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The *NFP* locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation

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Summary

Establishment of the *Rhizobium*–legume symbiosis depends on a molecular dialogue, in which rhizobial nodulation (Nod) factors act as symbiotic signals, playing a key role in the control of specificity of infection and nodule formation. Using nodulation-defective (Nod$^-$) mutants of *Medicago truncatula* to study the mechanisms controlling Nod factor perception and signalling, we have previously identified five genes that control components of a Nod factor-activated signal transduction pathway. Characterisation of a new *M. truncatula* Nod$^-$ mutant led to the identification of the *N*od *F*actor *P*erception (*NFP*) locus. The *nfp* mutant has a novel phenotype among Nod$^-$ mutants of *M. truncatula*, as it does not respond to Nod factors by any of the responses tested. The *nfp* mutant thus shows no rapid calcium flux, the earliest detectable Nod factor response of wild-type plants, and no root hair deformation. The *nfp* mutant is also deficient in Nod factor-induced calcium spiking and early nodulin gene expression. While certain genes controlling Nod factor signal transduction also control the establishment of an arbuscular mycorrhizal symbiosis, the *nfp* mutant shows a wild-type mycorrhizal phenotype. These data indicate that the *NFP* locus controls an early step of Nod factor signal transduction, upstream of previously identified genes and specific to nodulation.

Keywords: symbiosis, *Rhizobium*, Nod factor, signal transduction, calcium.

Introduction

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Mesorhizobium* (collectively known as rhizobia) induce the formation of nitrogen-fixing nodules on the roots of legume plants. This symbiotic association is initiated with a molecular dialogue in the rhizosphere. Flavonoids excreted by host plant roots induce the expression of bacterial *nod* genes, which encode proteins involved in the synthesis and excretion of lipochitooligosaccharide signalling molecules, the Nod factors (NFs) (for reviews, see Dénarié *et al.*, 1996; Long, 1996; Schultze and Kondorosi, 1998). In different cell layers of the root (mainly the epidermis and the cortex), purified NFs induce many of the responses induced by bacteria themselves (for reviews, see Cárdenas *et al.*, 2000; Downie and Walker, 1999; Geurts and Bisseling, 2002). Nod factor responses in root hairs include ion fluxes and associated depolarisation of the plasma membrane, calcium spiking, phosphatidic acid and diacylglycerol formation, accumulation of reactive oxygen species, root hair deformation involving changes in the actin cytoskeleton, and early nodulin gene expression. In cortical cells, NFs induce nodulin gene expression and cell division leading to nodule primordium formation. The rapidity and the specificity of several of the responses in root hair and epidermal cells, which are in direct contact with NFs, suggest that NFs are directly perceived by these cells. Some of these responses are induced at concentrations as low as 10^{-12} M NF, suggesting that NFs are recognised by high affinity receptors and that the signal is subsequently transduced and amplified within the epidermal cells and in different cell layers. Furthermore,

the perception of NFs appears to be complex with plant responses having different NF structural requirements, so it has been suggested that multiple perception mechanisms might exist (for a recent discussion, see Geurts and Bisseling, 2002). Using a biochemical approach, several proteins have been identified and it has been proposed that they are implicated in NF perception. These include NF binding sites and a lectin nucleotide phosphohydrolase that binds NFs and has an apyrase activity (Cullimore *et al.*, 2001; Gressent *et al.*, 2002). In the model legume *Medicago truncatula*, a genetic approach aimed at identifying genes involved in NF signal perception and transduction has been adopted (Catoira *et al.*, 2000). This has led to the identification of four genes named *DMI1*, *DMI2, DMI3* (doesn't make infections) and *NSP1* (nodulation signalling pathway) that control early steps of an NF-activated signal transduction pathway leading to the induction of symbiotic responses and nodulation (Catoira *et al.*, 2000; Wais *et al.*, 2000). A fifth gene implicated in NF signal transduction, *NSP2*, has recently been identified (C. Gough unpublished data; Oldroyd and Long, 2003). *DMI2* and orthologous genes in other legume species (*M. sativa*, *Lotus japonicus, Pisum sativum* and *Melilotus alba*) have been cloned (Endre *et al.*, 2002; Stracke *et al.*, 2002). These genes encode a receptor-like kinase (RLK), consistent with previous results implicating *DMI2* in an early stage of NF signal transduction (Catoira *et al.*, 2000; Wais *et al.*, 2000). *dmi2* mutants are not only deficient for nodulation, but are also unable to establish an arbuscular mycorrhizal symbiotic interaction. Therefore, despite the fact that *DMI2* encodes an RLK, it is likely that other *M. truncatula* gene(s), acting upstream of *DMI2* and specifically controlling NF perception, remain to be identified. Considering that *dmi1* and *dmi2* mutants respond to NFs by root hair swelling, we hypothesised that mutants in an upstream gene might be deficient for NF-induced root hair deformation.

In this work, we describe the identification and characterisation of C31, the first nodulation-deficient (Nod^-) mutant of *M. truncatula* that does not respond to NFs by the induction of root hair deformation. The C31 mutant was also found to be deficient in other NF responses, and is the first Nod^- mutant of any leguminous species shown not to respond to NFs by a rapid calcium flux, the earliest detectable response of legume root hairs to NFs. C31 is still able to establish an arbuscular mycorrhizal symbiosis, suggesting that the corresponding locus, the *N*od *F*actor *P*erception (*NFP*) locus, is specifically implicated in NF perception.

Results

C31 does not respond to Nod factors by root hair deformation

Nod factor-induced root hair deformation is a typical response of legumes and, in the case of *M. truncatula*, is characterised by the formation of subapical root hair branches (the Hab phenotype) (Catoira *et al.*, 2000). All *M. truncatula* Nod^- mutants so far described respond to NFs by root hair deformation, although the response is not always a wild-type response. Thus, *nsp1* and *hcl* mutants show the Hab phenotype, while *dmi* mutants show root hair swelling (the Has phenotype) (Catoira *et al.*, 2000, 2001). To identify a mutant of *M. truncatula* that does not respond to NFs by root hair deformation, tests were performed on our collection of *M. truncatula* mutants that are Nod^- and show no visible bumps.

