Epidermal and cortical roles of NFP and DMI3 in coordinating early steps of nodulation in Medicago truncatula

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SUMMARY
Legumes have evolved the capacity to form a root nodule symbiosis with soil bacteria called rhizobia. The establishment of this symbiosis involves specific developmental events occurring both in the root epidermis (notably bacterial entry) and at a distance in the underlying root cortical cells (notably cell divisions leading to nodule organogenesis). The processes of bacterial entry and nodule organogenesis are tightly linked and both depend on rhizobial production of lipo-chitooligosaccharide molecules called Nod factors. However, how these events are coordinated remains poorly understood. Here, we have addressed the roles of two key symbiotic genes of Medicago truncatula, the lysin motif (LysM) domain-receptor like kinase gene NFP and the calcium- and calmodulin-dependent protein kinase gene DMI3, in the control of both nodule organogenesis and bacterial entry. By complementing mutant plants with corresponding genes expressed either in the epidermis or in the cortex, we have shown that epidermal DMI3, but not NFP, is sufficient for infection thread formation in root hairs. Epidermal NFP is sufficient to induce cortical cell divisions leading to nodule primordium formation, whereas DMI3 is required in both cell layers for these processes. Our results therefore suggest that a signal, produced in the epidermis under the control of NFP and DMI3, is responsible for activating DMI3 in the cortex to trigger nodule organogenesis. We integrate these data to propose a new model for epidermal/cortical crosstalk during early steps of nodulation.

KEY WORDS: Medicago truncatula, Root nodule symbiosis, Root epidermis/cortex, Crosstalk, Long distance signalling, LysM-RLK, CCaMK

INTRODUCTION
Legumes can establish a root endosymbiosis with soil bacteria called rhizobia. This association is of great agronomical and ecological interest because the rhizobia can fix atmospheric nitrogen and transfer it to the plant. During establishment of this interaction the plant starts a new developmental programme leading to the formation of a new organ, the root nodule, where the bacteria are hosted. The establishment and maintenance of this symbiosis are dependent on a molecular dialogue between the plant and the bacteria involving the production by rhizobia of lipo-chitooligosaccharide molecules called Nod factors (NFs). NFs are responsible for the induction of early responses in the plant, such as root hair (RH) deformation, calcium oscillations and expression of genes known as early ‘nodulin’ genes, such as MiENOD11 (Oldroyd and Downie, 2008). Bacteria then attach to a RH and underlying inner cortical cells are activated by the NF-producing rhizobia to initiate new cell divisions leading to nodule primordium formation (Timmers et al., 1999). Then the RH curls around rhizobia, while the underlying outer cortical cells prepare for bacterial entry by forming so-called pre-infection threads (PITs) (Timmers et al., 1999). Rhizobia enter an RH inside an infection thread (IT) that progresses through the epidermis into the cortex of the root, its direction of growth directed by PITs, until it reaches the dividing inner cortical cells (Timmers et al., 1999).

Thus, infection and nodule development are tightly coupled, although they are initiated in different cell layers of the root. How these two processes are coordinated remains elusive. Forward genetic screens have revealed key players of the NF signalling pathway in the two model plants Medicago truncatula and Lotus japonicus. In M. truncatula, mutants in the Nod Factor Perception (NFP) gene are the most dramatically impaired for the interaction with the symbiont Sinorhizobium meliloti, as they do not nodulate and do not show any symbiotic responses to either rhizobia or purified NFs (Ben Amor et al., 2003). NFP encodes a receptor-like kinase with three extracellular lysin motif (LysM) domains and an inactive kinase domain (Arrighi et al., 2006). LysM domains have been shown to bind to N-acetylglucosamine, chitooligosaccharides and peptidoglycan (Kaku et al., 2006; Iizasa et al., 2010; Petutschnig et al., 2010; Willmann et al., 2011) and it is believed that NFP is an NF receptor, although no direct binding of NFs to NFP has been shown. In addition to the complete loss of NF-induced responses in nfp mutant roots, knockdown of NFP by RNAi and genetic analysis have shown that NFP also controls bacterial entry (Arrighi et al., 2006; Bensmihen et al., 2011). Other symbiotic mutants of M. truncatula include the dmi (Does not Make Infections) mutants, which define genes acting downstream of NFP in the NF signalling pathway. These mutants are impaired in their interaction with both rhizobia and arbuscular mycorrhizal fungi, and as such the DMI genes (DMI1, DMI2 and DMI3) control a common symbiotic pathway leading to establishment of both root endosymbioses (Catoira et al., 2000). DMI1 and DMI2 are genetically upstream of DMI3 and are required for DMI3 activation. DMI3 encodes a calcium- and calmodulin-dependent protein kinase (CCaMK) that is required to ‘decipher’ the calcium oscillations (known as calcium spiking) triggered by the perception of NFs. dmi3 mutants show no rhizobial entry, no nodule formation...
and limited response to NFs. Gain-of-function approaches have shown (both in *M. truncatula* and in *L. japonicus*) that DMI3 controls nodule organogenesis, as autoactive forms of DMI3 can induce the spontaneous formation of nodules in the absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2006). The calmodulin-binding domain of the product of the *L. japonicus* orthologue of DMI3, CCaMK, is important for rhizobial infection (Shimoda et al., 2012). Moreover, spontaneous nodules formed in *L. japonicus* mutants carrying a constitutively active form of a cytokinin receptor cannot be infected in the absence of CCaMK (Hayashi et al., 2010; Madsen et al., 2010). Finally, a DMI3 gene from rice can complement a *M. truncatula dmi3* mutant for nodule organogenesis, but does not restore normal infection (Godfroy et al., 2006), indicating that legume-specific features of DMI3 are required for rhizobial infection.

