Insights into Nod factor signaling mediated by Medicago truncatula LysM receptor-like kinases, MtNFP and MtLYK3

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CHAPTER 4

Protein kinase domain is indispensible for biological activity of Medicago truncatula NFP LysM receptor-like kinase.

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SUMMARY

In successful symbiosis between legume plants and nitrogen-fixing rhizobia, the bacteria are accommodated inside de novo formed plant organs (nodules), where they reduce dinitrogen for the plant’s use in exchange for carbohydrates. Perception of bacterial lipo-chitooligosaccharidic signals, termed Nodulation (Nod) Factors (NFs) is generally indispensable for establishing this interaction. However, little is still known about the activation and signaling mechanisms of the putative NF receptors. Our previous results (presented in Chapter 2) indicated a functional interaction of two *Medicago truncatula* (*Medicago*) putative NF receptors, MtNFP and MtLYK3, resulting in cell death (CD) induction upon their simultaneous production in *Nicotiana benthamiana* (*Nicotiana*) leaf. Here, we present a detailed structure-function study on MtNFP, using the ability of MtNFP to induce CD in the presence of MtLYK3 as readout of its biological activity. The MtNFP intracellular region (InR) seemed to be relatively tolerant to changes in the sequence. Nevertheless, substitution of the conserved Lys (in a β-strand 3) and Gly (in an α-helix F), possibly required for the correct tertiary structure of the kinase domain (KD), abolished MtNFP biological activity. Dissection of the MtNFP InR demonstrated that the KD itself was necessary for MtNFP signaling in *Nicotiana*, whereas the flanking sequences, i.e. a C-tail and a juxtamembrane region were dispensable or not sufficient for CD induction, respectively. Our results imply similar requirements for MtNFP biological activity in *Medicago* and *Nicotiana* with respect to the structure of the MtNFP InR.
INTRODUCTION

Perception of NFs is generally indispensable for nodule organogenesis and regulation of the infection process (Giles & Oldroyd 2008; Madsen et al., 2010). Up to now, several plasma membrane (PM)-spanning receptor-like kinases (RLKs) that possess three Lysin Motif (LysM) domains in their extracellular region (ExR), and a Ser/Thr protein KD in their InR are postulated to function as putative NF receptors. Among those, Nod Factor Perception (MtNFP) in Medicago and Nod Factor Receptor 1 and 5 (LjNFR1 and LjNFR5) in Lotus japonicus (Lotus) are indispensable for triggering of early symbiotic signaling upon perception of compatible NF, and for the host root infection by rhizobia via root hairs (RHs) (Ben Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; El Yahyaoui et al., 2004; Mitra et al., 2004a; Arrighi et al., 2006; Miwa et al., 2006b; Høgslund et al., 2009; Madsen et al., 2010; Nakagawa et al., 2010; Bensmihen et al., 2011). In contrast, Medicago LysM domain-containing RLK/Root Hair Curling (MtLYK3/HCL; from now on referred to as MtLYK3) is specifically required at the infection step, i.e. an entrapment of rhizobia in RH curls, and formation of specialized infection structures, termed infection threads [ITs], through which the bacteria colonize the nodule primordium (Catoira et al., 2001; Limpens et al., 2003; Smit et al., 2007). In addition, detection of MtNFP and MtLYK3 transcripts/ promoter activity in a nodule primordium and the invasion zone of a mature nodule points out to a yet-unidentified role for these genes in nodule development and/or accommodation of rhizobia inside nodule cells (Limpens et al., 2005; Arrighi et al., 2006; Mbengue et al., 2010; Haney et al., 2011).