Exploiting the same assay system used by Catoira *et al.* (2000) and 10^{-8} M *Sinorhizobium meliloti* NFs, three classes of mutants were identified. The first class of mutants were all Hab^+, including new *nsp1* and *hcl* alleles, and a new complementation group named *NSP2* (data not shown; C. Gough unpublished data; Oldroyd and Long, 2003). The second class of mutants were all Has^+ and corresponded in each case to new *dmi2* alleles (data not

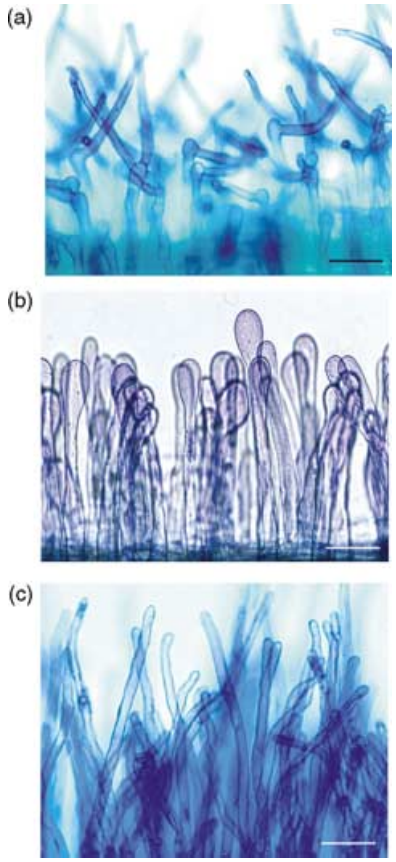

Figure 1. Wild-type and mutant root hair responses to Nod factors. Roots were treated with *S. meliloti* NFs at 10^{-8} M and stained with methylene blue 16 h later.
(a) Wild type showing the root hair branching (Hab) phenotype. Bar: 85 μm.
(b) P1 (*dmi2–3*) showing the root hair swelling (Has) phenotype. Bar: 70 μm.
(c) C31 showing no root hair deformations (neither the Hab nor the Has phenotype). Bar: 65 μm.

shown). A single mutant, C31, represented the third phenotypic class, and showed no NF-induced root hair deformations. Figure 1 illustrates the Hab and Has phenotypes of the wild type (Figure 1a), and a *dmi2* mutant (P1) (Figure 1b), respectively, and the absence of root hair deformations in the C31 mutant (Figure 1c). To exclude the possibility that this result was caused by the particular assay conditions used, in which plants are grown on the surface of agar-solidified medium, a novel system was developed, in which seedlings were grown in liquid medium (see Experimental procedures). In this new system, wild-type seedlings exhibited a typical Hab phenotype in response to 10^{-8} M NFs, while C31 seedlings still exhibited no root hair deformations (data not shown). From this, we conclude that C31 does not respond to NFs by root hair deformation.

To provide evidence that a single mutation is responsible for the absence of both nodulation and root hair deformation, the co-segregation of these two phenotypes was analysed in an F_2 population (see below) of 211 plants. All (58) Nod^- plants were Hab^- Has^- and all (153) Nod^+ plants were Hab^+. These data suggest that the mutation responsible for the Nod^- phenotype in the C31 mutant is also responsible for the absence of root hair deformation.

We checked the infection phenotype of the Nod^- C31 mutant using the strain GMI6526 of *S. meliloti* carrying a constitutively expressed *lacZ* gene (Table 1). C31, like *dmi* and *nsp1* mutants, is completely defective for marked root hair curling and infection thread initiation (data not shown). In response to rhizobial inoculation, the C31 mutant was also defective for the induction of cortical cell divisions, a typical response of wild-type plants that accompanies infection initiation. To exclude the possibility that the C31 mutant was Nod^- because of the inability to secrete rhizobial *nod* gene-inducing flavonoids, we verified that no nodules were formed on C31 plants inoculated with a constitutive NF producing strain of *S. meliloti*, GMI6390 (Table 1) (data not shown).

C31 represents a new complementation group

To study the genetic determinism of the Nod^- phenotype, C31 plants were crossed to wild-type plants. F_1 progeny of these crosses were all Nod^+ (Table 2), indicating a recessive nature of the Nod^- phenotype. The F_2 segregation data were consistent with a nuclear, monogenic mutation (Table 2). Allelism tests were performed to determine whether C31 falls into one of the five previously described complementation groups of infection-defective *M. truncatula* mutants, defining the genes *DMI1, DMI2, DMI3, NSP1* and *HCL* (Catoira *et al.*, 2000, 2001) and a sixth recently identified complementation group, *NSP2* (C. Gough unpublished data; Oldroyd and Long, 2003). F_1 progeny from these crosses were all Nod^+ (Table 3), showing that C31 falls into a new complementation group.

C31 is blocked for the induction of early nodulin gene expression in response to Nod factors

To determine whether the *C31* locus controls the same NF transduction pathway defined by the *DMI* and *NSP1* genes, we tested other NF responses previously shown to be defective in *dmi* and *nsp1* mutants, starting with expression of the early nodulin genes *rip1*, *MtENOD11* and *MtENOD40*. When gene expression was studied in the C31 mutant, either by RT-PCR (*MtENOD11*) or by Northern blot analysis (*rip1* and *MtENOD40*), no induction of these genes could be detected (Figure 2). As a more sensitive means of studying gene expression, we analysed transgenic C31 mutant plants carrying a fusion between the promoter of *MtENOD11* and the β-glucuronidase (GUS) reporter gene.

Table 1 Bacterial strains, plasmids and plants used in this study

Designation	Relevant characteristics	Reference/source
Sinorhizobium meliloti		
GMI6526	2011(pXLGD4), Nod^+ Fix^+ on *M. truncatula*	Ardourel *et al.* (1994)
GMI6390	2011(pMH682), Nod^+ Fix^+ on *M. truncatula*	Roche *et al.* (1991b)
GMI6702	2011*nodA*::Tn5#2208(pXLGD4), Nod^- on *M. truncatula*	Debellé *et al.* (1986)
Plasmids		
pXLGD4	pGD499 prime (IncP) carrying a *hemA*::*lacZ* fusion, Tc^R	Leong *et al.* (1985)
pMH682	pWB85a prime (IncP), carrying *nodD3* and *syrM* of *S. meliloti*, Tc^R	Honma *et al.* (1990)
Medicago truncatula		
Jemalong A17	Wild-type, Nod^+Fix^+ with *S. meliloti*	Penmetsa and Cook (1997)
Jemalong(p*MtENOD11-GUS*)	Jemalong A17 carrying a p*MtENOD11-GUS* fusion construct	Journet *et al.* (2001)
DZA315.16	Algerian accession, Nod^+ Fix^+ with *S. meliloti*	Tirichine *et al.* (2000)
C31	EMS Nod^- mutant of Jemalong	This study
C31(p*MtENOD11-GUS*)	EMS Nod^- mutant of Jemalong carrying a p*MtENOD11-GUS* fusion construct	This study

Tc^R, tetracycline resistant.