Thus, both NFP and DMI3 control rhizobial infection and nodule organogenesis, and NFP also controls NF perception. Here, we investigated further the respective roles of NFP and DMI3 in these symbiotic processes in the different cell layers of the root. Although intracellular infection and nodule organogenesis need to be tightly associated for the establishment of the root nodule symbiosis, these two developmental processes can be genetically uncoupled (Oldroyd and Downie, 2008). Therefore, crosstalk between the epidermis and the cortex is necessary for spatially coordinating and synchronizing nodule organogenesis with bacterial infection. However, the tissue-specific contributions of players of NF signalling in this crosstalk have never been addressed. Moreover, very little is known about the mechanisms that synchronise cortical cell division (CCD) in the inner cortex and IT formation in overlying RHs.

In this study, we have shown that both NFP and DMI3 are expressed in the epidermis and in the root cortex. Based on this observation, we studied their respective contributions in the control of epidermal and cortical events. To do so, we complemented *nfp* and *dmi3* mutants using tissue-specific expression of NFP and DMI3, respectively, and looked for the restoration of an early NF signalling event (*MtENOD11* induction), IT formation and nodule organogenesis.

### MATERIALS AND METHODS

#### Plant growth conditions

*nfp-2 pMtENOD11:GUS* (Arrighi et al., 2006), *dmi3-1* [TRV25 allele, (Lévy et al., 2004)] and *dmi3-1 pMtENOD11:GUS* lines (Catoire et al., 2000) in the *M. truncatula* cv. Jemalong A17 wild-type background were used for all complementation experiments. *pNFP:GUS* stable lines were obtained by transforming *M. truncatula* cv. 2HA by *Agrobacterium tumefaciens* strain AGL1 carrying the pLP100-pNFP-GUS plasmid described by Arrighi et al. (Arrighi et al., 2006). The *pNFP:GUS* expression profile was tested in three independent T3 lines.

Surface-sterilised seeds were sown on agar plates and placed for 3 days in the dark at 4°C then left overnight at 25°C to germinate.

For root transformation, we used *ARqua1* *Agrobacterium tumefaciens* as described by Boisson-Dernier et al. (Boisson-Dernier et al., 2001). *A. rhizogenes* transformed roots were selected by GUS expression and kanamycin resistance conferred by the pCambia2202 binary vector (http://www.cambia.org/daisy/cambia/5855/dsy585_Description).

For nodulation assays, transformed plants were transferred to sepiolite (Agrauxine, Quimper)/sand (2:1 volume mix) pots and grown at 25°C with 18 hour light/6 hour dark cycles (Catoire et al., 2000).

#### Rhizobial strains and inoculation

Wild-type *S. meliloti* RCR2011 (pXLGD4) (GMi6526) was grown at 28°C in tryptone yeast medium supplemented with 6 mM calcium chloride and 10 μg/ml tetracycline.

The bacteria were ‘scratched’ from the plate after 2 days and resuspended in sterile water. Plants were inoculated twice, at 7 days and 14 days after transfer to pots, with 2 ml of inoculum, adjusted to OD₆₀₀=0.2, per plant.