RLKs function as PM-localized protein kinases and signal transducers. Direct or indirect perception of a cognate extracellular signal induces specific conformational
changes in the ligand-bound RLK (often accompanied by a modulation of its oligomerization state) that, in turn, leads to enhanced activity of its KD and downstream signaling (Wang et al., 2008; Lemmon & Schlessinger 2010). All protein kinases share a conserved KD characterized by a distinctive tertiary fold composed of an N-terminal and a C-terminal lobe. In the past 20 years, crystal structures of many eukaryotic protein kinases (ePKs) have been solved, greatly contributing to our understanding of the exact role of various conserved residues for either catalysis or maintenance of the KD structure (Kornev & Taylor 2010; Klaus-Heisen et al., 2011; Taylor & Kornev 2011; Yang et al., 2012). In short, the N-lobe contains three distinctive motifs/structural elements crucial for kinase activity: a Gly-rich loop, an Ala-x-Lys (AxK, where x is any amino acid) motif, and an α-helix C. Within the C-lobe, a catalytic loop and an activation segment (AS) are crucial for catalysis and substrate binding, and an α-helix F serves as an organizing element for the entire KD. In most ePKs, ATP binds in the deep cleft between the two lobes through contacts with: amino acids in the Gly-rich loop (G193 through to G198 in IRAK4) and the immediately proceeding Val (V200 in IRAK4), the Lys (K213 in IRAK4) in the AxK motif; the conserved Lys (K313 in IRAK4) in the catalytic loop; and the Asp (D329 in IRAK4) in an Asp-Phe-Gly (DFG) motif at the start of the AS. Activation of ePKs typically involves translocation of the α-helix C and of the activation segment resulting in a specific

1 In Figure S1 we present a tertiary structure of a human (Homo sapiens) Interleukin Receptor-Associated Kinase 4 (HsIRAK-4) reported by Wang et al. (2006). HsIRAK-4 was chosen because it was used to generate a homology model of the MtLYK3 KD (see Klaus-Heisen et al., 2011). The selected conserved residues discussed in this Chapter are highlighted in the depicted structure of HsIRAK-4, in order to orient the reader in their positions in a 3D structure of a KD.
positioning of the two lobes optimally for catalysis (Huse & Kuriyan 2002; Nolen et al., 2004; Shi et al., 2006; Zhang et al., 2012). The recently postulated dynamic formation/disruption of two non-consecutive hydrophobic structures, termed regulatory (R) and catalytic (C) spines (Kornev & Taylor 2010; Taylor & Kornev 2011; see Fig. S1 for a detailed description), explains how the two previously established regulatory elements, the α-helix C and the AS, govern the activity of many ePKs.

ePKs function as crucial regulatory components in a multitude of cellular processes where they provide reversible phosphorylation of their protein substrates, resulting in a rapid and specific modulation of protein catalytic activity, oligomerization status, and/or subcellular localization (e.g. Wang et al., 2008; Lew et al., 2009; Jaillais et al., 2011; Oh et al., 2012). In addition, the sequenced genomes of model organisms (Boudeau et al., 2006; Castels & Casacuberta 2007) reveal a significant complement of genes encoding atypical ePKs lacking one or more key catalytic residues. Remarkably, some of these ePKs display phosphorylation activity, indicating that the sheer absence of certain conserved residues does not preclude kinase activity (Zegiraj & Aalten 2010). Other atypical ePKs are truly kinase-inactive and are classified as pseudokinases. Interestingly, pseudokinases have recently been implicated in the allosteric regulation of true kinases with which they interact (Zegiraj & Aalten 2010). Alternatively, they function as molecular scaffolds, facilitating the formation of multiprotein complexes (Zegiraj & Aalten 2010).

In contrast to MtLYK3 and LjNFR1 that possess an active KD capable of auto-and trans-phosphorylation (Arrighi et al., 2006; Mbengue et al., 2010; Klaus-Heisen et al., 2011; Madsen et al., 2011), MtNFP and LjNFR5 possess an atypical KD in which certain conserved motifs/substructures are either lacking or are significantly modified (Madsen et al., 2003; Arrighi et al., 2006). For instance, the Gly-rich loop
is absent except for the first Gly (G323 in MtNFP) and the AS is abnormally short and contains an Asn (N453 in MtNFP) in place of the conserved Asp in the DFG motif. In addition, the conserved Lys (K339 in MtNFP) in the AxK motif and the conserved Asp (D435 in MtNFP) in the catalytic loop are dispensable for MtNFP function in nodulation (Lefebvre et al., 2012). Therefore, it seems that MtNFP and LjNFR5 do not display nor rely on intrinsic kinase activity to signal. Analogously to the function of several known pseudokinases, a regulatory role of MtNFP over another kinase-active RLK presents an intriguing possibility. However, such interaction of MtNFP with MtLYK3 or any other RLK implicated in the RL symbiosis in Medicago (Endre et al., 2002; Limpens et al., 2003; Arrighi et al., 2006) remains to be shown. Similarly, LjNFR5 is hypothesized to interact and activate (via an unspecified mechanism) LjNFR1 (Radutoiu et al., 2003, 2007; Madsen et al., 2011), although a convincing proof for such mechanism is still lacking.

