Analysis of Differences Between *Sinorhizobium meliloti* 1021 and 2011 Strains Using the Host Calcium Spiking Response

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In the *Rhizobium*–legume symbiosis, compatible partners recognize each other through an exchange of signals. Plant inducers act together with bacterial transcriptional activators, the NodD proteins, to regulate the expression of bacterial biosynthetic nodulation (*nod*) genes. These genes direct the synthesis of a lipochito-oligosaccharide signal called Nod factor (NF). NFs elicit an early host response, root hair calcium spiking, that is initiated in root hair cells within 15 min of NF or live *Rhizobium* inoculation. We used calcium spiking as an assay to compare two closely related strains of *Sinorhizobium meliloti*, Rm1021 and Rm2011, derived from the same field isolate. We found that the two strains show a kinetic difference in the calcium spiking assay: Rm1021 elicits calcium spiking in host root hairs as rapidly as purified NF, whereas Rm2011 shows a significant delay. This difference can be overcome by raising expression levels of either the NodD transcriptional activators or GroEL, a molecular chaperone that affects expression of the biosynthetic *nod* genes. We further demonstrate that the delay in triggering calcium spiking exhibited by Rm2011 is correlated with a reduced amount of *nod* gene expression compared with Rm1021. Therefore, calcium spiking is a useful tool in detecting subtle differences in bacterial gene expression that affect the early stages of the *Rhizobium*–legume symbiosis.

In the *Rhizobium*–legume nitrogen-fixing symbiosis, compatible bacterial and plant partners recognize each other through an exchange of chemical signals. Host plants produce compounds that act as inducers of nodulation (*nod*) genes. These genes encode the *Rhizobium* biosynthetic machinery which produces the morphogenetic signal called Nod factor (NF). The NF signal triggers a series of host responses culminating in the development of a symbiotic organ on the plant, the nodule, in which *Rhizobium* spp. convert atmospheric nitrogen to ammonium-containing compounds.

Expression of the *Rhizobium nod* genes, and the subsequent production of NF, is regulated by a family of LysR-type transcriptional activators called NodD, which act in conjunction with plant inducers. *Sinorhizobium meliloti*, the symbiont of *Medicago* hosts, encodes three NodD proteins: NodD1, NodD2, and NodD3 (Honma et al. 1990). NodD1 and NodD2 are inducer-dependent activators of *nod* gene expression responsive to luteolin and trigonelline, respectively (Phillips et al. 1992). In contrast, NodD3 shows inducer-independent behavior: it

requires no plant inducers to activate *nod* gene expression (Maillet et al. 1990; Mulligan and Long 1989; Swanson et al. 1993). NodD-dependent transcription also is modulated by other cellular proteins. Two proteins in particular, NolR and GroEL, have been shown to affect *nod* gene expression. NolR acts as a repressor by binding to regions upstream of *nod* promoters and preventing NodD-dependent expression (Kondorosi et al. 1989). GroEL, a molecular chaperone, co-purifies with NodD proteins and is required for NodD activity (Ogawa and Long 1995).

S. meliloti cells grown in culture do not show measurable levels of expression from *nod-lacZ* fusions unless grown in the presence of inducers. In free-living bacteria, *nodD1* is constitutively expressed, whereas *nodD3* expression cannot be detected above background by means of reporter fusions (Mulligan and Long 1985; Swanson et al. 1993). *nodD3* expression is positively regulated by another LysR-type transcriptional activator, *syrM*, which also is not detectable above background in culture-grown cells (Swanson et al. 1993) though it is strongly expressed in nodules. Thus, we expected NF should be produced only when cells are exposed to plant inducers.

