Research Note

Requirements for *syrM* and *nodD* Genes in the Nodulation of *Medicago truncatula* by *Rhizobium meliloti* 1021

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Medicago truncatula, a relative of alfalfa (*Medicago sativa*), has been proposed as a model plant system for study of its interaction with the symbiont *Rhizobium meliloti*. Differences in *M. truncatula* and alfalfa may result in distinct symbiosis behaviors for the two hosts when in association with wild-type or mutant bacterial strains. We have found that *M. truncatula* has a more stringent requirement for *syrM* and *nodD3* than is seen in alfalfa. In particular, the lack of *syrM* is associated with arrested nodule development.

Rhizobium meliloti, the nodulation symbiont of the alfalfa family, has been extensively studied and many *nod* genes important in the regulation of Nod factor production have been elucidated (Dénarié et al. 1996). The major agronomically important host for this bacterium, alfalfa (*Medicago sativa*), is useful for studies of nodule physiology and development, but is not promising for future studies of host genetic determinants because it is tetraploid and not amenable to inbreeding. *Medicago truncatula*, a related species with a small, diploid genome, is also a host of *R. meliloti*, and has been proposed as a model system (Barker et al. 1990).

During the course of initial inoculation tests of *M. truncatula* on agar, we discovered a requirement for an *R. meliloti* regulatory gene, *syrM*, that was not apparent in previous studies with alfalfa. On *M. truncatula*, we observed primarily the formation of small swellings that never developed into nodules.

Determination of the requirement for the gene was confounded by the fact that, when *M. truncatula* is cultured on agar, it does not form nodules at high frequency. Under our conditions, *M. truncatula* roots exhibited the thick and short root phenotype (Tsr) observed on vetch by Zaat et al. (1989). That phenotype was suppressed and nodulation restored by inhibition of ethylene synthesis or perception. This finding parallels numerous reports that inhibition of ethylene can partially overcome nodulation deficiencies in wild-type plants and bacteria as well as in certain plant or bacterial mutants

(Peters and Crist-Estes 1989; Fearn and LaRue 1991; Guinel and LaRue 1992; Lee and LaRue 1992; van Spronsen et al. 1995; van Workum et al. 1995). Ethylene insensitivity has been linked to hypernodulation in *M. truncatula* (Penmetsa and Cook 1996). In comparisons of *M. truncatula* nodulation on agar containing 1.0 μ M silver sulfate (Ag_2SO_4), an ethylene perception inhibitor, or 1.0 μ M L-2-aminoethoxyvinyl glycine (AVG), an ethylene synthesis inhibitor, AVG yielded higher and more consistent nodulation (Table 1); therefore, we chose AVG for use in future experiments.

Even when *M. truncatula* roots were grown on AVG, nodulation did not occur after inoculation with the *syrM* mutant, indicating a possible host specificity since alfalfa will nodulate with the same mutant, although slightly more slowly (Swanson et al. 1987, 1993). We examined the regulatory requirements for nodulation of *M. truncatula* by scoring number of roots that formed nodules (percent nodulation) and number of nodules formed after infection with several *syrM* and *nodD* mutants of *R. meliloti* 1021 (Rm1021).

Seeds of *M. truncatula* cv. Jemalong (Purkiss Seeds, Armidale NSW, Australia) were surface sterilized in 70% ethanol followed by 30 min in undiluted Clorox + Tween 20. Seeds were rinsed well, imbibed at room temperature for at least 4 h with 2 to 3 water changes, and germinated in inverted petri plates by removing excess water and allowing seeds to cling to the plate surface by tension of the residual water. Sprouts were planted the next morning in individual culture tubes containing buffered nod medium (BNM) (2 mM CaSO_4 , 1 mM MgSO_4 , 0.5 mM K_2HPO_4 , 1 mM 2-[N-morpholino] ethane sulfonic acid, pH 6.5, and Murashige and Skoog minor

Table 1. Comparison of root growth and nodulation of Rm1021-inoculated *Medicago truncatula* in the presence of two different ethylene inhibitors and no inhibitor

Characteristic	No inhibitor ^a	Ag_2SO_4 ^a	AVG ^a
Root length, 6 DAP ^b	6.7 cm	9.4 cm	12.8 cm
Percent nodulation, 21 DAI ^c	67%	89%	99%
Average #nodules/root, 21 DAI	1.5	3.9	8.2

^a Each value is the average of 120 roots on 4 plates (30/plate). AVG = L-2-aminoethoxyvinyl glycine.