We recently revealed a functional interaction between MtNFP and MtLYK3 in a heterologous system of Nicotiana leaf (see Chapter 2), supporting their postulated co-functioning during nodulation. Since the Nicotiana system provided rapid and robust readout of the functional interaction between these proteins, we decided to further characterize the similarity between MtNFP-mediated signaling in Medicago and Nicotiana. Here, we present a detailed structure-function study using various truncated and mutated variants of MtNFP. We demonstrate that the requirements of nodulation and CD induction show significant similarities with respect to the MtNFP InR sequence, supporting the relevance of the Nicotiana system for structure-function studies on this and potentially other LysM-RLKs. In addition, our results indicate that MtNFP signaling role is likely dependent on the conserved fold of its KD.
RESULTS

Lys 339 and Gly 474 are essential for MtNFP biological activity in Nicotiana

In our structure-function study on MtNFP, we first focused on the residues characterized in the recent work of Lefebvre et al. (2012) (listed in Table 1). These included the conserved Lys 339, Asp 435, and Gly 447 (located in the α-helix F), as well as phosphorylation sites predicted in the MtNFP InR by NetPhos programme: Thr 281, Ser 282 and Ser 283 in a juxtamembrane (JM) region (an intracellular sequence that flanks the N-terminus of the KD); Thr 459, Ser 460 and Thr 461 in the AS; and Thr 578 and Ser 579 in a C-tail (an intracellular sequence that flanks the C-terminus of the KD) (see Fig. 1 for their position in the MtNFP InR). All MtNFP constructs carrying (a) mutation(s) were generated as C-terminal fusions to the sequence encoding super yellow fluorescent protein (FP) 2 (sYFP2) (Kremers et al., 2006). We used a constitutive 35S promoter of the cauliflower mosaic virus (CaMV) to drive the expression of all constructs in study. In order to test biological activity of MtNFP-sYFP2 mutated variants, they were produced alone or co-produced with MtLYK3-mCherry (a C-terminal fusion of MtLYK3 to a monomeric red FP [mCherry]) (Shaner et al., 2004) in Nicotiana leaves via Agrobacterium-mediated transient transformation (Agro TT). Concomitant mock infiltration (with Agrobacterium strain carrying an empty vector) and control co-expression of MtNFP-sYFP2 and MtLYK3-mCherry were performed on every leaf. Development of CD was monitored between 36 and 72 hours after infiltration (hai), and in case of the absence of or weakly pronounced macroscopic symptoms, the absence/presence of CD was further scrutinized with an exclusion dye (Evans blue) staining.
Figure. 1 Alignment of the InR sequences of MtNFP and its orthologs from *Pisum sativum*, *PsSYM10*, and *Lotus japonicus*, *LjNFR5*.

The KD sequences of MtLYK3 and human (*Homo sapiens*) IRAK4 are included in order to compare MtNFP with sequences of active ePKs. Conserved residues are indicated in the underlining consensus sequence as (*). The predicted start and end of the core KDs are indicated with black vertical lines. Motifs and structural features of interest are boxed in black and named underneath. MtNFP residues analyzed for their role in CD phosphorylation sites found in the LjNFR5 InR are highlighted in grey boxes, and their positions are given above. Conserved residues with a putative role in the assembly of “active” conformation of the MtNFP KD are underlined.