In the early stages of the *Rhizobium*–legume interaction, NF triggers a number of responses in host root hair cells (Cardenas et al. 2000; Downie and Walker 1999). One of these responses, cytoplasmic calcium oscillations (calcium spiking), is a rapid and very sensitive response: in *Medicago truncatula* root hairs, purified *S. meliloti* NF triggers calcium spiking after approximately 10 min at doses as low as 1 pM (Ehrhardt et al. 1996; Oldroyd et al. 2001; E. Engstrom and S. R. Long, unpublished data). A recent study demonstrated that live *S. meliloti* bacteria elicit calcium spiking as rapidly as purified NF, and that the response is dependent on the presence of the biosynthetic *nod* genes (Wais et al. 2002). This calcium spiking behavior is found in several *Rhizobium* spp.–legume pairs. These results suggested that calcium spiking constitutes a very sensitive assay for *nod* gene expression and regulation.

In the present study, we used the calcium spiking response to compare the early nodulation behavior of a test strain pair, Rm1021 and Rm2011. Rm1021 and Rm2011 are two closely related *S. meliloti* strains capable of forming nitrogen-fixing nodules in *Medicago* hosts. These strains are independent streptomycin-resistant derivatives of the wild-type *S. meliloti* isolate SU47 separated by 25 years of growth under different media conditions in different laboratories (Meade and Signer 1977; J. Dénarié, personal communication). Because they were derived from the same parent, Rm1021 and Rm2011 were thought to be functionally interchangeable and were expected to trigger identical responses in host plants. However, unpublished evidence suggests that these strains differ. In at

least one case, Rm2011 was slower to induce host gene expression than was Rm1021 (J. Cohn and G. Stacey, *personal communication*). We show here that the calcium spiking assay detected measurable differences in the behavior of the two strains and that these differences are attributable to activity of the NodD proteins. A careful comparison of *nod* gene expression in the two strains confirmed the trend uncovered by the calcium spiking behavior of Rm1021 and Rm2011. Based on this study, we propose that calcium spiking can be used as an assay for detecting subtle strain- and species-specific differences in the early kinetics of bacterial signaling to the host.

RESULTS

S. meliloti strains Rm1021 and Rm2011 differ in their ability to elicit root hair calcium spiking.

Calcium spiking is triggered by NF, which is thought to be produced only by bacteria that have been exposed to the appropriate plant inducer. Thus, we expected that only induced bacteria—that is, bacteria pretreated with a plant inducer—would elicit a response. We first examined the behavior of uninduced Rm1021 and Rm2011 to establish baselines for comparison between strains. Bacteria were tested on *M. truncatula* plants by assaying Oregon Green-injected root hairs for calcium spiking (Wais et al. 2000, 2002). Surprisingly, we found that Rm1021 cells that had not been exposed to plant inducers, such as luteolin or trigonelline, elicited prompt calcium spiking in more than 80% of host root hairs (Wais et al. 2002). Calcium spiking started approximately 10 min after inoculation with uninduced Rm1021 cells (Fig. 1A).

In contrast, uninduced Rm2011 cells failed to elicit calcium spiking in *M. truncatula* root hairs. In 35% of the root hair cells (5 of 14), Rm2011 elicited periodic fluctuations distinct from calcium spiking after 30 to 40 min (Fig. 1B). There is a clear difference between the response to uninduced Rm2011 and the subsequent response to purified NF. Use of a single

wavelength dye to detect changes in calcium concentrations prohibits assessment of the absolute amplitude of calcium levels from cell to cell. However, within individual cells, it is possible to compare the response to different treatments. We did not count the periodic behavior triggered by uninduced Rm2011 as calcium spiking because it lacked the characteristic rapid rises elicited in the same cells by purified NF. Thus, in the absence of pretreatment with plant inducer, Rm1021 and Rm2011 differ in their ability to trigger calcium spiking.

NodD3 is required for the inducer-independent calcium spiking behavior of Rm1021.

S. meliloti strains contain three different *nodD* genes, encoding proteins with different inducer specificities. Of these, only NodD3 can activate *nod* gene transcription in the absence of plant inducers. We hypothesized that the inducer-independent activity of Rm1021 in the calcium spiking assay was due to NodD3. We tested CSB222, a *nodD3*' derivative of Rm1021, and found that it fails to trigger calcium spiking when uninduced (Fig. 2; Table 1). The inducer-independent behavior of Rm1021 was still present when either NodD1 or NodD2 was inactivated (Table 1). Moreover the inducer-independent behavior of Rm1021 in the calcium spiking assay required *syrM*, a positive transcriptional activator of *nodD3* expression (data not shown). Thus, the behavior of Rm1021 in the absence of induction requires NodD3 and its associated regulatory circuit.