#### Plasmid constructs

All NFP cloning was performed using the Multisite Gateway technology (Invitrogen), with BP and LR reactions according to the manufacturer’s instructions. Promoter sequences were introduced in pDONR P4-P1R, NFP coding sequence was reconstructed with the extracellular domain (up to the juxtamembrane domain) in pDONR 221 and the kinase domain in pDONR P2R-P3. All fragments were amplified using either TaKaRa LaTaq (Takara Bio, Japan) or Phusion High Fidelity DNA polymerase (Finzymes, Finland). All NFP amplifications used p2201-NFP (Arrighi et al., 2006) as a template. The Δgen1-1:GUS construct described by Bucher et al. (Bucher et al., 2002) was used as a template for pLeEXT1 and the pGHL124-PCO2-HYFT construct (kindly provided by R. Heidstra, Department of Molecular Genetics, Utrecht University, Utrecht, The Netherlands) for PCO2. The different PCR fragments were obtained using the primers specified in supplementary material Table S1. Each fragment was subcloned and sequenced in the pGEM-T vector (Promega, USA) and subsequently introduced in the corresponding pDONR vector (Invitrogen) by BP reaction. The three ‘ENTRY clones’ generated in this way were then used for a LR recombination using pAMP-pat-multi as a destination vector (kindly provided by L. Deslandes, Laboratoire des Interactions Plantes Micro-organismes (LIPM), Toulouse, France). The recombined sequence obtained was then taken out of pAMP-pat-multi by Ascl and Pmel digestion and subcloned in a pCambia2202 vector, modified for its multiple cloning sites to introduce an *Ascl* site, using the *Ascl* and *SmaI* restrictions sites.

The plasmid containing both the pLeEXT1:NFP and the pCO2:NFP constructs was obtained by ligating two *Ascl/Pmel* fragments (containing pLeEXT1:NFP and pCO2:NFP, respectively) in a pCambia2202 vector digested by *Ascl* only.

pCO2 and pLeEXT1 promoters, amplified with pCO2forEcoRI/pCO2revKpnl and pLeEXT1forEcoRI/pLeEXT1rev-Kpnl, respectively, were also introduced by a classical (restriction enzyme) cloning method in the *EcoRI/Kpnl* sites of pCambia2202, giving the pCambia2202:pLeEXT1 and pCambia2202:pCO2 vectors. NFP versions with these constructs were also generated and gave similar results to the Gateway constructs.

The pDMI3:DMI3 construct (p135H) was described by Lévy et al. (Lévy et al., 2004). To place the cDNA of the full length DMI3 sequence (denoted cDMI3) under the control of tissue-specific promoters, an XbaI/SalI fragment of the plasmid pGreen49:p35S:c-DMI3 (C.R., unpublished data) was introduced in pCambia2202:pLeEXT1 or pCambia2202:pCO2 vectors digested by the same enzyme combination.

For the plasmid containing both pLeEXT1:DMI3 and pCO2:DMI3, a *XhoI/PsaI* fragment of the pCO2:DMI3 construct was introduced in the pCambia2202:pLeEXT1-DMI3 vector digested by *SalI/PsfI*

pCO2-GUS and pDMI3-GUS constructs were obtained by LR recombination in the Gateway destination vector pKGWFS7.0 (Karimi et al., 2002). Promoter sequences were obtained with the attBfor PCO2/attB2rev PCO2 and attBpDMI3F/attBpDMI3R primers and subcloned by BP reaction in pDONR 221.

pLeEXT1:GUS corresponds to the Δgen1-1:GUS described by Bucher et al. (Bucher et al., 2002) and was kindly provided by M. Bucher to A. Niebel (LIPM, Toulouse, France).

#### NF treatment

Purified NFs from the *S. meliloti* wild-type strain RCR2011 were provided by F. Maillet (LIPM, Toulouse, France) and used at 10⁻⁸ M working solution in sterile water.

Transformed roots were transferred to pouch paper/Farhaeus agar plates and incubated for 16 hours in a growth chamber at 25°C with 16 hour light/8 hour dark cycle after either water or NF treatment as described previously (Andriankaja et al., 2007).
Microscopy methods

Histochemical β-glucuronidase (GUS) tests were performed as described previously (Journet et al., 2001). Whole root segments were stained for GUS activity for 3 hours at 37°C with the magenta-glucA (5-bromo-4-chloro-3-indolyl glucuronide, cyclohexylammonium salt) substrate (Duchefa Biochemie, The Netherlands). To visualise bacterial infection, whole roots were fixed under vacuum for 15 minutes with 1.5% glutaraldehyde phosphate-buffered solution followed by one hour fixation in the same solution. Histochemical staining of β-galactosidase activity expressed by the plasmid pxLGD4 was performed as described previously (Ardourel et al., 1994). Whole root segments were observed with a Leica MZFLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany).