**gly-rich loop**

| **MtNFP:** | 270 | YCLMKMKRLNR5 | **PsSYM10:** | 520 | DPKLESPYPDNSLALAENVCTADKSLRSRPTVEAEIVLCLSLLMPSSEPLERLS-TS |
| **MtNFP:** | 270 | YCLMKMKRLNR5 | **PsSYM10:** | 520 | DPKLESPYPDNSLALAENVCTADKSLRSRPTVEAEIVLCLSLLMPSSEPLERLS-TS |
| **LjNFR5:** | 270 | YCRRRKALN5 | **MtLYK3:** | 310 | FDQVLKNVTNPFDKDRKVKQHNLVEQGF |
| **MtNFP:** | 580 | GLDAEA-THVVTAVIR |
| **NsLYK3:** | 579 | GLDVEA-THVVTAVIR | **LjNFR5:** | 579 | GLDVEA-THVVTAVIR | **MtNFP:** | 580 | GLDAEA-THVVTAVIR |

**α-helix F**
Twenty four hai, a complex subcellular localization in *Nicotiana* leaf epidermal cells was observed for all MtNFP-sYFP2 mutated protein fusions that resembled the reported subcellular localization of the wild-type (WT) MtNFP fusion protein (see Lefebvre et al., 2012 and Chapter 2). Approximately 36 to 48 hai, clear co-localization of all but one mutated MtNFP-sYFP2 protein fusions with the PM marker (HVR-mCherry, see Chapter 2) was observed, and indicated their efficient production and PM localization in *Nicotiana* leaf epidermal cells (Fig. 2–demonstrated for MtNFP [K339A]-sYFP2, rest not shown). In case of MtNFP [G474E]-sYFP2, pronounced localization of the protein fusion in the endoplasmic reticulum (ER) (indicated with the arrows in Fig. 2) was still observed 48 hai, although some cells showed a more uniform pattern of fluorescence at the cell boundary, indicating that some protein fusion had reached the PM. In addition, strong fluorescent puncta (see the arrowhead in Fig. 2 bottom panel) were visible at the cell boundary of many cells (sometimes in association with nuclei). Such fluorescent puncta were not observed in leaf regions co-producing WT MtNFP-sYFP2 and HVR-mCherry protein fusions. The CD induction assay demonstrated that the G474E substitution abolished MtNFP-sYFP2 biological activity in *Nicotiana* leaf (Table 1). On the contrary, MtNFP [D435A]-sYFP2 and MtNFP-sYFP2 mutated protein fusions carrying Ala substitutions of the predicted phosphorylation sites showed WT-like CD induction when co-produced with MtLYK3-mCherry in *Nicotiana* leaf (Table 1). Co-production of MtNFP [K339A]-sYFP2 and MtLYK3-mCherry resulted in confluent CD of (nearly) the entire infiltrated region in only 3 out of 11 infiltrations, whereas the remaining regions displayed only a patch or spot of dead tissue (Table 1). This surprising observation led us to investigate the role of additional residues that might be important for MtNFP biological activity in *Nicotiana*. We chose to test the effect of Ala substitution of the conserved Gly 323,
HVR-mCherry

NFP [K339A]-sYFP2

NFP [K339A K340A]-sYFP2

NFP [G474E]-sYFP2

Figure 2. Subcellular localization of various MtNFP-sYFP2 mutated protein fusions.


HVR-mCherry, encoding the PM marker, was co-expressed with (from top to bottom): MtNFP [K339A]-sYFP2; MtNFP [K339A K340A]-sYFP2; MtNFP [G474E]-sYFP2 in Nicotiana leaf epidermal cells via Agro TT, and the fluorescence (viewed from abaxial side) was imaged 48hai using confocal laser scanning microscopy. YFP channel presents green fluorescence of sYFP2; mCherry channel present orange fluorescence of mCherry; merged channel superimposes green, orange, red (chlorophyll autofluorescence), and differential interference contrast (DIC) image. Note co-localization of the PM marker with MtNFP [K339A]-sYFP2 or MtNFP [K339A K340A]-sYFP2. Localization of MtNFP [G474E]-sYFP2 in ER is indicated with arrows, and in puncta structures – with an arrowhead. Bars are 20 µm.

the Asn 453, and the Lys 340 immediately downstream from the AxK motif (the latter mutation was introduced into MtNFP [K339A]-sYFP2 construct). Again, all three protein fusions were efficiently produced and correctly localized to the PM in Nicotiana leaf epidermal cells (Fig. 2–demonstrated for MtNFP [K339A K340A]-
sYFP2, rest not shown) but only MtNFP [G332A]-sYFP2 and MtNFP [N453A]-sYFP2 were able to induce efficient CD upon co-production with MtLYK3-mCherry (Table 1). On the contrary, MtLYK3-mCherry and MtNFP [K339A K340A]-sYFP2 co-production resulted in very limited death in 3 out of 11 infiltrated regions, and a confluent CD of the entire infiltrated region was not observed (Table 1). In addition, no CD was observed after separate expression of any of the MtNFP-sYFP2 constructs carrying the above mutations (Table 1). Taken together, these results indicated that many residues were apparently dispensable, whereas the Lys 339 and Gly 474 were essential for MtNFP biological activity in *Nicotiana*.