Induced Rm2011 triggers calcium spiking more slowly than Rm1021.

NodD-dependent *nod* gene expression is inducer dependent except when NodD3 activates gene expression (Maillet et al. 1990; Mulligan and Long 1989; Swanson et al. 1993). We asked whether, in the absence of NodD3, Rm1021 would show the same calcium spiking behavior as Rm2011. Knowing that, in the absence of induction, neither Rm2011 nor CSB222 triggers calcium spiking, we tested whether induction with 3 μ M

Fig. 1. Calcium spiking phenotypes of uninduced Rm1021 and Rm2011. Calcium spiking response in single root hairs treated with **A**, uninduced Rm1021 or **B**, uninduced Rm2011. The point-to-point change in fluorescence is shown. Solid triangles mark the initiation of calcium spiking. The open triangle marks the periodic fluctuations observed occasionally in the root hairs exposed to uninduced Rm2011 (**B** only). The dashed vertical line indicates the time at which Nod factor (1 nM) was applied in **B**.

luteolin allowed Rm2011 and CSB222, a *nodD3*⁻ derivative of Rm1021, to elicit the response. We reasoned that, if NodD3 were the sole cause of the difference between the two strains, then Rm2011 and CSB222 should behave identically when induced. Induced Rm2011 cells initiated calcium spiking in 45% of root hairs tested (9 of 20) after a delay, or lag, of 35 to 40 min (Fig. 2A). We also pretreated Rm2011 with trigonelline, an inducer of NodD2-mediated transcription, but found no improvement in calcium spiking compared with treatment with luteolin (Table 1). In a parallel experiment, CSB222 induced with either luteolin or trigonelline triggered calcium spiking within 10 to 15 min, twofold more rapidly than induced Rm2011 and just as rapidly as purified NF or uninduced Rm1021 (Fig. 2B, Table 1). Thus, Rm2011 differs from Rm1021 and its derivatives in several respects. First, Rm2011 requires pre-induction to elicit calcium spiking, whereas uninduced Rm1021 triggers the response as rapidly as purified NF. Second, even in the presence of inducer, the percent of root hairs that respond to Rm2011 is lower and the lag to the onset of calcium spiking is longer than what is observed in cells responding to Rm1021. In fact, induced Rm2011 triggers calcium spiking more slowly than induced CSB222, the *nodD3*⁻ derivative of Rm1021. From this, we concluded that *nodD3* genotype alone does not suffice to explain the difference in behavior between Rm2011 and Rm1021.

***nod* gene expression is higher in Rm1021 than in Rm2011, in both the uninduced and the induced state.**

The low percentage of responding root hairs and longer lag times seen with Rm2011 in the calcium spiking assay are similar to the response observed when plants are exposed to very low levels of NF or to fewer bacteria (Oldroyd et al. 2001; G. E. D. Oldroyd, R. J. Wais, and S. R. Long, *unpublished data*). These observations suggest that NF production might be lower

in Rm2011 than in Rm1021, possibly due to lower *nod* gene expression. We examined expression of a NF biosynthetic gene, *nodF*, using an integrated transcriptional fusion to *uidA* (β-glucuronidase), in either an Rm1021 background (DW386) or in the Rm2011 background (DW396). In the uninduced state, *nodF* expression is twofold higher in Rm1021 than in Rm2011 (Fig. 3). Furthermore, when the strains are induced with luteolin, DW386, the Rm1021 derivative, shows greater than fourfold higher expression than DW396, the Rm2011-derived strain. These data demonstrate that *nod* gene expression is not only higher in Rm1021 than in Rm2011 but also that Rm1021 displays a significantly greater responsiveness to induction than Rm2011. These results are consistent with the observed differences in the calcium spiking behavior of the two strains.