For further analysis, 70 μm- or 50 μm-thick and 5 μm-thick sections were made using a vibratome (Leica VT1000S) or a microcut (2040 Reichert Jung), respectively. Fragments were embedded in 4% agarose solution for the 70 μm-thick sections and in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) for the 5 μm sections. Observations were performed on a Zeiss Axioplan2 imaging microscope.

Statistical analyses

Non-parametric Kruskal-Wallis tests were performed using the Statgraphics Centurion software (Sigma Plus).

RESULTS

NFP and DMI3 are expressed both in the epidermis and in the cortex of the root during nodulation

The NFP expression pattern in Medicago truncatula was previously reported using Agrobacterium rhizogenes transformed roots (Arrighi et al., 2006), but no root sections were performed. To analyse this expression pattern further, we generated stable pNFP:GUS plant lines and made sections on both non-inoculated roots and roots inoculated with Sinorhizobium melliloti. In young non-inoculated roots, pNFP:GUS expression was strong (Fig. 1A) and we observed pNFP:GUS expression in the epidermis, as previously shown (Arrighi et al., 2006), but also in cortical layers (Fig. 1A). Upon inoculation at 4 days post inoculation (dpi), pNFP:GUS was strongly and locally expressed in cortical cells underlying IT progression and before any nodule ‘outgrowth’ could be observed (Fig. 1B) but was weaker in the epidermis, as previously shown by Arrighi et al. (Arrighi et al., 2006). Subsequent pNFP:GUS expression was also observed, as previously shown (Arrighi et al., 2006), in the infection zone of mature nodules 10 dpi (data not shown).

The DMI3 tissue expression pattern in M. truncatula has so far been described only in RHs (Smit et al., 2005) and nodules (Limpens et al., 2005) but no other root cell layer or spatiotemporal expression data during nodulation are available. Thus, we introduced a pDMI3:GUS construct in wild-type roots by A. rhizogenes transformation and looked for its tissue expression pattern in non-inoculated roots and upon infection with S. melliloti. Whereas in non-inoculated roots (Fig. 1C) GUS expression was faint in the epidermis and in the cortex, it increased upon inoculation at 2 dpi, especially in RH cells (Fig. 1D). During infection, pDMI3:GUS expression was stronger in cortical cells where ITs progressed and in the underlying layers in front of ITs (Fig. 1E). In young nodules, pDMI3:GUS activity was detected in the whole nodule cortex (data not shown). In mature nodules, pDMI3:GUS was expressed in the infection zone at 12 dpi (Fig. 1F), as shown by Limpens and colleagues using in situ hybridisation (Limpens et al., 2005).

Thus, before and during early steps of the M. truncatula/S. melliloti symbiosis, both DMI3 and NFP were found to be expressed in the epidermis and in the cortex and both were upregulated in dividing cortical cells, ahead of IT progression. To understand their respective contributions to the establishment of symbiosis in the root epidermis and in the root cortex, we decided to complement mutant plants by expressing DMI3 or NFP specifically either in the epidermis or in the cortex.

pLeEXT1 and pCO2 as tissue-specific promoters in M. truncatula roots

We chose pLeEXT1 for epidermal expression. This promoter, originally described in tomato (Bucher et al., 2002), was shown to be expressed specifically in M. truncatula RH cells (Mirabella et al., 2004). Using a pLeEXT1:GUS construct, we checked for this tissue-specific expression, before and after S. melliloti inoculation. In non-inoculated plants, the observation of whole roots and root sections revealed that pLeEXT1 was active in RH and non-RH epidermal cells whereas no expression could be detected in the root cortex (Fig. 2A,C). The expression of pLeEXT1:GUS decreased in mature RHs and was absent in older parts of main roots, as described by Mirabella et al. (Mirabella et al., 2004) (Fig. 2E).

pLeEXT1:GUS was thus expressed in the ‘NF susceptible zone’
To check further the validity of both promoters for our tissue-specific complementation assays, we took advantage of a very sensitive reporter gene, pMtENOD11:GUS, which is only expressed in the epidermis in response to purified NFs (Charron et al., 2004; Journet et al., 2001). As NFP is a presumptive NF receptor, we used pLeEXT1:NFP and pCO2:NFP to complement nfp pMtENOD11:GUS mutant plants and looked for the ability of each construct to restore pMtENOD11:GUS activity upon application of purified NFs. As shown in Fig. 3, pLeEXT1:NFP (Fig. 3C, n=26/54), but not pCO2:NFP (Fig. 3B, n=39/249), could complement for pMtENOD11:GUS expression upon NF application at 10^{-7} M, as did the pNFP:NFP construct (Fig. 3D, n=26/45). This indicates that pLeEXT1:NFP is correctly expressed in the epidermis to allow NF perception. By contrast, the absence of pMtENOD11:GUS induction by the pCO2:NFP construct strongly indicates that there is no NFP expression in the epidermis, or that it is not sufficient to lead to pMtENOD11:GUS expression. Therefore, the use of the pLeEXT1 and pCO2 promoters seemed appropriate, as they were able to restrict the expression of a reporter gene in the specific cell types.