^b Day after plating.

^c Day after inoculation.

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salts without the KI, solidified with purified agar from Sigma [St. Louis, MO] plus 0.88 μ M AVG. Plants were grown at 24 to 26°C, with a 16 h light/8 h dark photoperiod. Mutant strains of Rm1021 used were all previously isolated in this lab and are listed in Table 2. One day after planting, each seedling was inoculated with approximately 10^8 bacteria from overnight cultures that had been washed and resuspended in 1/2× BNM. Tubes were capped loosely to allow maximum gas exchange. Twenty-six to 27 seedlings were used for each bacterial strain. Control seedlings were inoculated with the resuspension medium containing no bacteria.

Percent nodulation and number of nodules per plant were recorded 19 days after inoculation. Nodules were also scored for size and color. Some treatments resulted in apparent nodule primordia (bumps) that did not continue to develop and often turned dark. We interpreted the formation of bumps to indicate initiation without continuing support of nodulation. Only those structures that were distinct from the root were considered nodules and included white as well as pink nodules. Figure 1 shows the relative numbers of the different types of structure elicited by the various bacterial genotypes.

Four trends were evident in this experiment (Fig. 1): (i) *syrM* was necessary for efficient nodulation; (ii) *nodD3*, while not essential, contributed to optimal nodulation; (iii) *nodD2* was unable to initiate nodulation in the presence of *syrM* alone or *nodD3* alone; and (iv) constructs containing only one *nodD* gene in the presence of *syrM* were unable to support or, in the case of *nodD2*, to initiate nodulation.

The stringent requirement for *syrM* for nodulation of *M. truncatula* is shown in Figure 1A (compare columns 1, 2, 3, and 4 with 8, 9, 10, and 11). Several constructs lacking or with only basal levels of SyrM apparently were able to initiate nodulation, as evidenced by formation of bumps that did not continue to develop. The kinetics of development of these structures indicated that they were arrested, not early stages of nodules that were developing slowly. This suggests *syrM* is important in sustaining, rather than initiating, nodulation. Since empty nodules and incomplete development are characteristic of Exo mutants, we tested for exopolysaccharide production by plating on 0.02% Calcofluor White. We found

all mutant strains used in this study were Calcofluor-bright (Leigh and Walker 1994) and looked identical in colony morphology to Rm1021 on M9 sucrose, indicating no apparent exopolysaccharide deficiency (data not shown).

Fig. 1. Sprouted seeds of *Medicago truncatula* were planted in culture tubes containing agar + L-2-aminoethoxyvinyl glycine (AVG). Plants were inoculated with wild-type Rm1021 and strains containing mutations yielding the given genotypes (x-axis). **A**, Total number of nodules (bars) and percent nodulation (above bars) were scored as stated in the text. **B**, Bars represent total apparent nodulation starts (bumps) that did not continue to develop. There were 26 or 27 plants for each strain. Numbers were normalized to 26 for comparison. Letters indicate intact genes present. Parentheses indicate low product expected due to absence of *syrM* or *nodD3*. M = *syrM*, D1 = *nodD1*, D2 = *nodD2*, D3 = *nodD3*.

Table 2. Bacterial strains used in inoculation experiments with L-2-aminoethoxyvinyl glycine (AVG)

Strain	Genotype				Mutation	Source or reference
	D1	M	D3	D2		
Rm1021	+	+	+	+	Str ^r SU47	Meade et al. 1982
JAS111	-	+	+	+	<i>nodD1::Tn5</i>	This lab
JM139	+	+	+	-	<i>nodD2-uidA</i>	Mulligan and Long 1989
JAS302	+	+	-	+	<i>nodD3-uidA</i>	Swanson et al. 1993
JM98	-	+	+	-	<i>nodD1::Tn5</i> , <i>nodD2-uidA</i>	Fisher et al. 1988
MB504	-	+	-	+	<i>nodD1::Tn5</i> , <i>nodD3-uidA</i>	This lab
MB505B	+	+	-	-	<i>nodD2-uidA</i> , <i>nodD3-uidA</i>	This lab
JAS105	+	-	+	+	<i>syrM::Tn5</i>	Swanson et al. 1993
JM86	-	-	+	+	<i>syrM::Tn5</i> , <i>nodD1-lacZ</i>	Mulligan and Long 1989
JM79	+	-	+	-	<i>syrM::Tn5</i> , <i>nodD2-uidA</i>	This lab
JAS136	+	-	-	+	<i>syrM::Tn5</i> , <i>nodD3-uidA</i>	Swanson et al. 1993