Rm2011 carrying extra copies of *nodD1* or *nodD3* elicits rapid and robust calcium spiking.

We hypothesized that the difference in calcium spiking behavior could be due to the observed difference in *nod* gene expression between Rm1021 and Rm2011. In order to address this, we introduced extra copies of *nodD* genes into Rm2011 to raise the level of *nod* gene expression and examined the ability of the resultant strain to trigger calcium spiking. We tested Rm2011 strains overproducing either NodD1 or NodD3 and found that an Rm2011 strain in which *nodD1* is overexpressed (RJW16), when induced with luteolin, rapidly triggers calcium spiking (Fig. 4A and B, Table 1). When NodD3 is overproduced in Rm2011, the resultant strain, RJW10, triggers calcium spiking as rapidly as Rm1021 and without being induced (Fig. 4C, Table 1). Thus, the difference in behavior between Rm2011 and Rm1021 can be overcome by raising the levels of either NodD1 or NodD3, thereby increasing *nod* gene expression in Rm2011, in an inducer-dependent or inducer-independent behavior, respectively.

Fig. 2. Calcium spiking response of induced Rm2011 and CSB222, a *nodD3* mutant of Rm1021. Traces represent individual root hair cells. Solid triangles mark the initiation of calcium spiking. **A**, Root hair treated with induced Rm2011. Rm2011 cells were induced for 16 h with 3 μM luteolin. **B**, Root hair treated with uninduced (solid line) and induced (dashed line) CSB222 cells. Bacteria were induced for 16 h with 3 μM luteolin.

The difference between Rm1021 and Rm2011 is not due to *NolR*, a repressor of *nod* gene expression.

The results above show that overexpression of *nodD1* or *nodD3* allows Rm2011 to trigger calcium spiking as rapidly as Rm1021, yet mutation of these genes in Rm1021 is not sufficient to cause the long lag times seen with Rm2011. We inferred that the strains could differ in some other factor or factors affecting NodD activity, and consequent *nod* gene expression, and examined candidate genes that might confer the observed behavior. Kondorosi and associates (1989, 1991) described a repressor that interferes with the ability of NodD to induce *nod* gene expression by binding to sequences upstream of *nod* genes and preventing NodD-mediated transcription. The repressor, *NolR*, has been found in many *Sinorhizobium* and *Rhizobium* spp. (Kiss et al. 1998). Strains that contain an intact *nolR* show lower levels of *nod* gene induction and NF production (Cren et al. 1995; Kondorosi et al. 1989). In Rm1021, however, the *nolR* gene contains a single nucleotide insertion that inactivates the protein (Cren et al. 1994). We asked whether Rm2011 differs from Rm1021 in possessing a wild-type *nolR*. We cloned and sequenced the *nolR* genes from Rm1021 and Rm2011 and found that the two strains are genetically identical, and both mutated, at this locus (data not shown). Therefore, the difference between Rm1021 and Rm2011 is not attributable to *nolR*.

GroEL, a chaperone required for NodD activity, confers the ability to rapidly trigger calcium spiking in Rm2011 when overexpressed and is required for Rm1021-elicited calcium spiking.

GroEL, a molecular chaperone encoded by at least four different *groEL* genes in *S. meliloti*, has been shown to affect *nod* gene expression in Rm1021. GroEL affects the in vivo activity of NodD1 and NodD3 as well as SyrM and is required for in vitro NodD DNA-binding activity (Ogawa and Long 1995). Mutation of one copy of *groEL* in Rm1021 results in significantly lower *nod* gene induction by *nodD1* and *nodD3*, as well as a reduction in *nodD3* induction by SyrM (Ogawa and Long 1995). We hypothesized that the differing levels of *nod* gene expression in Rm1021 and Rm2011 may be the result of varying GroEL activity in the two strains. Thus, decreasing GroEL levels in Rm1021 might result in Rm2011-like behavior, whereas increasing GroEL levels in Rm2011 might confer Rm1021-like activity. We tested strain B4T1, a *groEL_c* derivative of Rm1021, and found that, 45 min after application, the strain still is unable to elicit calcium spiking (Fig. 5B). Addition of NF after 50 min of treatment with B4T1 results in a prompt calcium spiking response (Fig. 5B). Next, we introduced *groEL* on a low copy number plasmid into Rm2011.