**Epidermal expression of DMI3 can rescue bacterial infection whereas epidermal expression of NFP rescues nodule organogenesis**

Next, we tested the ability of the pLeEXT1:NFP and pLeEXT1:DMI3 constructs to complement nfp pMtENOD11:GUS and dmi3 pMtENOD11:GUS plants, respectively. We examined plants at 7 dpi and 21 dpi both for infection (i.e. presence of ITs) and for different steps of nodule organogenesis [initial CCDs leading to visible bumps, small round nodules and pink (functional) elongated nodules]. We also analysed pMtENOD11:GUS expression in inoculated plants because pMtENOD11:GUS is also expressed in response to bacterial infection. Indeed, Journet et al. have shown that the expression of pMtENOD11:GUS is widespread along the root epidermis in response to NF-producing rhizobia whereas it becomes restricted to infection sites when the bacteria start entering RHs. Later on, pMtENOD11:GUS expression
is also associated with nodule organogenesis in the root cortex (Journet et al., 2001).

Whereas nfp and dmi3 mutants were completely deficient for infection, cell division and pMtENOD11:GUS expression at any time after inoculation (Fig. 4A, Fig. 5A), as previously described (Ben Amor et al., 2003; Catoira et al., 2000), the pNFP:NFP nfp or pDMI3:Dmi3 dmi3 control plants showed young nodule formation by 7 dpi (Fig. 4B). In the case of nfp pMtENOD11:GUS roots complemented by pLeEXT1:NFP, strong epidermal pMtENOD11:GUS expression could be seen at 7 dpi (Fig. 4C), but rhizobin were only rarely entrapped in curled RHs, and, neither IT initiation nor CCD could be observed (Fig. 4C,D). By contrast, both pMtENOD11:GUS expression (Fig. 4E) and frequent IT formation in RHs (Fig. 4F, Table 1) were observed with the pLeEXT1:Dmi3 construct in dmi3 pMtENOD11:GUS roots at 7 dpi, but no CCD was observed (Fig. 4F).

At 21 dpi, whereas the control pNFP:NFP and pDMI3:Dmi3 constructs led to good nodule organogenesis and infection in their corresponding mutant backgrounds (Fig. 5B, Fig. 6), pLeEXT1:NFP nfp plants mainly showed bumps with typical pMtENOD11:GUS expression (forming in 53/59 plants) (Fig. 6A) and bacteria were entrapped in RHs but ITs were still absent (Fig. 5C). In pLeEXT1:Dmi3 dmi3 plants, IT formation was frequently observed but no CCD was ever seen opposite the ITs (36 plants) (Fig. 5D, Fig. 6B). These ITs progressed within the epidermal cell but were blocked at the junction with the cortical cell (Fig. 5D). Therefore, epidermal NFP could trigger nodule organogenesis but no IT formation, whereas epidermal DMI3 did not restore nodule organogenesis but could restore IT formation restricted to the epidermis.

No significant complementation for nodulation, infection or pMtENOD11:GUS expression of the mutant phenotypes was observed with the pCO2:NFP (n=59) or pCO2:Dmi3 (n=18) constructs (Fig. 6) (data not shown). Therefore, we investigated whether expression of either gene simultaneously under both the epidermal and the cortical promoters (i.e. pLeEXT1+pCO2) could improve the restoration of infection and nodule organogenesis.

**Combining epidermal and cortical expression restores nodule organogenesis for DMI3**

Constructs consisting of pLeEXT1:NFP and pCO2:NFP, or pLeEXT1:Dmi3 and pCO2:Dmi3 cloned on the same plasmid were introduced in A. rhizogenes for complementation studies in nfp or dmi3 mutants.

