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Does SUNN-SYMRK Crosstalk occur in *Medicago truncatula* for regulating nodule organogenesis?

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Recently we reported that overexpression of intracellular kinase domain of Symbiosis Receptor Kinase (SYMRK-kd) hyperactivated spontaneous nodulation in *Medicago truncatula* indicating the importance of SYMRK ectodomain in restricting nodule number. To clarify whether *sun*n and *sickle* pathways were overcome by SYMRK-kd for hyperactivation of nodule organogenesis, we overexpressed SYMRK-kd in these mutants and analyzed for spontaneous nodulation in absence of rhizobia. Spontaneous nodulation in *skl*/SYMRK-kd roots was 2-fold higher than A17/SYMRK-kd roots indicating nodule organogenesis induced by SYMRK-kd to be ethylene sensitive. Intriguingly, *sun*n/SYMRK-kd roots failed to generate any spontaneous nodule which directly indicate the LRR-RLK SUNN to have a role in SYMRK-kd mediated nodule development under non-symbiotic conditions. We hypothesize a crosstalk between SUNN and SYMRK receptors for activation as well as restriction of nodule development.

Rhizobia-legume root nodule symbiosis is an energy consuming process and is therefore highly-regulated. Several strategies are adapted by the host plant to control the number of nodules in tune with the physiological status of the plant to adjust the nitrogen availability and carbon drain.

One such strategy adapted in response to internal cues is autoregulation of nodulation or AON^{1,2} which is a long-distance autoregulatory negative feedback system involving root–shoot communication.³ Insight into this mechanism has been obtained by the identification of supernodulation mutants. Several such mutants

were defective in genes encoding for leucine-rich repeat receptor kinases that are required in shoots for AON, for instance, *Glycine max nts* mutant encoding NTS/NARK⁴; *Lotus japonicus har1* encoding HAR1,⁵ *Pisum sativum sym29* encoding SYM29,⁵ *Medicago truncatula sunn* encoding SUNN.^{6,7} These LRR-RLK receptors are activated by CLE (CLAVATA3/ENDOSPERM SURROUNDING REGION) related small peptides that are speculated to function as long-distance root-derived mobile signals.^{8–11} The transcription factor NODULE INCEPTION (NIN) is projected as the central coordinator, as it is essential for both nodule primordium formation and CLE peptide generation that results in systemic suppression of nodulation.^{12–14}

Several plant hormones are associated with the control of nodule number by AON. For example, long distance auxin transport is defective in *sun*n-1¹⁵ and supernodulation in *har1* can be suppressed by cytokinin receptor mutations.¹⁶ Methyl jasmonate and brassinosteroid are projected as the shoot derived inhibitors for AON.^{17,18} In *M. truncatula*, both ABA and ethylene are well characterized internal cues that serve as local negative regulators. In the ethylene-insensitive mutant, *sickle*¹⁹ where SICKLE encodes for the *M. truncatula* ortholog of Arabidopsis ethylene signaling protein, EIN2²⁰ the number of successful bacterial infections and nodules are substantially increased. This hyperinfection of rhizobia in *sickle* mutant maintains the AON systemic regulation indicating that ethylene functions in an AON-independent manner.¹⁹ Therefore *sun*n and *sickle* define distinct genetic pathways for negative feedback control of number of nodules

Keywords: autoregulation of nodulation, ethylene, *sickle*, spontaneous nodulation, SUNN, supernodulation, SYMRK

Abbreviations: SYMRK, symbiosis receptor kinase; kd, kinase domain; AON, autoregulation of Nodulation; *sun*n, super numeric nodule; LRR, Leu-rich repeat; *nts*, nitrate-tolerant symbiotic; *skl*, *Sickle*; *har-1*, hypernodulation aberrant root formation; CLE (CLAVATA3/ENDOSPERM SURROUNDING REGION); *snf*, spontaneous nodule formation; TR25 (*symrk* knock out); A17, wild-type *Medicago truncatula* plant.

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which is in accordance with *sunn/skl* double mutants having an additive super-nodulation phenotype.⁶

Symbiosis Receptor Kinase (SYMRK), an upstream LRR-RLK of the Nod factor signaling pathway, is indispensable for nodule organogenesis.^{21,22} Recently, we have demonstrated that overexpression of the intracellular kinase domain of SYMRK (SYMRK-kd) from *M. truncatula* or *Arachis hypogaea* in *M. truncatula* roots induces nodule organogenesis in the absence of rhizobia.²³ Spontaneous nodulation in presence of SYMRK-kd was 6-fold more than rhizobia induced nodulation supported by full length SYMRK in *M. truncatula* TR25 (*symrk* knock out) roots suggesting the importance of SYMRK ectodomain in regulating nodule number. In accordance, spontaneous nodulation in SYMRK-kd transformed roots of wild-type *M. truncatula* (A17) plant under identical conditions was always lower than what was observed in TR25 suggesting a dominant negative effect of the endogenous SYMRK presumably mediated through its ectodomain. In this report our objective was to understand how control over nodule number by SYMRK ectodomain was related with the *sunn* (super numeric nodule) and *sickle* pathways that regulate nodule number in *M. truncatula*. Both Sickle and SUNN pathways are evidenced to negatively influence Nod signaling^{14,24} and therefore in absence of these pathways an increase in SYMRK-kd induced spontaneous nodule organogenesis was expected. SUNN encodes an LRR receptor-like kinase and is a key component of autoregulation of nodulation, which regulates nodule number by a long-distance negative feedback system.⁷ On the other hand, ethylene mediate local inhibition of nodulation and insensitivity to ethylene in the sickle mutant is thought to be causal to its super-nodulation phenotype.¹⁹