The kinase domain but not the C-tail is necessary for MtNFP biological activity in *Nicotiana*

Previously tested MtNFP truncated variant with almost the entire InR deleted, i.e. MtNFP [ΔInR] (see Lefebvre *et al.*, 2012 and Chapter 2), still retained part of the MtNFP JM region, including three predicted phosphorylation sites (i.e. Thr 281, Ser 282 and Ser 283). Recently, the InR of MtNFP ortholog from *Lotus*, LjNFR5, was found to be phosphorylated *in vitro* by LjSYMRK (for Symbiotic Receptor Kinase) of *Lotus* on four sites located in the JM region (Madsen *et al.*, 2011): the Thr 280, Ser 282, Ser 292 and Ser 295 (the last two residues were wrongly annotated by Madsen *et al.* [2003, 2011] as being located within the LjNFR5 KD). The same conserved (Ser 282, Ser 292, Ser 295) or corresponding (Thr 281) residues are present in MtNFP (see Fig. 1) but the Ser 292 and Ser 295 are missing from the MtNFP [ΔInR]-sYFP2 protein fusion. In order to study whether a truncated MtNFP variant possessing the entire JM region was capable of CD induction in *Nicotiana*, we generated another truncated protein fusion, MtNFP [full JM]-sYFP2 (amino acids [aa’s]: 1-312; prediction
Table 1. Cell death induction upon (co-)expression of \textit{MtNFP} mutated and truncated constructs and WT \textit{MtLYK3} in \textit{Nicotiana} leaves.

<table>
<thead>
<tr>
<th>\textit{MtNFP-sYFP2} construct</th>
<th>Nodulation *</th>
<th>Co-expression with \textit{MtLYK3-mCherry}</th>
<th>Separate expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>48/52</td>
<td>0/9</td>
</tr>
<tr>
<td>[G323A]</td>
<td>NT</td>
<td>10/11</td>
<td>0/9</td>
</tr>
<tr>
<td>[K339A] (AxK motif)</td>
<td>+</td>
<td>3/11</td>
<td>(impaired)</td>
</tr>
<tr>
<td>[K339A K340A] (catalytic loop)</td>
<td>NT</td>
<td>1/11</td>
<td>0/9</td>
</tr>
<tr>
<td>[D435A] (NFG motif)</td>
<td>+</td>
<td>11/11</td>
<td>0/9</td>
</tr>
<tr>
<td>[G474E] (α-helix F)</td>
<td>-</td>
<td>0/15</td>
<td>0/9</td>
</tr>
<tr>
<td>T281A/S282A/S283A (JM)</td>
<td>+</td>
<td>12/12</td>
<td>0/9</td>
</tr>
<tr>
<td>T459A/S460A/T461A (AS)</td>
<td>+</td>
<td>12/12</td>
<td>0/9</td>
</tr>
<tr>
<td>T578A/S579A (C-tail)</td>
<td>+</td>
<td>11/12</td>
<td>0/9</td>
</tr>
<tr>
<td>full JM</td>
<td>NT</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>ΔC-tail</td>
<td>NT</td>
<td>13/13</td>
<td>0/9</td>
</tr>
</tbody>
</table>
The designated constructs were expressed alone or co-expressed with MtLYK3-mCherry in Nicotiana leaves via Agro TT, and the infiltrated regions were marked. Macroscopic symptoms of CD were scored 48hai; only infiltrations that resulted in confluent death of (nearly) the entire infiltrated region were scored as a fraction of total independent infiltrations performed. In case of no or weak macroscopic symptoms for a particular (pair of) construct(s), three leaves were stained with Evans blue to confirm the lack of CD (data not shown), and macroscopic symptoms in the remaining infiltrated regions were scored again 72hai, after which point a weak unspecific chlorosis could be observed. For selected pairs of constructs, macroscopic symptoms of CD 48hai and subsequent Evans blue staining are presented.