Table 1. Calcium spiking response of Rm1021 and Rm2011 mutants

Strain	Genotype	Calcium spiking behavior ^a	
		Uninduced cells	Induced cells
Rm1021	Wild type	+	...
TJ9B8	<i>nodD1</i> ⁻ (Rm1021)	+	...
RmD2	<i>nodD2</i> ⁻ (Rm1021)	+	...
CSB222	<i>nodD3</i> ⁻ (Rm1021)	-	L: +, T: +
B4T1	<i>groEL_c</i> ⁻ (Rm1021)	-	...
Rm2011	Wild type		L: ±, T: ±
RJW16	Rm2011 (<i>pnodD1</i>)	-	L: +
RJW10	Rm2011 (<i>pnodD3</i>)	+	+
RJW17	Rm2011 (<i>pgroEL_a</i>)	+	...

^a Symbols: + denotes a robust calcium spiking response (more than 50% of root hairs tested, with a maximum lag time of 15 min), - denotes a failure to trigger calcium spiking, ± indicates that calcium spiking was observed in fewer than half the cells tested, L = luteolin, 3 µM; and T = trigonelline, 20 µM.

The resultant strain, RJW17, was tested for the ability to elicit calcium spiking. Uninduced RJW17 caused calcium spiking within 15 min of inoculation (Fig. 5A). Of 11 cells tested, 9 (81%) on three plants showed calcium spiking; each plant had at least one responsive cell. The observation that RJW17 triggers calcium spiking in the absence of induction suggests that *nodD3* is expressed in free-living Rm2011, as it is in Rm1021, further demonstrating that the difference in behavior between the two strains is not solely attributable to *nodD3*. Altering the levels of GroEL is sufficient to change the calcium spiking behavior of both strains; raising GroEL levels in Rm2011 leads to more rapid induction of calcium spiking, while a mutation that reduces the available pool of GroEL in Rm1021 leads to Rm2011-like behavior (Fig. 5B, Table 1).

DISCUSSION

In the present study, we demonstrate that host root hair calcium spiking can be used to detect differences between strains with relation to the kinetics of NF production. Rm1021 and Rm2011, two strains derived from the same parent (SU47), served as the test pair. Both strains form nitrogen-fixing nodules on host plants, yet unpublished evidence suggested that these strains might differ in subtle ways. Calcium spiking provided a sensitive and quantitative assay for comparing the two strains and enabled us to demonstrate that even closely related strains can differ in their ability to elicit a rapid host response. We identified genes that may contribute to the strain difference observed in the assay and show that the observed behavior is dependent on NodD regulatory proteins that affect *nod* gene expression. These results indicate that, for early host responses triggered by low levels of NF, subtle differences in *nod* gene expression or NF production significantly affect the timing and extent of the response.

Our findings using this assay are generally consistent with the established model for the regulation of *nod* genes derived from experiments with *nod* gene fusions; however, we also detected behavior not previously distinguished with transcriptional fusions. In our assay, NodD1 showed luteolin-dependent induction of calcium spiking and NodD3 acted in an inducer-independent fashion. These results are consistent with initial studies using *lacZ* fusions to *nod* promoters which showed that NodD1 and NodD2 activate gene expression in an inducer-de-

Fig. 3. *nod* gene expression in Rm1021 and Rm2011. Cultures of Rm1021 and Rm2011 strains carrying a transcriptional fusion between *nodF* and *uidA* (*gus*) were diluted and grown to late exponential phase (optical density at 600 nm [OD₆₀₀] ≈ 0.8) in the absence (white columns) or presence (gray columns) of luteolin (at a concentration of 3 µM). Expression values (β-glucuronidase activities) are represented by Miller units. Background β-glucuronidase activity for Rm1021 (which has no endogenous *uidA* gene) is 0. Strains assayed: DW386 (Rm1021, *nodF::uidA*), DW396 (Rm2011, *nodF::uidA*). There was no detectable difference in CFU/OD₆₀₀ between the two strains.