Table 2 Genetic analysis of the C31 mutant

Cross	F_1[a]		F_2[a]			
	Nod^+	Nod^-	Nod^+	Nod^-	Albino (%)	χ^2 [b]
C31 × Jemalong(p*MtENOD11-GUS*)	10	0	222	80	0	0.36
C31 × DZA315.16	10	0	329	107	5.55	0.05

[a]Nodulation was scored in plants of the F_1 and F_2 generations, 3 weeks after inoculation with *S. meliloti* GMI6526; numbers indicate the number of plants found to be Nod^+ or Nod^- and the percentage of F_2 plants found to be albino.
[b]χ^2 calculated for a 3 : 1 ratio of Nod^+/Nod^- phenotype, $P > 0.05$ when $\chi^2 < 3.84$. Albino plants were not taken into account in calculating the χ^2 for the cross C31 × DZA315.16.

These plants were generated by crossing C31 to wild-type *M. truncatula* plants carrying the construction p*MtENOD11-GUS,* Jemalong(p*MtENOD11-GUS*) (Journet *et al.*, 2001). *MtENOD11* expression could be detected in root hair and non-root hair epidermal cells in response to 10^{-8} M *S. meliloti* NFs in wild-type transgenic plants (Figure 3a), whereas in the C31(p*MtENOD11-GUS*) plants no such *MtENOD11* expression could be detected (Figure 3b). In contrast, the non-symbiotic pattern of *MtENOD11* expression in root cap cells of root apexes, was unaffected in the C31 mutant (data not shown).

These data indicate that the *C31* locus of *M. truncatula* is required for NF-induced expression of *MtENOD11, rip1* and *MtENOD40*. The phenotype of C31 plants for nodulin gene expression is similar to that of *dmi* and *nsp1* mutants, except that gene induction was completely absent in C31 plants, whereas very slight *rip1* induction is detected in *dmi* and *nsp1* mutants, and a low level of *MtENOD11* induction is detected in *nsp1* mutants (Catoira *et al.*, 2000).

The C31 locus appears to act upstream of a mastoparan-responsive signalling element in the Nod factor transduction pathway

The application of a pharmacological approach to studying NF transduction has provided evidence for the participation of a heterotrimeric G protein in NF signalling (den Hartog *et al.*, 2001; Pingret *et al.*, 1998; Vernoud *et al.*, 1999). To determine where the *C31* locus acts with respect to a potential G protein signalling element, we exploited the results of Vernoud *et al.* (1999) that the heterotrimeric G protein agonist mastoparan can induce the p*MtENOD11-GUS* construct in *M. truncatula* transgenic plants. We used both mastoparan and the more active analogue of mastoparan, Mas-7 (Pingret *et al.*, 1998), and as these two agonists gave similar results (data not shown), we only present data obtained using Mas-7. Mas-7 induced GUS activity in wild-type transgenic plants, in the root hair and the epidermal cells of the region of root hair emergence and development (Figure 3c). Mas-7 treatment of C31(p*MtENOD11-GUS*) plants also resulted in GUS activity that was comparable, both in quantitative and qualitative terms, to that induced by Mas-7 in wild-type transgenic plants (Figure 3d). Compared to GUS activity induced by NFs in wild-type transgenic *M. truncatula* plants (Figure 3a), however, Mas-7 induction both in wild-type and mutant plants was relatively weak. No *MtENOD11* expression could be detected with the inactive analogue of mastoparan, Mas-17 (data not shown).

These results indicate that the C31 mutant is not defective in NF signalling leading from the site of Mas-7 action to the expression of *MtENOD11*. The *C31* locus therefore appears to act upstream of the mastoparan-responsive signalling element.

Table 3 Allelism tests

Cross	Number of crosses	Phenotypes[a]	
		Nod^+	Nod^-
C31 × Y6 (*dmi1–3*)	4	15	0
C31 × TR25 (*dmi2–1*)	4	16	0
C31 × TRV25 (*dmi3–1*)	3	17	0
C31 × B85 (*nsp1–1*)	2	17	0
C31 × D11 (*nsp2–3*)	8	35	0
C31 × B56 (*hcl-1*)	3	16	0

[a]The nodulation phenotype was scored in the F_1 generation, 3 weeks after inoculation with *S. meliloti* GMI6526. Numbers represent the number of plants found to be Nod^+ or Nod^-. Note that the allele name of the B85 mutant has been changed from *nsp-1* to *nsp1–1*.

Like DMI1 *and* DMI2*, the C31 locus controls Nod factor-induced calcium spiking*

Calcium spiking is the response of legume root hairs to NFs, in which sharp oscillations of the cytoplasmic calcium ion concentration are induced (Cárdenas *et al.*, 2000; Downie and Walker, 1999; Ehrhardt *et al.*, 1996). Compared to early nodulin gene expression, the induction of calcium spiking is rapid, being initiated within 10–15 min following NF application. Furthermore, *dmi1* and *dmi2* mutants of *M. truncatula* are defective for NF-induction of calcium spiking, while *dmi3, nsp1* and *hcl* mutants display calcium spiking (Wais *et al.*, 2000).

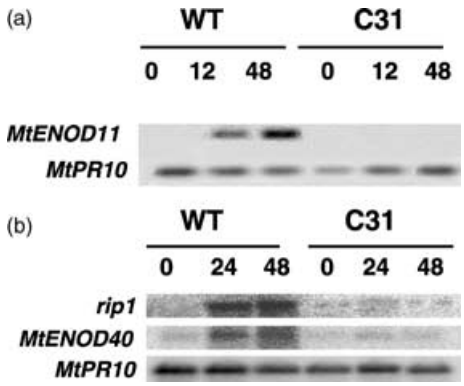

Figure 2. Analysis of early nodulin gene expression in wild-type (WT) and C31 mutant plants in response to Nod factors. Roots were treated with *S. meliloti* NFs at 10^{-8} M and harvested at the indicated times (hours post-treatment). *MtPR10* RNA was amplified as a control for the quality and quantity of the RNA samples.
(a) RT-PCR analysis of *MtENOD11* expression.
(b) Northern blot analysis of *rip1* and *MtENOD40* expression.