In the case of NFP, at 7 dpi, the pLeEXT1:NFP+pCO2:NFP construct did not significantly improve complementation compared with the pLeEXT1-driven version alone. Thus, the pLeEXT1:NFP+pCO2:NFP construct did not restore infection in nfp plants as bacteria were still only entrapped in curled RHs and
no ITs could be seen (data not shown). In the later stages, at 21 dpi, the pLeEXT1:NFP+pCO2:NFP nfp plants showed CCD leading to bump formation together with plant (Fig. 6A). However, no IT formation could be detected with the pLeEXT1:NFP construct, with an average of 1.5 versus 0.54 per pLeEXT1:NFP+pCO2:NFP construct versus 12/59 plants for the uninfected nodules (Fig. 5F) were observed than with the pLeEXT1:NFP construct: on 12/46 plants for the uninfected nodules (white bars) and pink elongated nodules (black bars), small uninfected nodules (dotted bars), small infected and cortical contributions to the control of rhizobial infection and nodule organogenesis.

**DISCUSSION**

The coordinated control of rhizobial infection and nodule organogenesis leading to successful establishment of root nodule symbiosis is poorly understood. Previous data have shown that the cortex prepares for IT progression [with PITs (Timmers et al., 1999) and calcium spiking (Sieberer et al., 2012)] and it has been proposed that secondary signalling transmits information from the site of bacterial entry in RHs to trigger cell divisions within the cortex for nodule organogenesis (for a review, see Oldroyd et al., 2011). However, no studies have uncoupled the epidermal and cortical contributions to the control of rhizobial infection and nodule organogenesis.

**NFP and DMI3 are expressed in both the epidermis and the cortex**

NFP and DMI3 were fused to the GUS reporter gene to analyse their spatiotemporal expression patterns during early symbiotic steps. For both pNFP:GUS and pDMI3:GUS, we detected constitutive expression (prior to rhizobial inoculation) that was uniform in the root epidermis and in the root cortex. This is compatible with very early symbiotic roles of NFP and DMI3. Upon rhizobial inoculation, pNFP:GUS expression was quickly and strongly upregulated and became restricted to cortical cells underlying ITs growing in RHs. pDMI3:GUS expression was first upregulated in RHs, prior to IT formation (at 2 dpi), and then in cortical cells underlying or containing ITs. For both promoters, the enhanced expression levels appeared to precede actual infection and CCD, suggesting that these genes control early steps of both processes and have important contributions to the control of rhizobial infection and nodule organogenesis.

**Table 1. Quantification of infection thread formation in root hairs in dmi3 pMTENOD11:GUS plants complemented by the pDMI3:DMI3, pLeEXT1:DMI3 or pLeEXT1:DMI3+pCO2:DMI3 constructs at 7 dpi with S. meliloti**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Infection threads/cm (mean)</th>
<th>Number of roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDMI3:DMI3</td>
<td>3.7a</td>
<td>37</td>
</tr>
<tr>
<td>pLeEXT1:DMI3</td>
<td>13.5b</td>
<td>91</td>
</tr>
<tr>
<td>pLeEXT1:DMI3+pCO2:DMI3</td>
<td>8.1c</td>
<td>75</td>
</tr>
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Results are averages of three experiments. a,b,cStatistically different means, as shown by Student’s t-test (P<0.001).
Fig. 7. Model for the control of nodule organogenesis by NFP and DMI3. Cortical cell division (CCD) can be triggered through a signalling cascade that is activated by NFP and DMI3 in the epidermis. This involves calcium spiking and leads to the activation of MiENOD11 expression in the epidermis. The nature of the secondary signal is unknown and is unlikely to be cytokinins but would lead to the activation of DMI3 in the cortex. In turn, cortical DMI3 would activate the cytokinin production/signalling pathway (via the cytokinin receptor CRE1) leading to CCD and nodule organogenesis.

Symbiotic roles not only in the epidermis for initial NF perception, but also in the cortex for subsequent steps.

pLeEXT1 and pCO2 are useful promoters for tissue-specific complementation approaches in M. truncatula

As described by Mirabella et al. (Mirabella et al., 2004), we demonstrated that the tomato promoter pLeEXT1 has good root epidermis specificity in M. truncatula. Moreover, the pLeEXT1:GUS epidermal pattern and its absence of expression in the cortex were conserved during nodulation. Furthermore, pLeEXT1::NFP could complement nfp mutants for the well-characterised epidermal NF-specific response that is the induction of pMtENOD11::GUS. pCO2::GUS activity was detected in all cortical layers of the root, but not in epidermal cells. Upon nodulation, pCO2 activity was also clearly restricted to internal parts of the root, notably dividing cortical cells. Furthermore, the fact that no CCD and no pMtENOD11::GUS activity could be observed with pCO2::NFP or pCO2::DMI3 alone in the corresponding mutant backgrounds showed that pCO2 activity in the epidermis of M. truncatula roots was either absent or too weak to allow any induction of these symbiotic responses.

