNN* coding sequence up to and including the next genetic landmark, a genomic MITE insertion. The construct was transformed into wild type (A17) *M. truncatula* using the protocol of Zhou, et al.[6] and detection of GUS was via the protocol of Jefferson et al.[7]

The expression of this construct in T3 plants gave a very similar pattern of expression to that reported for the *RDN1* reporter construct. The GUS staining pattern indicated expression in the vasculature of many tissues including leaves, petioles, stems and roots (**Fig. 1**). The staining was often in cell layers adjacent to phloem cells in tissues examined under higher magnification. For example, staining was seen in the procambium in petioles (1B) and stems (1D, 1E), and the cells surrounding the primary phloem in roots (1F). Despite the fact that SUNN regulates nodule formation, nodule expression was limited to the vasculature (1G and H). No staining was observed in shoot meristematic tissue with faint staining in the vasculature leading to the

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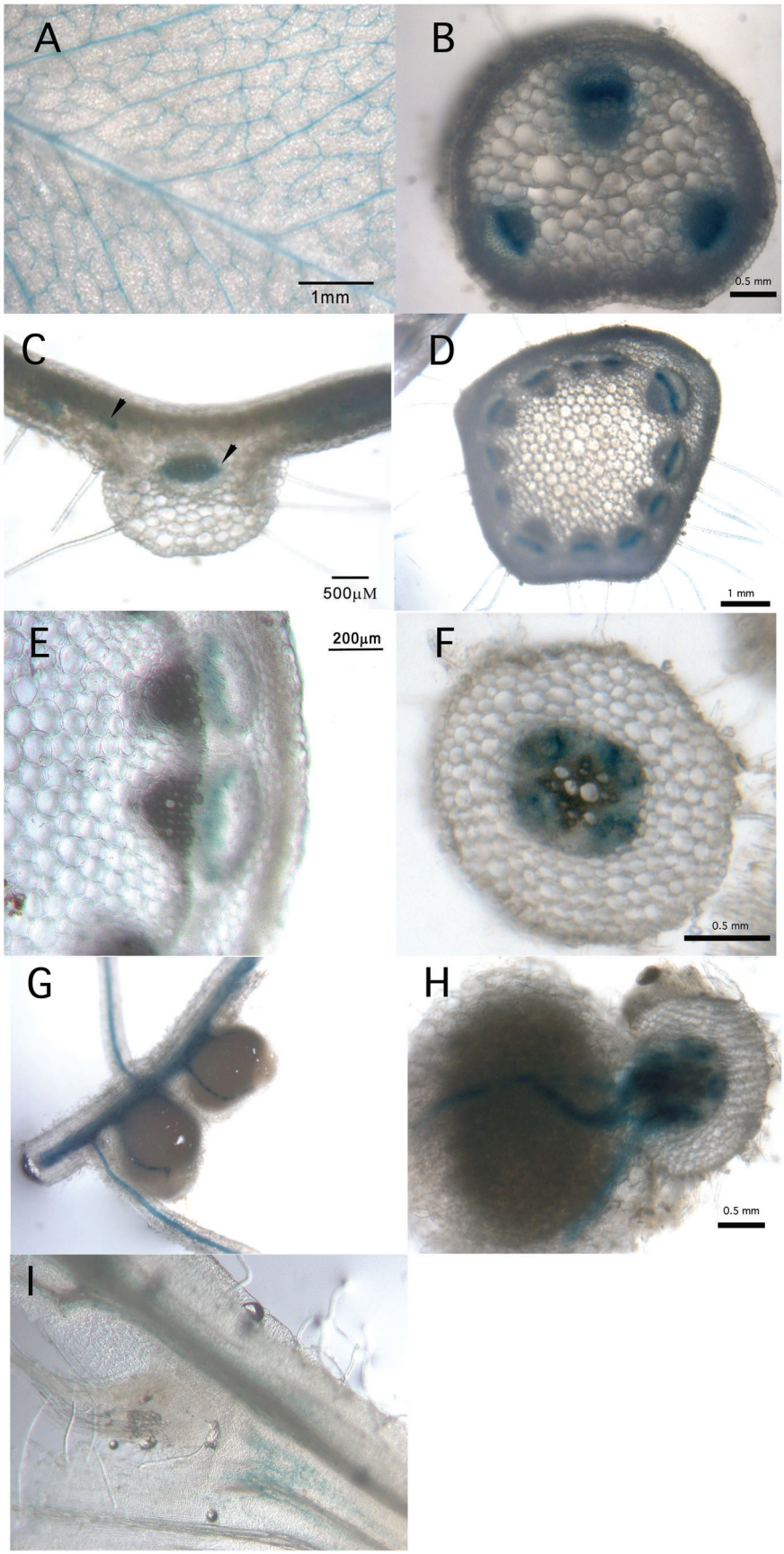