To clarify whether *sunn* and *sickle* pathways were overcome by SYMRK-kd for hyperactivation of spontaneous nodule organogenesis, we overexpressed SYMRK-kd in these mutants and analyzed the transformed roots for spontaneous nodulation in absence of rhizobia. Nodulation score in these mutants would be same as A17, if SYMRK-kd had either overcome or had no

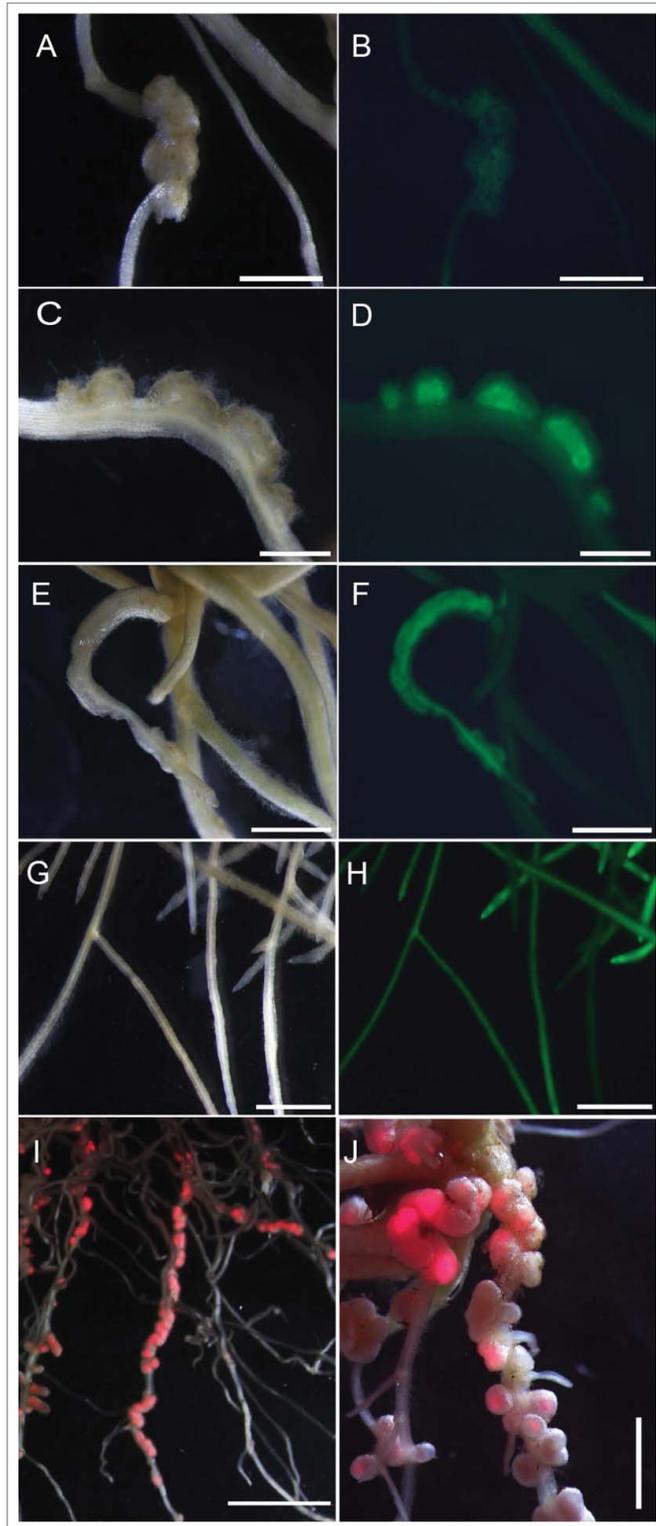


Figure 1. Overexpression of SYMRK-kd in hypernodulating mutants *skl1-1* and *sunn-4* of *M. truncatula*. A17, *skl1-1* and *sunn-4* roots were transformed with SYMRK-kd derived from *AhSYMRK* (*Arachis hypogaea* SYMRK). Spontaneous nodulation was scored 3 weeks after transplantation in sterile agar plates in A17/ 35S::AhSYMRK-kd -GFP (A–B), *skl1-1*/ 35S::AhSYMRK-kd-GFP (C–F) or *sunn-4* /35S::AhSYMRK-kd -GFP where transformed roots failed to generate spontaneous nodules (G–H). Both bright-field (left) and GFP fluorescence (right) images are shown. Distribution of mRFP-*S.melliloti* infected nodules in *skl1-1* (I) and *sunn-4* (J) mutant plants 3 weeks after infection is shown as merged images of bright-field and mRFP fluorescence. Scale bar 500μm (A, B); 1mm (C–D); 2mm (E–J).