pendent manner. In these same studies, NodD3 was shown to be inducer-independent and regulated at the transcriptional level by SyrM (Mulligan and Long 1989; Swanson et al. 1993). In the original *lacZ* fusion tests, *nod* gene expression was not detectable in the absence of induction, suggesting that *nodD3* is not expressed in free-living bacteria (Maillet et al. 1990; Mulligan and Long 1989). However, the sensitivity of the calcium spiking response allowed us to detect behavior that strongly suggests there is NodD3 activity in free-living bacteria. The assay also revealed that Rm2011 showed a quantifiable delay and reduced effectiveness in triggering calcium spiking compared with Rm1021. A significant feature of previous studies with transcriptional fusions was the need for multiple copies of either the reporter gene or *nodD* transcriptional activators to obtain measurable levels of *lacZ* activity. By constructing a single copy transcriptional fusion between *nodF* and *uidA* (β -glucuronidase), we demonstrated that the two strains show a significant difference in *nod* gene expression, which is exacerbated when the bacteria are induced. Thus, the calcium spiking assay enabled us to identify quantifiable differences in gene expression that had not been revealed in previous studies. Future uses of this assay could address inducer specificity of NodDs or uncharacterized *Rhizobium* strains. The rapidity of the calcium spiking response also provides an assay for defining the kinetics of the bacterial response to host plant inducers.

Using calcium spiking as an assay, we have shown that there is a measurable difference in the behavior of Rm1021 compared with that of Rm2011. Cells of Rm1021 and its derivatives trigger a robust calcium spiking response, defined by a 10- to 15-min lag time and more than half of the root hairs responding, which is comparable to the response seen with purified NF (Table 1) (Wais et al. 2002). In contrast, Rm2011 does

not trigger a strong calcium spiking response, even when induced, unless multiple copies of *nodD* are present. Robust behavior in the calcium spiking assay also was restored in Rm2011 by overproducing GroEL, a molecular chaperone implicated in NodD activity (Ogawa and Long 1995). Introduction of additional copies of *nodD* into Rm2011 is expected to have a direct effect on biosynthetic *nod* gene expression, whereas the effect on *nod* gene expression of changing the amount of GroEL in the cell is most likely complex. Identification of the molecular mechanism through which GroEL affects Rm2011 behavior is complicated by the existence in the *S. meliloti* (Rm1021) genome (Galibert et al. 2001) of at least four copies of *groEL*, of which two have been shown to be functionally interchangeable (Ogawa and Long 1995). It is likely that both Rm1021 and Rm2011 have developed different behavior from the original SU47 through years of culturing in the lab; thus, it is unclear which, if either, represents the behavior of the progenitor strain. Our results attest to the complexity of *nod* gene regulation and indicate that *nod* gene expression levels probably depend not simply on total amount of NodD protein in the cell, but rather on the amount of active protein available upon exposure to inducer molecules.

Calcium spiking provides a useful and unique diagnostic tool for studies of the regulation of *nod* gene expression. The present study indicates that differences between strains that affect NF production can alter the timing and extent of a host response in ways that cannot be ascertained by experiments with purified NF. Attention is increasingly directed to early events in nodulation, defining new host responses such as ionic fluxes, cytoskeletal rearrangements, and changes in host gene expression triggered within the first 24 h after inoculation (Cardenas et al. 2000; Oldroyd 2001). Identification of plant

Fig. 4. Calcium spiking responses of Rm2011 strains overexpressing NodD1 (RJW16) or NodD3 (RJW10). Traces show calcium response in individual root hairs. Solid triangles mark the initiation of calcium spiking. **A**, Root hair calcium spiking response to uninduced RJW16. **B**, Root hair calcium spiking response to induced RJW16. Cells were induced for 16 h with 3 μ M luteolin. **C**, Root hair calcium spiking response to uninduced RJW10.