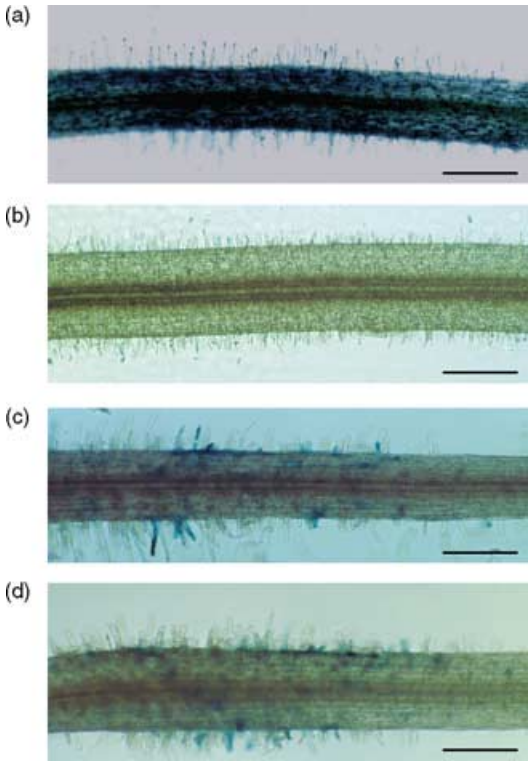

Figure 3. *MtENOD11* expression in transgenic wild-type and C31 mutant plants in response to Nod factors or Mas-7. The histochemical localisation of GUS activity is shown in roots of transgenic plants carrying an *MtENOD11* promoter–GUS fusion. Roots were treated with 10^{-8} M *S. meliloti* NFs or 0.2 μM Mas-7 for 6 and 16 h, respectively, and then coloured for GUS activity.
(a) Primary root of the wild type treated with NF. Bar: 180 μm.
(b) Primary root of C31 treated with NF. Bar: 180 μm.
(c) Secondary root of the wild type treated with Mas-7. Bar: 200 μm.
(d) Secondary root of C31 treated with Mas-7. Bar: 160 μm.

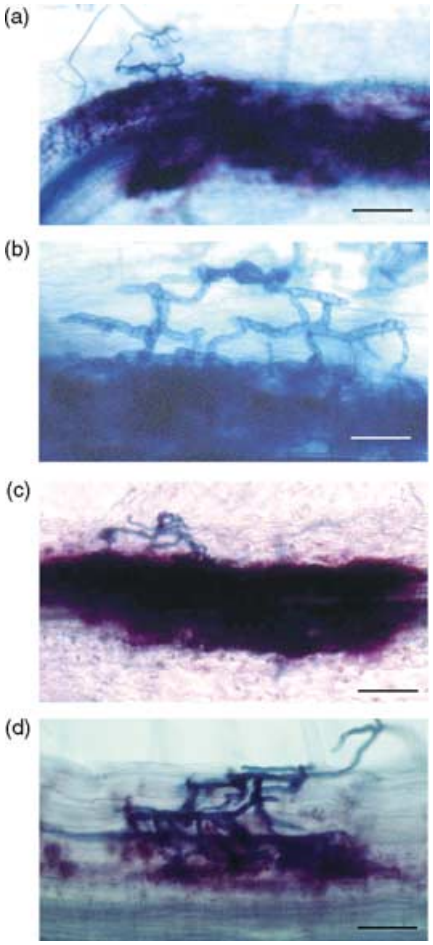

Figure 6. Mycorrhizal colonisation and associated *MtENOD11* expression of wild-type and C31 mutant plants.
Transgenic plants carrying a fusion between the promoter of *MtENOD11* and the GUS reporter gene were grown in test tubes with spores of *Gigaspora margarita* for 4 weeks. Photos are of mycorrhizal roots after single (a and b) or double (c and d) staining with ink and vinegar for fungal structures (in blue) and Magenta-GlucA for GUS activity (in red). Fungal hyphae can be seen penetrating into the roots, and GUS expression is associated with fungal infection in the inner cortex. (a, b and d) C31 mutant; (c) Wild-type plant. Bars: 150 μm (a); 45 μm (b); 110 μm (c); 55 μm (d).

The C31 mutant was tested for NF-induction of calcium spiking, and was found to be completely defective for this response: 0 of 37 root hairs on nine plants showed calcium spiking. A wild-type plant was treated in parallel to C31 in all these experiments and in every case the wild-type plant showed calcium spiking. Figure 4 shows representative traces for the C31 mutant and the wild type. These data indicate that the *C31* locus, like *DMI1* and *DMI2,* is required for NF-induced calcium spiking and acts upstream of calcium spiking, *DMI3* and *NSP1* in the NF signal transduction pathway.

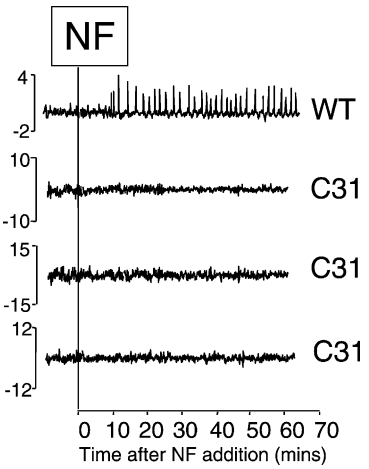

Figure 4. Calcium spiking response of wild-type and C31 mutant plants in response to Nod factors. A single wild-type (WT) trace is shown and three mutant traces, each trace represents an individual cell. Roots were treated with 10^{-9} M *S. meliloti* NFs. Traces represent the change in fluorescence intensity from one time point to the next ($x_{n+1} - x_n$) of a single root hair injected with Oregon green-dextran. The amplitude of the fluorescence change is given in arbitrary units and cannot be compared from one cell to another, given that Oregon green-dextran is a non-ratiometric dye.

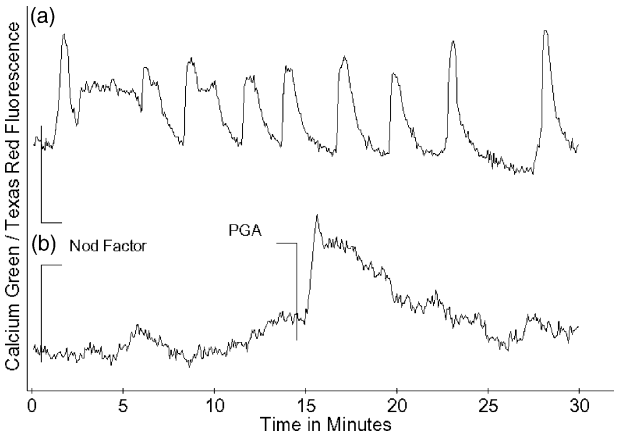

Figure 5. Analysis of Nod factor-induced calcium flux in wild-type and C31 mutant plants. *S. meliloti* NF (10^{-8} M final bath concentration) was applied to seedlings and changes in calcium were recorded using a ratiometric imaging protocol.
(a) Wild-type plants show an immediate, biphasic calcium flux followed by calcium spiking, at about 9 min after NF addition.
(b) C31 mutant shows no changes in calcium concentration in response to NF. A rapid change in calcium concentration is observed in C31 root hair cells in response to the polysaccharide elicitor polygalacturonic acid heptamer (PGA) at 1 μg ml^{-1} final bath concentration.