Table 1. Spontaneous nodule development in hypernodulating mutants (*sun-4* and *skl1-1*) and A17 (wild type) of *Medicago truncatula* by overexpression of SYMRK-kd

Medicago line	Spn+	Nodules/root system	Nodules/nodulated plant
A17	20% (11/56)	3.4 ± 2.48	7.40 ± 3.58
skl1-1	29% (7/24)	9.0 ± 4.18	17.14 ± 7.55
sun-4	0/32	0	0

Spn+: Spontaneous nodulation scored 3 weeks after transplanting the plants in sterile agar plates. Nodule numbers have been indicated as mean ± Std. Deviation. Numbers are compiled results of 3 independent experiments.

overlap with the *sun* and *sickle* mediated inhibitory pathways. On the other hand nodulation score could increase in the mutants as compared to A17, if the *sun* and *sickle* pathways actively inhibited the SYMRK-kd induced hyperactivation of spontaneous nodulation. As indicated in **Figure 1**, *skl1-1*/AhSYMRK-kd transformed roots successfully developed spontaneous nodules and the efficiency of nodulation in *sickle* background was at least 2-fold higher than what was noted in wild type A17 roots (**Table 1**).

This indicates that *sickle* mediated pathway has a negative role on spontaneous nodule organogenesis. Earlier reports indicated that ethylene affects rhizobia induced nodulation both at the level of rhizobial infection¹⁹ as well as nodule organogenesis.²⁵ For spontaneous nodulation, the signaling pathway originating from rhizobial infection is bypassed. This might explain why there is only 2-fold increase in spontaneous nodules in absence of rhizobia in *skl* background. Overall, the data indicates that the pathway triggered by deregulated SYMRK for inducing spontaneous nodulation is sensitive to ethylene. Or in other words SYMRK-kd could not overcome the inhibitory effect of an ethylene mediated pathway for hyperactivation of nodule organogenesis.

Unexpectedly, in *sun-4*/SYMRK-kd roots neither of the expected possibilities turned out to be true and as shown in **Figure 1** spontaneous nodules completely failed to develop in absence of SUNN receptor. This observation was intriguing and suggested a role of SUNN in nodule organogenesis under nonsymbiotic conditions which is completely in contrast to the widely evidenced role of SUNN as a negative regulator of nodule number through AON under symbiotic conditions. Since the root length and nodule

number phenotypes are noted to be in opposition in SUNN mutants, a positive role of SUNN receptor in developmental processes was already evident. These positive roles of SUNN in differentiation and development is consistent with its high similarity with CLAVATA1, which maintains the shoot apical meristem.⁷

Based on our observations, we propose that SUNN receptors apart from acting systemically from shoots also involve in a cross talk with SYMRK for regulating nodule number. This could be local because SUNN receptors express in roots as well. The proposition of SUNN-SYMRK crosstalk is consistent with absence of autoregulation in spontaneous nodule organogenesis by SYMRK-kd in *M. truncatula*, where absence of SYMRK ectodomain could have affected the cross talk and caused hyperactivation of nodule organogenesis.²³ Accordingly, the proposition is also in consistency with autoregulated spontaneous nodule formation under deregulated activation of CCaMK (calcium / calmodulin dependent protein kinase) or LHK1 (cytokinin receptor) in *snf1* and *snf2* mutants respectively of *L. japonicus* where the proposed cross talk between the corresponding receptors is expected to keep the nodule number under control.^{26,27} Interestingly, SYMRK's role in regulating nodule numbers was already evident when SYMRK mutants were identified as suppressors of hypernodulation in *L. japonicus*²⁸ and the proposed crosstalk between SUNN-SYMRK receptors could be a possible mechanism by which SYMRK exercises its control over nodule number. At this point, it may be relevant to mention that the pattern of rhizobia induced nodule development in SUNN mutants resemble the pattern of spontaneous nodulation in SYMRK-kd transformed roots.²³ In both

cases nodules appear to be arranged in parallel files along the length of the root suggesting that spatial restriction of nodulation is controlled both by SUNN and SYMRK receptors. Finally, the inability of SYMRK-kd to induce spontaneous nodulation in absence of SUNN receptor (this study) hints at a positive role of their interaction in nodule development and therefore also serves as an evidence in favor of a crosstalk between these 2 receptors. Any attempt to integrate SUNN-SYMRK crosstalk within the present models of AON or hormonal pathways would be immature without a proper understanding of their level of interactions.

Concluding Remarks

We hypothesize a crosstalk of SUNN-SYMRK receptors for regulating nodule number in *Medicago truncatula*. Our results highlight a pleiotropy in SUNN and SYMRK function both of which has the intriguing possibility of being repressive or activating toward nodule organogenesis depending on their context.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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