Taken together, these data support the suitability of pLeEXT1 and pCO2 promoters for tissue-specific complementation approaches during symbiotic events in M. truncatula.

Nodule organogenesis is controlled by epidermal NFP and by both epidermal and cortical DMI3

Our observation that restricting NFP expression to the epidermis was sufficient to induce nodule organogenesis suggests that NFP acts in a non-cell-autonomous manner to trigger cell divisions in the cortex. Because multiple cell layers separate the site of initial bacterial contact at the RH surface from the site of cell division in the inner cortex, the role of a secondary signal has been suggested for the induction of these cell divisions (Oldroyd et al., 2011). Our observation that epidermal NFP expression is sufficient to induce nodule organogenesis supports this hypothesis. NFP would thus be required to perceive NFs in the epidermis and generate this secondary signal, but would not be necessary in inner tissues for induction of CCD and nodule organogenesis (Fig. 7).

Unlike NFP, epidermal DMI3 expression was not sufficient to restore induction of CCD. No CCDs were observed either in dmi3 mutants complemented by the pCO2::DMI3 construct, showing that cortical expression of DMI3 is not sufficient. By contrast, when the dmi3 mutant was complemented by the double construct with both promoters, cell divisions occurred in the inner cortex leading to the formation of small, uninfected nodules. These results show that the induction of CCD requires expression of DMI3 both in the epidermis and in the cortex (Fig. 7). Although the key role of DMI3 in controlling CCD has already been demonstrated using gain-of-function alleles (Gleason et al., 2006; Tirichine et al., 2006), no data has so far shown the need for DMI3 in the cortex. If nodule organogenesis requires secondary signalling between the epidermis and the cortex, our results indicate that DMI3 is involved at two different levels in this signalling mechanism. This CCaMK would thus be required in the epidermis for the generation of a secondary signal in response to NFs, and in the cortex for the perception/transduction of this secondary signal (Fig. 7).

Epidermis-cortex signalling leading to nodule organogenesis

The nature of the signal generated by epidermal NF perception and which would trigger the organogenesis programme in the root cortex is not known, but the hormones cytokinin and abscisic acid are possible candidates as they are involved in nodule organogenesis (Ding and Oldroyd, 2009; Frugier et al., 2008; Oldroyd and Downie, 2008). In L. japonicus, genetic studies have shown that the cytokinin receptor gene LHK1 acts downstream of CCaMK (Hayashi et al., 2010; Heckmann et al., 2011; Tirichine et al., 2007). Taken together, we propose that cortical DMI3 acts upstream of cytokinin perception to activate nodule organogenesis (Fig. 7) and that cytokinin is probably not the secondary signal generated in the epidermis as DMI3 would not be necessary in the cortex in this case (Fig. 7). This is consistent with reports showing that cytokinin signalling is restricted to cortical cells (Oldroyd et al., 2011; Plet et al., 2011).

Some authors have suggested that this secondary signal could involve GRAS or ‘Notch-like’ transcription factor movement (Hirsch and Oldroyd, 2009; Oldroyd and Downie, 2008). However, as these transcription factors are genetically downstream of DMI3 both for epidermal and cortical responses (Gleason et al., 2006; Marsh et al., 2007), this hypothesis is not compatible with the need for DMI3 in the cortex. Calcium is also an interesting candidate, but L. japonicus mutants with a very low epidermal calcium-spiking response can induce cortical responses leading to nodule organogenesis (Groth et al., 2010; Kosuta et al., 2011). If calcium is not itself the secondary signal, the need for a CCaMK in the cortex strongly suggests that the signalling pathway activated in the cortex involves a calcium signal. New players such as nitric oxide could also play a role in different steps of symbiosis establishment (for a review, see Meilhoc et al., 2011).

Epidermal expression of DMI3, but not of NFP, is sufficient for root hair infection