Figure 1. Expression of a *SUNN*$_{pro}$*:mGFP-GUS* fusion in wild type plants. A Nikon E600 microscope with a Retiga EXi-FAST monochrome CCD 12-bit camera was used for visualization and imaging of some stained samples and a Zeiss Lumar.V12 stereoscope equipped with AxioCAM MRC and AxioVision software (Zeiss) for others. Leaf, stem and root transverse sections were made by hand using a razor blade. The sections were routinely ~0.2mm in thickness. (A) Staining in the veins in leaflets; (B) cross section of petioles; (C) cross section of leaflet with arrows indicating staining in vasculature; (D) cross section of stem; (E) close up of stem showing staining in phloem; (F) cross section of root; (G) mature nodules; (H) cross section of root and nodule and (I) faint staining in the vasculature leading to the meristem from the same plant used for (E), but no meristematic staining.

meristem (1I). These results were consistent with those of Nontachaiyapoom et al. who examined the orthologous soybean *NARK* and *Lotus HAR1* promoters in a *L. japonicus* background.[8] They reported sequence conservation between the promoters of the *Lotus*, *Medicago* and *Glycine* orthologs including a sequence element driving vascular specific expression and our results confirm this experimentally in the *Medicago* background.

Because both the *RDN1* and *SUNN* genes were expressed in the vasculature and regulate nodule number, we asked if *SUNN* or *RDN1* transcription had similar expression level patterns in response to rhizobia. We examined *SUNN* and *RDN1* expression in wild type shoots and roots over a time course of early nodulation with Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR), using protocols from our previous work.[9] We saw little change in expression levels in either gene in root and shoot tissue at 24, 48, or 72 h after addition of rhizobia (**Fig. 2**). Values show no statistical difference with $p > 0.05$ for all comparisons by Student's t test.

The presence of *SUNN* and *RDN1* in vascular tissue has mechanistic implications. We speculate that the function of the SUNN kinase and/or RDN1 may affect phloem loading/unloading of auxin or other molecules. Outside of legumes, the SUNN kinase is most closely related by sequence to the Arabidopsis CLV1 kinase and related

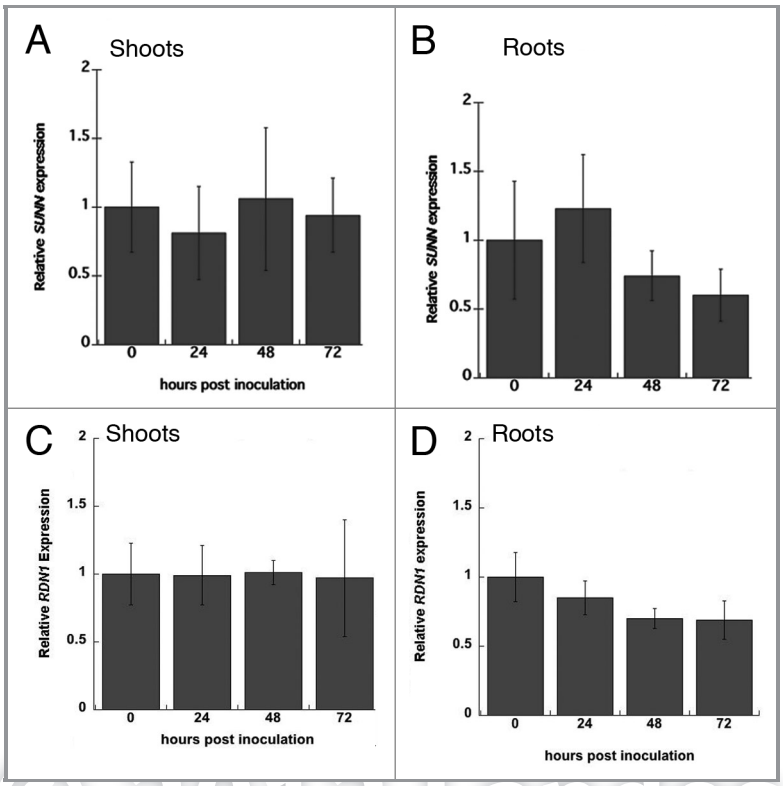

Figure 2. Quantification of gene expression. Results of reverse transcription quantitative PCR of *SUNN* and *RDN1* gene expression in shoots and roots of wild type plants during early nodulation signaling. Expression is shown relative to expression at time 0 (no rhizobia) for *SUNN* in shoots (A) and roots (B) as well as for *RDN1* in shoots (C) and roots (D). Mean of three independent biological replicates of 3–5 plants per time point and three technical replicates. Error bars are standard error of the mean.

proteins in monocots involved in meristem maintenance,[5] and *AtCLV1*, in addition to its well characterized expression in apical meristems, is expressed in the phloem companion cells.[10] Combined with the observation that the RDN1 protein is completely unknown in function but highly conserved across all green plants including moss,[4] the functions of RDN1 and SUNN cannot be limited to signal transduction in the autoregulation of nodulation. The fact that they are expressed in the vasculature with little change in expression in response to rhizobia could suggest the two proteins are involved in the same signal transduction event and we are investigating this further. The observation that SUNN regulates from the shoot while RDN1 regulates from root, yet both are expressed in both shoot and root tissues, most likely indicates there are other as yet undiscovered genes involved in long distance nodulation signaling events.

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