C31 is the first Nod$^-$ mutant that is blocked for the induction of a rapid calcium flux in response to Nod factors

To have a better idea of when the *C31* locus acts in NF signal transduction, we sought to test an earlier NF response. Prior to calcium spiking, several NF-induced ionic fluxes are detected, the most commonly described of which appears to be a rapid calcium influx (Cárdenas *et al.*, 2000; Downie and Walker, 1999; Müller *et al.*, 2000; Yokoyama *et al.*, 2000).

Using dual-dye ratiometric calcium imaging, a rapid, biphasic rise in cytosolic free calcium has recently been detected in *M. truncatula* in response to 10^{-8} M NFs (Shaw and Long, 2003). This response initiates 1–2 min after NF application and consists of an initial phase of calcium elevation followed by a sustained rise in cytoplasmic calcium concentration until calcium spiking starts (Figure 5a). In *M. truncatula dmi1* and *dmi2* mutants, NF application induces a monophasic calcium flux, while a wild-type biphasic response is induced in a *dmi3* mutant (Shaw and Long, 2003).

When the C31 mutant was tested for this rapid calcium response, no perceptible changes in cytoplasmic calcium concentration in response to 10^{-8} M NF were detected when compared to changes in wild-type plants ($n = 9$ cells representing four plants) (Figure 5b). In all cases for the C31 mutant, polygalacturonic acid (heptamer at 1 μg ml^{-1}) was added subsequent to NF to demonstrate that the root hairs were competent to respond to exogenous signals with a change in cytoplasmic calcium levels (Figure 5b). These results provide evidence that the *C31* locus (i) acts upstream of the rapid calcium flux, (ii) acts upstream of *DMI1* and *DMI2*, and (iii) controls a step of NF signal transduction that is shared in common between calcium spiking and the rapid increase in cytosolic calcium concentration.

The C31 mutant is able to establish arbuscular mycorrhization and MtENOD11 *is expressed during mycorrhizal colonisation*

All three *DMI* genes of *M. truncatula* control not only NF transduction, but also the establishment of arbuscular mycorrhizal symbiotic associations (Catoira *et al.*, 2000), and, in contrast to the *Rhizobium*–legume association, arbuscular mycorrhizal symbioses show very little host specificity. That there should be an NF-specific branch of the transduction pathway upstream of the *DMI* genes is, therefore, likely and has already been proposed (reviewed recently by Kistner and Parniske (2002)).

The ability of the C31 mutant to establish an arbuscular mycorrhizal symbiosis with mycorrhizal fungi was studied using an *in vitro* mycorrhizal system developed in our laboratory (see Experimental procedures). This system was clearly shown to differentiate wild-type plants (Myc$^+$ phenotype) and *dmi* mutants (Myc$^-$ phenotype) (data not shown). For C31 plants, the arbuscular mycorrhizal fungus *Gigaspora margarita* was found to penetrate roots and develop internal infection structures, as for wild-type plants, demonstrating an Myc$^+$ phenotype (Figure 6a,b). This result, together with the fact that the *C31* locus appears to act upstream of the *DMI* genes, suggests that the *C31*

locus is on a branch of the NF signal transduction pathway specifically activated by NFs. If this is true, we can predict that any response that can be induced in *M. truncatula* both by NFs and during the arbuscular mycorrhizal symbiosis, can still be induced during mycorrhizal colonisation of the C31 mutant. To test this hypothesis, we studied the expression of *MtENOD11* in roots of C31 colonised by *G. margarita*, as this early nodulin gene is expressed in arbuscule-containing cortical cells of *M. truncatula* roots colonised by mycorrhizal fungi (Journet *et al.*, 2001). *MtENOD11* was found to be expressed in roots of C31 plants colonised by *G. margarita*, and no difference could be detected in the pattern or level of expression compared to wild-type plants (Figure 6c,d). From this, we can conclude that the signalling pathway leading to *MtENOD11* expression during mycorrhizal colonisation is not affected by mutation in the *C31* locus. As the C31 mutant is blocked for all NF responses tested and has a normal mycorrhizal phenotype, the corresponding locus has been named *NFP* for *N*od *F*actor *P*erception.

The NFP *locus is located on chromosome 5 of* Medicago truncatula

As a first step towards the map-based cloning of the *NFP* locus, a molecular marker-based approach was used to map the locus on the *M. truncatula* genetic map. As this genetic map was derived from a cross between *M. truncatula* Jemalong and the accession DZA315.16 of *M. truncatula* (Thoquet *et al.*, 2002), we first generated an F_2 population between the C31 mutant (Jemalong derivative carrying the C31 mutation) and DZA315.16. The nodulation phenotypes of F_1 and F_2 plants are indicated in Table 2.

Ten AFLP markers linked to the *NFP* locus were identified using the Bulked Segregant Analysis method (Michelmore *et al.*, 1991). Distances between the *NFP* locus and the linked markers were evaluated by genotyping 12 Nod^+ individuals and 93 Nod^- individuals of an F_2 subpopulation derived from the cross C31 $\times$ DZA315.16. Two markers (PK12 and B07494), which had already been localised on the *M. truncatula* genetic map (Thoquet *et al.*, 2002), were also found to be linked to the *NFP* locus. Consequently, we were able to map the *NFP* locus at the Northern extremity of linkage group 5, corresponding to chromosome 5. Of the 10 newly identified AFLP markers, the one found to be localised at the closest genetic distance to the *NFP* locus, PV02, was mapped on the *M. truncatula* genetic map. This allowed us to build a 3.8 cM local map of the *NFP* region, including three markers (Figure 7).

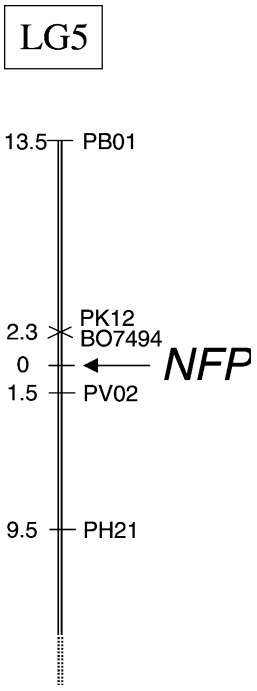

Figure 7. Local genetic map of the *NFP* locus.
Localisation of the *NFP* locus on linkage group 5 (LG5) of the *M. truncatula* genetic map. Markers PK12, B07494 and PV02 were found to be linked to the *NFP* locus. PV02 was mapped on LG5, while the other markers were already mapped on LG5 by Thoquet *et al.* (2002). PB01 is the Northern-most genetic marker on LG5. Genetic distances are indicated on the left (Kosambi cM).