of the MtNFP KD was done before the identification of an α-helix B as a possible integral part of a Ser/Thr KD; see Klaus-Heisen et al., [2011] and Fig. 1). MtNFP [full JM]-sYFP2 was not able to induce CD either in the absence or presence of MtLYK3-mCherry (Table 1), despite being efficiently produced and correctly localized to the PM of Nicotiana leaf epidermal cells (data not shown). Since this result suggested that MtNFP KD or the entire MtNFP InR was required for MtNFP biological activity in Nicotiana, we tested CD induction ability of MtNFP after removal of its C-tail (MtNFP [ΔCtail], aa’s: 1-568). MtNFP [ΔCtail]-sYFP2 was efficiently produced and localized to the PM of Nicotiana leaf epidermal cells (data not shown), and displayed WT-like ability to induce CD when co-produced with MtLYK3-mCherry (Table 1). Taken together, we showed that MtNFP KD played a crucial role in CD induction, whereas the JM region alone was not sufficient, and the C-tail was dispensable for MtNFP biological activity in Nicotiana.

DISCUSSION

Similarities between MtNFP signaling in Medicago and Nicotiana

Nicotiana has proved to be a useful model for heterologous production and structure-function studies on multiple proteins, providing invaluable insights
into their biological activity that guided their subsequent analyses in the respective homologous systems (e.g. dissection of tomato (Solanum lycopersicum) Pto [for Pseudomonas syringae pv. tomato]-mediated signaling; see Oh & Martin 2010). In a similar fashion, Nicotiana was useful for efficient production of MtNFP and MtLYK3, facilitating characterization of their subcellular localization, and homo(oligo)merization in vivo (see Chapter 2 and 3). The use of Nicotiana as a possible system to investigate signaling mediated by the symbiotic LysM-RLKs present important practical advantages over the legume root system, especially in terms of rapidity and ease of protein production. Therefore, to further investigate the relevance of Nicotiana system in this respect, we compared the requirements of nodulation in Medicago and CD induction in Nicotiana in respect to the structure of MtNFP InR. Remarkably, we showed that out of eleven residues tested, nine (Thr 281, Ser 282, Ser 283, Asp 435, Thr 459, Ser 460, Thr 461, T578, and S579) were equally dispensable, and one (Gly 474) was equally crucial for MtNFP biological activity in Nicotiana. In case of the K339A substitution, the observed impairment of biological activity of the MtNFP [K339A] in Nicotiana agreed with the partial impairment of this mutated variant in nodulation (B. Lefebvre, personal communication). This significant overlap indicates that requirements of CD induction and nodulation in respect to the structure of MtNFP IR are similar, supporting our notion of the relevance of Nicotiana system for studies on this, and potentially other symbiotic LysM-RLKs.

**MtNFP might not require trans-phosphorylation by another kinase to signal**

The LjNFR5 IR was shown to be trans-phosphorylated in vitro by the LjNFR1 and LjSYMRK (for Symbiotic Receptor Kinase) IRs, although the importance of these phosphorylation sites for LjNFR5 function in nodulation has not been shown (Madsen
et al., 2011). On the contrary, evidence is lacking for trans-phosphorylation of the MtNFP InR by MtLYK3 (Klaus-Heisen et al., 2011), despite the fact that the phosphorylation sites found in the LjNFR5 JM region (Thr 280, Ser 282, Ser 292, and Ser 295) are conserved in the MtNFP sequence (respectively, Ser 280, Ser 282, Ser 292, and Ser 295). The effect of S280A, S292A or S295A substitution on MtNFP biological activity has not been analyzed, and therefore it is formally possible that these residues might be important for MtNFP signaling. However, Ala substitution of other predicted phosphorylation sites, i.e. T281A, S283A, T459A, S460A, T461A, T578A, and S579A did not affect MtNFP biological activity (Lefebvre et al., 2012 and Table 1). Therefore, transphosphorylation of these sites, if it occurs in vivo, does not seem to be crucial for MtNFP signaling in either Medicago or Nicotiana.