While *nodD3* was not as essential as *syrM*, it contributed to optimal nodulation, possibly due to up-regulation of *syrM* (Fig. 1A, compare columns 1, 2, and 3 with 4). However, *nodD3* also showed a synergistic effect with *nodD1*, but not with *nodD2*, in the absence of *syrM* (Fig. 1A, columns 8, 9, and 10). The enhancement was particularly strong for nodule initiation (Fig. 1B, columns 8, 9, and 10).

nodD2 was completely incapable of initiating nodulation in strains containing *syrM* alone or *nodD3* alone, in which cases the expression of the lone gene is very low (Fig. 1B, columns 6 and 9). Roots infected with these constructs had no swellings and looked identical to negative controls. This was also true in three other experiments performed under different conditions: two in which plants were grown in culture tubes of agar + Ag₂SO₄, and one in which plants were grown in tubes filled with vermiculite (data not shown). It would be interesting to see whether very early events such as calcium spiking or root hair deformation and curling are induced by these constructs.

Although *nodD3* and *syrM* appear to be necessary for optimal nodulation, they are not sufficient (Fig. 1A and B, column 5). One more *nodD* gene is required (Fig. 1A, compare columns 2 and 3 with 5). Nodulation was extremely poor when constructs containing only one of any of the *nodD* genes were used. This parallels the report of Göttfert et al. (1986) of significant delays in nodulation on alfalfa with a *nodD1-nodD2* double mutant, leading them to conclude that at least two *nodD* genes are necessary for efficient nodulation. It is not known what mechanisms are deficient for each of the constructs, but they may include the requirement for either *nodD1* or *nodD2* for interaction with plant inducers, and the requirement for *nodD3* in order to achieve high *syrM* expression.

It is difficult to evaluate the effects of *syrM* or *nodD3* in the absence of the other due to the mutual regulatory loop they constitute (Mulligan and Long 1989; Maillet et al. 1990; Kondorosi et al. 1991; Swanson et al. 1993). However, the evidence of Figure 1B indicates *nodD1* is of prime importance for initiation, with synergistic effects from *nodD3* or, to a lesser extent, *nodD2* enhancing efficiency. Figure 1A suggests that *syrM* is necessary for continued nodule development; it is possible that *syrM* is important in its own capacity and *nodD3* important in up-regulation. The latter theory would be supported by evidence of Swanson et al. (1993) that *syrM* is expressed later in nodule development in the central tissue. *syrM* is known to interact with other genes such as *syrA*, although this appears not to be required for nodulation (Barnett et al. 1998). Perhaps *syrM* also interacts with additional nodulation-specific genes that are more important for nodulation in *M. truncatula* than in alfalfa. While it is possible that *nodD3* is necessary for sustaining infection independently of its influence on *syrM*, several reports indicate that the *nod* genes themselves are not expressed during later stages of nodule development (Sharma and Signer 1990; Schlaman et al. 1991; Swanson et al. 1993).

Redundancy of the *R. meliloti nodD* genes has been suggested to play a role in host-range specificity (Honma and Ausubel 1987). Differences in the responses of the *nodD* genes to flavonoids produced by different host plants suggest that harboring three copies of *nodD* is advantageous for increasing host range by optimizing interactions with different host plants (Györgypal et al. 1988; Honma et al. 1990). It is also

possible that different combinations of genes are optimal for nodulation under different environmental conditions or at different stages of development. We found *M. truncatula* to have more stringent requirements for *nodD3* and *syrM* genotypes than does alfalfa. This observation suggests that it may be fruitful to elucidate the regulatory and structural genes downstream of *nodD3* and *syrM*, as such genes may control events in the intermediate stages of nodulation.

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