Discussion

In this study, we have used the model legume *M. truncatula* to further dissect the mechanisms by which NFs induce symbiotic responses and nodulation. We hypothesised that mutants in a gene acting upstream of *DMI1* and *DMI2* might be deficient for NF-induced root hair deformation, and screened for this phenotype. A novel *M. truncatula* mutant, C31, was identified that does not respond to NFs by root hair deformation, a rapid calcium flux, calcium spiking or early nodulin gene expression. This pleiotropic phenotype strongly suggests that the *NFP* locus controls NF signal transduction. Previous work had identified five genes (*DMI1*, *DMI2*, *DMI3, NSP1* and *NSP2*) that are required for an NF-activated signal transduction pathway, with *DMI1* and *DMI2* apparently acting upstream of the other three genes (Catoira *et al.*, 2000; Oldroyd and Long, 2003; Wais *et al.*, 2000; C. Gough, unpublished results). Like the *nfp* mutant, *dmi1* and *dmi2* mutants are unable to respond to NFs by calcium spiking and early nodulin gene expression (Catoira *et al.*, 2000; Wais *et al.*, 2000). This indicates that *DMI1*, *DMI2* and the *NFP* locus all control steps of the same NF-activated signal transduction pathway. Furthermore, while *dmi1* and *dmi2* mutants show modified root hair deformation and a modified rapid calcium flux response (Catoira *et al.*, 2000; Shaw and Long, 2003), the *nfp* mutant is completely deficient for both these responses. This provides good evidence that the *NFP* locus acts upstream of the *DMI1* and *DMI2* genes in this signal transduction pathway. Unlike *dmi* mutants, the *nfp* mutant

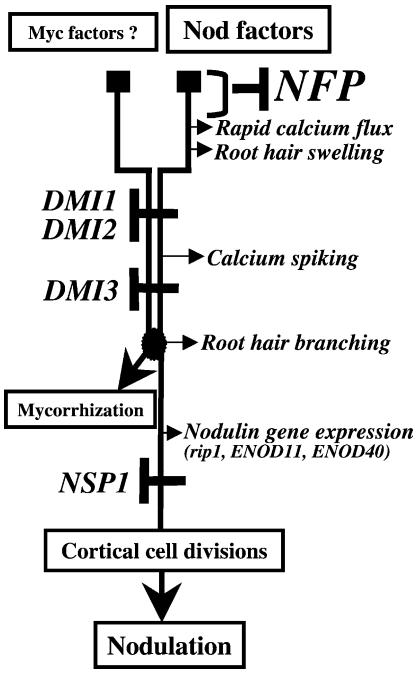

Figure 8. Model for the intervention of *NFP* in Nod factor signal transduction.
An adaptation of the model presented by Catoira *et al.* (2000), for an NF signal transduction pathway leading to cortical cell divisions and nodulation. *DMI1*, *DMI2, DMI3* and *NSP1* were previously ordered from the phenotypes of corresponding mutants (see Catoira *et al.*, 2000). The *NFP* locus would intervene upstream of *DMI1* and *DMI2,* with the bracket indicating that the *NFP* locus either encodes an NF receptor or controls a very early step of NF signal transduction. Our data do not enable us to distinguish between these two possibilities. NF responses characterised in *nfp, dmi* and *nsp1* mutants are indicated in italics with arrows, indicating that these responses are not necessarily components of this NF signal transduction pathway. Note that the *NSP1* gene is a re-naming of the *NSP* gene. Myc factors are hypothetical mycorrhizal signals, which would play a role in the preparation of the plant for infection by arbuscular mycorrhizal fungi.

is still able to establish a symbiotic association with mycorrhizal fungi. Furthermore, we find that symbiotic expression of *MtENOD11* is normal in arbuscule-containing cortical cells of *nfp* roots colonised by a mycorrhizal fungus. The mechanism for *MtENOD11* induction during mycorrhizal colonisation is therefore independent of the *NFP* locus, supporting the idea that *NFP* is specifically involved in NF signalling (see model presented in Figure 8).

For the genetic interpretation of the pleiotropic phenotype of the *nfp* mutant, complete linkage was shown between the absence of both nodulation and NF-induced root hair deformation. Also, the NF responses which are absent in the *nfp* mutant, are all absent or modified in *dmi* and *nsp1* mutants, for which there is genetic evidence that a single mutated gene is responsible in each case for the multiple defects (Catoira *et al.*, 2000). Furthermore, the *NFP* locus does not map on the same linkage group as any of the three *DMI* genes (Ané *et al.*, 2002). In conclusion, it is probable that the multiple defects in NF responsiveness of the *nfp* mutant are the result of only one mutated gene, although we cannot rule out that mutations in two closely linked genes are involved.

The NFP *locus controls the Nod factor-induced calcium flux and calcium spiking*

The calcium ion is firmly established as an important component of a diverse array of plant signal transduction pathways and is often implicated as a primary step in signalling (Sanders *et al.*, 2002). In response to NF, a host legume experiences a rapid change in cytoplasmic calcium concentration, followed by periodic calcium oscillations (reviewed in Cárdenas *et al.*, 2000; Downie and Walker, 1999). These responses are dependent on those NF structures responsible for conferring host specificity, suggesting a role in the transduction of the NF signal. Pharmacological studies in *M. sativa* suggest a critical role for calcium elevation in the initiation of downstream responses to NF (Donaire *et al.*, 1999; Felle *et al.*, 1998, 1999a). Analysis of these calcium changes in *M. truncatula* Nod$^-$ mutants now provides an opportunity to explore the possible role of calcium signalling in the control of symbiotic infection (Catoira *et al.*, 2000; Oldroyd *et al.*, 2001; Shaw and Long, 2003; Wais *et al.*, 2000).

A rapid and biphasic increase in cytosolic calcium concentration was recently observed in *M. truncatula* with a dual-dye technique (Shaw and Long, 2003). The two phases of this rapid calcium flux appear to be genetically separable. Thus, the calcium flux response in *dmi1* and *dmi2* mutants has only a single phase, corresponding to the initial increase and drop in wild-type plants (Shaw and Long, 2003). In this work, the *nfp* mutant was found to be completely deficient for the NF-induced rapid calcium flux. The *NFP* locus therefore controls a step of NF signal transduction upstream of both phases of this calcium flux.