nfp and dmi3 mutant complementation by epidermal-driven genes led to striking differences in the infection process, from which we...
can conclude that NFP and DMI3 play distinct roles in the control of IT initiation and progression. In dmi3 plants complemented by pLeEXT1:DMI3, ITs were initiated and developed normally inside RHs. This showed an important role for epidermal DMI3 in IT formation, and is consistent with data obtained in L. japonicus in which epidermal infection of spontaneous nodules (obtained by activation of the cytokinin receptor LHK1) is not possible in a ccamk mutant background (Hayashi et al., 2010; Madsen et al., 2010). Furthermore, the fact that in dmi3 pLeEXT1:DMI3 plants ITs aborted when they reached the apposed cortical cell (where DMI3 was not expressed) indicates that DMI3 acts in a cell-autonomous manner to control IT formation. The absence of IT progression in the cortex is consistent with recent data showing that high frequency calcium oscillations occur in the outer cortical cell that will be infected, but not in the neighbouring cells (Sieberer et al., 2012). Taken together, these data suggest an involvement of DMI3 in the cortical infection process. Moreover, the leucine-rich repeat receptor-like protein kinase (LRR-RLK) MtDMI2, which is implicated in activating the CCaMK (Hayashi et al., 2010), is expressed in the cortex during nodule development (Bersoult et al., 2005).

In nfp plants complemented by pLeEXT1:NFP, bacteria could be entrapped in curled RHs, but no ITs were observed. It is unlikely that the expression of NFP under the pLeEXT1 promoter is incompatible with restoration of infection in RHs because wild-type plants expressing pLeEXT1:NFP are not impaired for nodulation (J. Cullimore, personal communication). The strong activity of the native NFP promoter in cortical cells ahead of and during infection (Fig. 1B) (Arrighi et al., 2006) is consistent with a need for NFP in the cortex, possibly to control PIT formation, which is tightly coupled with early steps of infection (Timmers et al., 1999). Therefore NFP could control IT formation in a non-cell-autonomous manner, which would be independent of the presence of DMI3 in the cortex.

Because we did not obtain infected nodules when either NFP or DMI3 expression was restricted to the epidermis or to the cortex, and given the fact that both DMI3 and NFP are expressed in both tissues when driven by their own promoters, we tested whether the simultaneous expression of these genes in both tissues could restore infection to mutant plants. The constructs combining the pLeEXT1 and pCO2 promoters were functional for nodule organogenesis, as shown by the restoration of nodule formation in dmi3 plants and increased nodule organogenesis in nfp plants. Also, in these complemented dmi3 plants, there were significantly fewer ITs in RHs compared with dmi3 pLeEXT1:DMI3 plants. This suggests a negative control from dividing cortical cells on the number of epidermal infection events, as described for cytokinin receptor mutants (Gonzalez-Rizzo et al., 2006; Murray et al., 2007).

Full infection was not restored in either mutant by these double constructs. An explanation for this could be that infection requires a strict control of the timing and/or level of expression of NFP and DMI3 between the epidermis and the cortex. Nevertheless, full complementation with formation of infected functional nodules can be obtained when NFP or DMI3 expression is driven by the CaMV 35S promoter (data not shown), which has been shown to be strongly and constitutively expressed in most root tissues (Auria and Timmers, 2007). An alternative hypothesis is that in the pLeEXT1+pCO2 double constructs, cortical expression of NFP and DMI3 driven by the pCO2 promoter, though functional for nodule organogenesis and the control of the number of infection events, does not reach the critical level required in sub-epidermal outer cortical cells for infection to proceed. This is supported by the observation that IT progression in outer cortical cells during early steps of nodulation is associated with a lower level of pC2O:GUS activity compared with the inner dividing cortical cells (Fig. 2F). Also, the pNFP:GUS and pDMI3:GUS constructs show increased GUS expression associated with infection (Fig. 1B,E) (Arrighi et al., 2006), suggesting that NFP and DMI3 have to be expressed more highly for cortical cells to become infected. However, as we cannot completely rule out other explanations, such as a need for these genes to be expressed in other tissues, we cannot formally conclude that cortical expression of NFP and DMI3 is required for the infection process.

Concluding remarks

In this work we have shown that NFP and DMI3, through non-cell-autonomous or cell-autonomous functions, control spatial crosstalks between the epidermis and the cortex during establishment of nodulation. We have also highlighted different mechanisms underlying infection and nodule organogenesis, and provided more data concerning the coordination of these symbiotic processes. LYK3 is another putative NF receptor that is expressed in both the epidermis and the cortex of M. truncatula roots (Haney et al., 2011; Mbengue et al., 2010), and lyk3 mutants are defective in both PIT formation and infection (Catoira et al., 2001). Hence, LYK3 is also likely to play a role in coordinating the infection process in both cell layers.

The approach that we used to uncouple the contributions of NFP and DMI3 in two different cell layers of the root was a tissue-specific complementation approach of symbiotic mutants. This type of approach could be extended in M. truncatula and other legume species by the discovery of endogenous tissue-specific promoters, using tissue-specific transcriptomics, for example. Such promoters should also be useful to study the involvement of different cell layers in other plant/microbe interactions.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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