genes induced early in the symbiosis have utilized a variety of bacterial strains (Cohn et al. 2001; Cook et al. 1995; Pichon et al. 1992; Vernoud et al. 1999), some of which are known to differ in overall nodulation kinetics (D. Cook, personal communication; L. Smith and S. R. Long, *unpublished data*). However, even among strains with indistinguishable nodulation kinetics, such as Rm1021 and Rm2011, our results demonstrate that differences between strains can significantly affect the profile of at least one early plant response. That such differences in behavior can occur even between closely related strains emphasizes the importance of strain choice and highlights the necessity of using bacterial mutants constructed in a common genetic background when studying early host responses.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. Rm1021 and Rm2011 were isolated in different labs at MIT (Massachusetts, U.S.A.) and INRA CNRS (Toulouse, France) by selecting for streptomycin-resistant variants of SU47, a *S. meliloti* wild type strain (Casse et al. 1979; Meade and Signer 1977). Plasmids were introduced into Rm2011 by triparental mating using pRK2013 (Ditta et al. 1980). CSB222 contains a hygromycin-marked deletion of *nodD3*. The *nodD3* coding region (*Bst*EII–*Stu*I, 1.035 kb) was replaced with the hygromycin-resistance gene, *hph*. A 2-kb fragment carrying the deletion then was subcloned into pJQ200. The resultant

Fig. 5. Contribution of GroEL to the ability of Rm1021 and Rm2011 to trigger calcium spiking. Traces represent individual cells on the same plant treated with **A**, uninduced RJW17 or **B**, uninduced B4T1. The solid line denotes when bacteria were added. The dashed line in panel B marks the time at which 1 nM Nod factor was applied.

Table 2. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or (reference)
<i>Sinorhizobium meliloti</i>		
Rm1021	Wild type, SU47 str21	(Meade et al. 1982)
Rm2011	Wild type, SU47	J. Dénarié
RJW10	Rm2011 pRmE65, Tc ^R	This study
RJW16	Rm2011 pRmE43, Tc ^R	This study
RJW17	Rm2011 pO37, Tc ^R	This study
DW396	Rm2011 <i>nodF::uidA</i> , Nm ^R	This study
CSB222	Rm1021 Δ <i>nodD3</i> , Hyg ^R	C. Starker
TJ9B8	Rm1021 <i>nodD1::Tn5</i> , Nm ^R	(Jacobs et al. 1985)
RmD2	Rm1021 <i>nodD2::Tm</i> , Tm ^R	(Honma and Ausubel 1987)
B4T1	Rm1021 <i>groELc::Tn5</i>	(Ogawa and Long 1995)
DW386	Rm1021 <i>nodF::uidA</i> , Nm ^R	This study
Plasmids		
pRmE65	<i>nodD3</i> in pTE3	(Fisher et al. 1988)
pRmE43	<i>nodD1</i> in pTE3	(Fisher et al. 1988)
pO37	<i>groEL_a</i> in pRK290, under native promoter	(Ogawa and Long 1995)
pTE3	pLAFR1 containing polylinker flanked by <i>trp</i> promoter and <i>rpoC</i> terminator, Tc ^R	(Egelhoff and Long 1985)
pRK290	IncP, Tc ^R	G. Ditta
pRK2013	Helper plasmid for mobilization of IncP plasmids, Km ^R	(Ditta et al. 1980)
pVO155	For generating transcriptional fusions to <i>uidA</i> (GUS) reporter, Ap ^R , Nm/Km ^R	(Oke and Long 1999)
pDW110	pVO155-derivative with <i>nodF</i> promoter	This study

plasmid, pCGS212, then was mated into Rm1021. Double recombination events, yielding a deletion of *nodD3* on pSymA, were selected for by screening for sucrose resistance and gentamycin sensitivity.