In contrast to the rapid calcium flux, calcium spiking is a very prolonged NF-induced response (Ehrhardt *et al.*, 1996), that is probably a signal transduced into a growth response of activated root hairs. The absence of calcium spiking in nodulation mutants of *M. sativa* (Ehrhardt *et al.*, 1996), *M. truncatula* (Wais *et al.*, 2000) and pea (Walker *et al.*, 2000), suggests a conserved role for calcium spiking in NF signal transduction. Our demonstration that the *nfp* mutant of *M. truncatula* is also deficient for calcium spiking (this work), consolidates the evidence that calcium spiking is an essential NF signalling element. Furthermore, these data show that the first phase of the rapid calcium response and calcium spiking share a common step controlled by the *NFP* locus. Interestingly, however, the induction of calcium spiking does not depend upon an initial rapid calcium flux, as calcium spiking can be induced independently using NF concentrations in the range of 10^{-9} to 10^{-12} M (Oldroyd *et al.*, 2001), while the rapid calcium flux response requires

10^{-7} to 10^{-9} M NFs (Felle *et al.*, 1999b; Shaw and Long, 2003). Also, chitin oligomers can induce calcium spiking in *M. truncatula* (Oldroyd *et al.*, 2001), and in pea (Walker *et al.*, 2000), but not rapid calcium fluxes in *M. sativa* (Felle *et al.*, 1999b). We therefore conclude that the calcium flux response is physiologically separable from the calcium spiking response, although both responses require *NFP*.

Finally, it can be deduced that the second phase of the rapid calcium flux is not necessary for root hair swelling, but that the first phase might be. Thus, the *nfp* mutant is completely defective in both properties, while *dmi1* and *dmi2* mutants show only the first phase of the rapid calcium flux and show root hair swelling (this work; Catoira *et al.*, 2000; Shaw and Long, 2003). The process by which NFs induce re-initiation of root hair growth involves a new tip-focussed calcium gradient that forms at the site of growth (de Ruijter *et al.*, 1998). Changes to the actin cytoskeleton are also probably involved, as rearrangements of actin filaments are induced rapidly by NFs (Cárdenas *et al.*, 1998; de Ruijter *et al.*, 1999), and actin plays an important role in root hair morphogenesis (Miller *et al.*, 1999). These changes are all likely to be compromised in the *nfp* mutant.

The NFP *locus and early Nod factor signalling*

The phospholipase C (PLC) and phopsholipase D (PLD) pathways are implicated in early NF signalling (Engstrom *et al.*, 2002; den Hartog *et al.*, 2001; Pingret *et al.*, 1998), and are probably activated subsequent to rapid ionic fluxes and membrane depolarisation. Calcium spiking is likely to be downstream of PLC activation, as inhibition of PLC activity in *M. truncatula* inhibits NF-induced calcium spiking (Engstrom *et al.*, 2002). The *NFP* locus, controlling as it does a rapid calcium flux, probably controls a step of NF signal transduction upstream of the PLC and PLD pathways.

Heterotrimeric G proteins have also been suggested to be implicated in early NF signalling (den Hartog *et al.*, 2001; Pingret *et al.*, 1998) and *DMI2* may act upstream of such a potential heterotrimeric G protein mediating NF signal transduction leading to *MtENOD11* expression (Vernoud *et al.*, 1999). As Mas-7 can induce *MtENOD11* expression in the *nfp* mutant, the *NFP* locus will also act upstream of this potential G protein signalling element. Alternatively, the mastoparan activities observed in *Medicago* spp. (Pingret *et al.*, 1998; Vernoud *et al.*, 1999; this work) and in *V. sativa* (den Hartog *et al.*, 2001), might be independent of NF signalling, which could explain the differences in activity levels between NFs and mastoparan. Despite the apparent paucity of prototypical heterotrimeric G proteins in plants, G protein-based signalling is certainly widespread (Assmann, 2002; Yang, 2002). Molecular characterisation of the very early steps of NF signal transduction will confirm or not the involvement of a G protein.

NFP *may be a Nod factor receptor or constitute part of a receptor complex*

The nature of several plant receptors and their corresponding signal transduction pathways have largely been elucidated using genetic screens for response-deficient mutants. By analogy, a plant mutant in an NF receptor would be expected to be insensitive to NFs. Although the list of NF responses tested in the *nfp* mutant is not exhaustive, the complete absence of rapid responses such as calcium flux, calcium spiking, root hair deformation and early nodulin gene expression, indicates that the *NFP* locus of *M. truncatula* might encode a receptor of an NF signal transduction pathway leading to symbiotic responses and nodulation, or at least controls a very early step of this pathway (see model presented in Figure 8).

DMI2 and orthologous genes from *M. sativa*, *L. japonicus*, *P. sativum* and *M. alba* have recently been cloned and shown to encode a receptor-like kinase (RLK) (Endre *et al.*, 2002; Stracke *et al.*, 2002). RLKs constitute a large family of plant proteins and there is growing evidence that plant RLKs are often part of receptor complexes. By analogy, it is possible that DMI2 forms part of a receptor complex. Moreover, considering that a functional *DMI2* gene is required for the establishment of both the *Rhizobium*-legume symbiosis and an arbuscular mycorrhizal symbiosis, such a complex might integrate symbiotic signals from both *Rhizobium* (NFs and possibly others) and arbuscular mycorrhizal fungi (hypothetical 'Myc factors'). In this scenario, *NFP* would encode an element of the receptor complex specifically involved in NF perception. Analogous functions might be performed by SYM10 of pea, and SYM1 and SYM5 of *L. japonicus*, as the corresponding mutants are Nod^-, are all still able to establish arbuscular mycorrhizal symbioses, all lack a root hair response to *Rhizobium*, and pea *sym10* mutants are deficient for NF-induced calcium spiking (Kistner and Parniske, 2002; Walker *et al.*, 2000). The *sym10* locus of pea has recently been mapped on pea linkage group I (Schneider *et al.*, 2002), and preliminary data from comparative mapping indicate that *SYM10* of pea and *NFP* of *M. truncatula* might be orthologous genes (F. Maillet and N. Ellis, unpublished results). Whether or not we can confirm this hypothesis will become apparent by cloning of *NFP* and NFP orthologues.

Experimental procedures

Bacterial strains and plants

Bacterial strains and plants are described in Table 1. The C31 mutant was screened from the ethyl-methyl sulphonate (EMS) mutagenesis described by Penmetsa (1998) and Penmetsa and Cook (2000).

