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

Besma Ben Amor1, Sidney L. Shaw2, Giles E. D. Oldroyd2,−1, Fabienne Maillet1, R. Varma Penmetsa3, Douglas Cook3, Sharon R. Long2, Jean Dénaire1 and Clare Gough1,−

1Laboratoire des Interactions Plantes-Microorganismes, INRA-CNRS, BP 27, 31326 Castanet-Tolosan, France, 2Howard Hughes Medical Institute, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA, and 3Department of Plant Pathology, University of California, Davis, CA 95616, USA

Received 10 December 2002; revised 28 January 2003; accepted 5 February 2003.

For correspondence (fax +33 561285061; e-mail gough@toulouse.inra.fr).

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 Nod Factor Perception (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; Schultz 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 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 as well as swelling (the Has phenotype) (Catoira et al., 2000). All the perception mechanisms 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 a very clear bump, and 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 shown). 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 symbiotic interaction, suggesting that the corresponding locus, the Nod Factor Perception (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.

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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 F₂ 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, 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. F₁ progeny of these crosses were all Nod⁺ (Table 2), indicating a recessive nature of the Nod⁻ phenotype. The F₂ 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). F₁ 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

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
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<tr>
<td>Sinorhizobium meliloti</td>
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<tr>
<td>GM16526</td>
<td>2011(pXLGD4), Nod⁺ Fix⁺ on M. truncatula</td>
<td>Ardourel et al. (1994)</td>
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<td>2011(pMH682), Nod⁺ Fix⁺ on M. truncatula</td>
<td>Roche et al. (1991b)</td>
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<td>GM16702</td>
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<td>Debèlè et al. (1986)</td>
</tr>
<tr>
<td>Plasmids</td>
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<tr>
<td>pXLGD4</td>
<td>pGD499 prime (IncP) carrying a hemA::lacZ fusion, TcR</td>
<td>Leong et al. (1985)</td>
</tr>
<tr>
<td>pMH682</td>
<td>pWB85a prime (IncP), carrying nodD3 and syrM of S. meliloti, TcR</td>
<td>Honma et al. (1990)</td>
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<td>Medicago truncatula</td>
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<tr>
<td>Jemalong A17</td>
<td>Wild-type, Nod⁻ Fix⁺ with S. meliloti</td>
<td>Penmetsa and Cook (1997)</td>
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<tr>
<td>Jemalong(pMtENOD11-GUS)</td>
<td>Jemalong A17 carrying a MtENOD11-GUS fusion construct</td>
<td>Journet et al. (2001)</td>
</tr>
<tr>
<td>DZA315.16</td>
<td>Algerian accession, Nod⁺ Fix⁺ with S. meliloti</td>
<td>Tirchine et al. (2000)</td>
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<td>C31</td>
<td>EMS Nod⁻ mutant of Jemalong</td>
<td>This study</td>
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<tr>
<td>C31(pMtENOD11-GUS)</td>
<td>EMS Nod⁻ mutant of Jemalong carrying a MtENOD11-GUS fusion construct</td>
<td>This study</td>
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TcR, tetracycline resistant.

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The nsp1 allele name of the B85 mutant has been changed from Nod$^+$ to Nod$^–$.

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$$\chi^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 $\chi^2$ for the cross C31 × DZA315.16.

These plants were generated by crossing C31 to wild-type M. truncatula plants carrying the construction pMtENOD11-GUS, Jemalong(pMtENOD11-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(pMtENOD11-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 pMtENOD11-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(pMtENOD11-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.

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 (Cádernas 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).

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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 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).
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 S. meliloti NFs. 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 $1 \mu g ml^{-1}$) 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

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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 Medicago 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 Medicago 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 Nod Factor Perception.

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 Medicago truncatula genetic map. As this genetic map was derived from a cross between Medicago truncatula Jemalong and the accession DZA315.16 of Medicago truncatula (Thoquet et al., 2002), we first generated an F2 population between the C31 mutant (Jemalong derivative carrying the C31 mutation) and DZA315.16. The nodulation phenotypes of F1 and F2 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 F2 subpopulation derived from the cross C31 × DZA315.16. Two markers (PK12 and B07494), which had already been localised on the Medicago 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 Medicago truncatula genetic map. This allowed us to build a 3.8 cM local map of the NFP region, including three markers (Figure 7).

Discussion

In this study, we have used the model legume Medicago 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 Medicago 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 7. Local genetic map of the NFP locus. Location of the NFP locus on linkage group 5 (LG5) of the Medicago 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).

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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 Cardenas 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 Ruiter 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 Ruiter 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 phospholipase 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, Jema-long (pMtENOD11-GUS) (Jouret 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 Fahraeus 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 with 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 (Jouret et al., 2001) cDNA was amplified (25 cycles) between positions 82 and 519 by using the forward primer 5'-GCTCATCCCCCAATATGGCTCCA-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'-CCGGAAAACATTCAAAAAAC-3' and reverse primer 5'-TGAAAAAGACCATCACCCTCC-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 GM16526. Control plants were treated with the strain GM16702, 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 H₂O 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 F₂ 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+ F₂ individuals were randomly chosen to constitute four DNA pools of five individuals each. DNA pools were screened with 64 primer combinations.
Acknowledgements

We are very grateful to David Barker for kindly providing a wild-
type line of M. truncatula carrying the construction pMENOD11-
GUS, and the MLENOD11 sequence, which were provided prior to
publication, and for helpful discussions concerning the use of
mastoparan and Mas-7. We thank Maria Harrison for preliminary
information on the mycorrhizal phenotype of the C31 mutant;
Guillaume Bécard and Patrice Galaup for help in developing the
in vitro mycorrhizal system; Thierry Huget for DNA from the F2
mapping population Jemalong × DZA315.16; Charles Rosenberg
for fruitful discussions and Romy Catoira for preliminary work on
the C31 mutant. Besma Ben Amor was financed by a grant from the
French Government in the frame of a Tunisian co-operation. This
work was supported by the European Union (Contract MEDICAGO
QLG2-CT-2000-00676); the Institut National de la Recherche Agro-
nomique (ATS 2000-P00241) and the French Ministry of Research
(ACI ‘Biologie du Développement et Physiologie Intégrative’).

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