Bacterial strains tested in the calcium spiking assay were grown in tryptone yeast (TY) liquid medium (Beringer 1974) under appropriate antibiotic selection. Antibiotics were used at the following concentrations: streptomycin 500 at µg/ml, kanamycin at 25 µg/ml, tetracycline at 10 µg/ml, hygromycin at 50 µg/ml, and neomycin at 50 µg/ml. Cultures were grown to mid- or late-log phase and cells were spun at 20,000 × g for 1 min, washed, and resuspended in buffered nodulation medium (BNM) to an optical density at 600 nm (OD₆₀₀) of 0.5. 100 µl of resuspended cells (approximately 10⁸ cells based on colony counts performed following each experiment) were added to the 2-ml bath containing the injected plants. Plant inducers were used at the following standard concentrations: luteolin at 3 µM and trigonelline at 20 µM (Phillips et al. 1992). Bacteria were induced by pretreatment with plant inducers for 16 h.

Plant growth and preparation.

Seeds of *M. truncatula* cv. Jemalong (Purkiss Seeds, Armidale, Australia) were surface-sterilized and germinated as previously described (Wais et al. 2000). Germinated seedlings were transferred to plates containing BNM (Ehrhardt et al. 1992) with 12% purified agar and 0.1 µM L-α-(2-aminoethoxyvinyl) glycine (Sigma-Aldrich, St Louis) and placed in a 24°C growth chamber with a photoperiod of 16 h light, 8 h dark and a light intensity of 60 photons per m² s⁻¹. The bottom half of the plates was covered with foil to shade the roots. For calcium spiking assays, 2-day-old seedlings were prepared as described (Wais et al. 2000).

GUS assays.

To generate a transcriptional fusion between *nodF* and β-glucuronidase (*uidA*), the entire upstream regulatory and coding regions of *nodF* were cut from pRFm792 (R. F. Fisher, unpublished data) using restriction enzymes *Bam*HI and *Xba*I. This fragment then was cloned in front of the promoterless *uidA* gene in pVO155 (Oke and Long 1999) using restriction sites *Bam*HI and *Spe*I, resulting in plasmid pDW110. pDW110, which does not replicate in *S. meliloti*, was introduced into Rm1021 by triparental mating, and a strain which contained pDW110 integrated into the native location of *nodF* was selected by neomycin resistance and confirmed by polymerase chain reaction. An N3 phage lysate (Martin and Long 1984) of this strain was subsequently used to transduce the *nodF::uidA* fusion into Rm1021 and Rm2011, resulting in strains DW386 and DW396, respectively. Cultures of the two strains in TY medium were diluted and grown (for 3 to 4 hours) to an OD₆₀₀ of approximately 0.8 either in the absence or presence of 3 µM luteolin. Three milliliters of each culture was collected and GUS assays were performed as described by Jefferson and associates (1986).

Calcium spiking assay.

Calcium dye injection and fluorescence imaging were performed as previously described (Wais et al. 2000). In cases where bacteria failed to induce calcium spiking, purified *S. meliloti* NF,NodRmIV (Ac,S), was added to a final concentration of 1 nM as a positive control for the root hairs' ability to initiate calcium spiking. Root hairs were imaged for a minimum of 30 min after addition of bacteria to determine whether or not calcium spiking was triggered.

Calcium spiking traces show the change in background-corrected fluorescence from one timepoint to the next in a single injected root hair, as described by Wais and associates (2000).

In brief, raw fluorescence data were corrected for background by dividing the average fluorescence of an injected cell by the average fluorescence of a background sector (Ehrhardt et al. 1996). Background-corrected fluorescence data were then transformed using the function $Y = X_{(n+1)} - X_n$, where Y is the point-to-point change in fluorescence, and $X_{(n+1)}$ and X_n are the intensity measurements at timepoints n and $n + 1$ (Wais et al., 2000). This data transformation emphasizes large changes in fluorescence that occur within single imaging intervals (5 s), a characteristic of calcium spikes. A cell was counted as positive for calcium spiking when five or more consecutive spikes were detected whose amplitudes more than doubled the standard deviation of baseline fluctuation.

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