Genetic analysis

Screening for the Nod$^-$ *M. truncatula* mutant C31, crosses and nodulation assays were as described by Catoira *et al.* (2000). Transgenic wild-type plants carrying a fusion between the *MtENOD11* promoter and the reporter gene encoding GUS, Jemalong(p*MtENOD11-GUS*) (Journet *et al.*, 2001) were used for genetic analysis of C31, as described by Catoira *et al.* (2000), to enable the identification of true hybrids.

Plant growth conditions

Plant growth conditions for nodulation tests, *MtENOD11* expression by RT-PCR and *MtENOD11* transgenic studies have been described (Catoira *et al.*, 2000). For RNA gel blot analysis and co-segregation tests, plants were grown in aeroponic medium (Catoira *et al.*, 2000). For root hair deformation studies, plants are either grown on agar (Catoira *et al.*, 2000) or in liquid medium, for which germinated seeds were put in grids and grown with their roots in Fähraeus liquid medium (Catoira *et al.*, 2000) for 1 day before being treated with NFs. For calcium spiking studies and the measurement of the rapid flux of calcium, plants were grown as described by Wais *et al.* (2000) and Shaw and Long (2003), respectively. For mycorrhizal experiments, we used an *in vitro* mycorrhizal system developed in our laboratory. Transgenic plants were grown in test tubes on slopes of 20 ml of sterile M medium (Bécard and Fortin, 1988), solidified with 5 g l^{-1} phytagel (Sigma). Four spores of *G. margarita* were put in the bottom of each slope near the roots of young (just germinated) seedlings. The plants were grown for 4 weeks and then stained for both β-glucuronidase (GUS) activity and fungal structures (see below).

Nod factor treatment

Nod factors (NFs) were prepared as described by Roche *et al.* (1991a). NFs were added directly to plants growing on agar or in liquid medium for root hair deformation studies, directly to plants growing in growth pouches for RT-PCR, and as described by Pingret *et al.* (1998) and Cook *et al.* (1995) for transgenic plants and RNA gel blot analysis, respectively. NF treatment of root hairs for the calcium spiking assay and the measurement of the rapid flux of calcium were performed as described by Wais *et al.* (2000) and Shaw and Long (2003), respectively.

RT-PCR assay

At 0, 12 and 48 h after NF addition, roots of 30 seedlings that were 11 days old, were frozen in liquid nitrogen and total RNA was extracted as described by Catoira *et al.* (2000). RT-PCR was performed using the SuperScript one-step RT-PCR kit would platinum Taq (Life technologies). For cDNA synthesis and pre-denaturation, 1 cycle of 30 min at 55°C and 2 min at 94°C were performed and for PCR amplification, the repeating cycle was 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min. *MtENOD11* (Journet *et al.*, 2001) cDNA was amplified (25 cycles) between positions 82 and 519 by using the forward primer 5′-CTCCATCCCACAATATGCCTCCA-3′ and reverse primer 5′-ATCGATGCTAGGTGGAGGCT-3′. As described by Catoira *et al.* (2000), we also performed PCR amplification of *MtPR10*, a gene constitutively and strongly expressed in roots of *M. truncatula* to control for equivalent cDNA synthesis levels. The *MtPR10* cDNA was amplified (20 cycles) between positions 143 and 433 by using the forward primer 5′-CCGGAACCATCAAAAAAC-3′ and reverse primer 5′-TGAAAAGACCATCACCCC-3′. PCR products were analysed by DNA gel blotting with radioactive probes made from a 341 bp PCR fragment of *MtENOD11* and with the complete cDNA of *MtPR10*.

Northern blot analysis

At 0, 24 and 48 h after NF addition, roots of 30 seedlings that were 15 days old were frozen in liquid nitrogen. Total RNA was extracted using the Extractol protocol (Eurobio). Northern blots were made from total RNA denatured with glyoxal and dimethyl sulphoxide, and transferred to Hybond N membranes (Amersham). Hybridisation of radioactive DNA probes for *rip1* (Cook *et al.*, 1995) and *MtENOD40* (Gamas *et al.*, 1996) was performed at 65°C in 0.5 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA, 0.1% BSA and 100 μg ml^{-1} denatured salmon sperm DNA.

Staining methods

Plants were observed for infection events by histochemical staining for β-galactosidase activity expressed by the plasmid pXLGD4 (Ardourel *et al.*, 1994). Root hair deformations were observed after methylene blue staining (Catoira *et al.*, 2000). GUS staining was as described by Pingret *et al.* (1998), except that plants were stained at 5 days after germination. To look for cortical cell divisions, seedlings were grown on agar plates for 3 days and were then spot inoculated with the *S. meliloti* strain GMI6526. Control plants were treated with the strain GMI6702, unable to produce NFs (Table 1). Roots were collected 3 days later, fixed in glutaraldehyde (2.5%), and treated as previously described (Catoira *et al.*, 2001). For mycorrhizal tests, plants were stained first for GUS activity using the substrate Magenta-glucA and then for fungal structures using ink and vinegar (Vierheilig *et al.*, 1998).

Assay for pMtENOD11-GUS activation in transgenic plants with pharmacological effectors

Transgenic plants were grown in the growth pouch system (Catoira *et al.*, 2000). The peptide effectors Mas-7 and Mas-17 were dissolved in H_2O and used at 0.2 μM (Mas-7) and 1 μM (Mas-17). Pharmacological studies were performed as described in Pingret *et al.* (1998). For each effector treatment, 10–25 plants were examined, and all experiments were performed at least thrice.

Calcium spiking assay

The assessment of calcium spiking was performed as described by Wais *et al.* (2000).

Rapid calcium flux assay

The rapid calcium flux response was assessed following co-injection with calcium green-dextran (10 kDa) and Texas Red dextran (10 kDa) (Molecular Probes, Eugene, OR, USA) as described by Shaw and Long (2003). A polygalacturonic acid heptamer (kind gift of Dr Mike Hahn, CCRC, Athens, GA, USA) was applied from 1 mg ml^{-1} stock in water.

Mapping

AFLP markers linked to the *NFP* locus were identified using the Bulked Segregant Analysis method (Michelmore *et al.*, 1991). The cross C31 × DZA315.16 resulted in 5.55% of albino plants in the F_2 generation that stopped growing after 3 days, and were thus not taken into account for the segregation data. Similar percentages of albinos have been observed in other crosses between *M. truncatula* Jemalong lines and ecotypes DZA315.16 (Ané *et al.*, 2002) and A20 (Penmetsa and Cook, 2000). For the Bulked Segregant Analysis, 10 Nod$^+$ and 10 Nod$^-$ F_2 individuals were randomly chosen to constitute four DNA pools of five individuals each. DNA pools were screened with 64 primer combinations.

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