The conserved fold of kinase domain might be required for MtNFP signaling

KD s found in divergent ePK subfamilies display not only a significant conservation of certain residues/motifs but also a remarkable similarity of their tertiary structures in an active state. More specifically, three hydrophobic elements (the α-helix F, the R spine, and the C spine) are postulated to constitute an “internal frame” for assuming the active conformation by ePKs (Kornev & Taylor 2010; Taylor & Kornev 2011). Interestingly, the α-helix F is conserved in the MtNFP sequence, and the G474E substitution abolishes MtNFP biological activity in vivo (Lefebvre et al., 2012 and Table 1). Kornev et al. (2008) postulated that this conserved Gly (G225 in protein kinase A [PKA], G374 in IRAK4) facilitates tight packing of α-helix F and α-helix H that is required for a correct tertiary structure of the KD of PKA, as well as of other ePKs. Incorrect folding of the KD is a likely explanation for the negative effect of various substitutions of this conserved Gly on in vitro
and/or in vivo activity reported for MtNFP and several other RLKs (Clark et al., 1997; Gomez-Gomez & Boller 2000). In addition, an Asp substitution of the conserved Val immediately downstream from this Gly (Gly 228 and Val 229) in the sequence of tomato Pto decreased accumulation of Pto [V229D] mutated variant in vivo (Dong et al., 2009). Therefore, we hypothesize that the impairment of the KD tertiary structure in MtNFP [G474E] mutated protein may affect its trafficking to and/or its stability at the PM. Both hypotheses agree with the recently demonstrated sensitivity of MtNFP to the ER quality control system in Medicago and Nicotiana, and the postulated requirement of the PM localization for MtNFP function in nodulation (Lefebvre et al., 2012). Analogously, the decreased PM occupancy by MtNFP [G474E] mutated protein might contribute to the lack of its biological activity in Nicotiana. However, as shown in Medicago (Lefebvre et al., 2012) and (with less certainty) in Nicotiana (Fig. 2), at least a fraction of MtNFP [G474E]-sYFP2 protein fusion is localized to the PM, suggesting that the G474E substitution has also a more direct effect on MtNFP biological activity, possibly via affecting the tertiary structure of its KD.

In this respect, we note the conservation of residues implicated in the orientation of the α-helix C (K339, E348, and F454 in MtNFP; see Fig. 1) in an active conformation and assembly of the R- (L352, L363, H433, and F454 in MtNFP) and C- (V326, A337, and L442 in MtNFP) spines in the MtNFP sequence (see Fig. 1). Therefore, we hypothesize that the assembly of the MtNFP KD in an active conformation, possibly driven by an analogous “internal frame” and stabilized by interactions between additional residues, might be required for MtNFP interaction with, and possible regulation of another (membrane-bound) protein. Different biological activities of various MtNFP truncated variants in the CD induction assay support the notion that the KD itself, rather than the JM region or the C-tail, is required
for MtNFP-mediated signaling in *Nicotiana*. This might also be true for MtNFP function in *Medicago*, as MtNFP mutated variants carrying substitutions in the JM region (T281A, S282A, and S283A) or C-tail (T578A and S579A) were active in nodulation (Lefebvre *et al.*, 2012). Active-like conformation (and analogous formation of R- and C-spines) has been reported for various pseudokinases, despite significant alterations of their sequence. In fact, it is thought to underlie their function as scaffold proteins or allosteric regulators (Kornev & Taylor 2009; Zegiraj & Aalten 2010). A similar active conformation of the MtNFP KD is not unlikely, although a crystal structure of an ePK with a similarly altered sequence that would confirm or falsify this notion is currently lacking.

**MtNFP binding of ATP is not excluded**

MtNFP KD possesses all residues required for a putative assembly of the R- and C-spines. However, whereas formation of the R-spine in the MtNFP KD might be readily possible (providing favorable positioning of the H433 and F454; see Fig. S1 for a detailed explanation), assembly of the C-spine is somewhat more speculative. It is postulated to be completed by the adenine ring of ATP (Kornev & Taylor 2010), whereas one of the structural elements implicated in ATP binding (i.e. the Gly-rich loop) is missing in the MtNFP KD. Nevertheless, recent demonstration of ATP binding or even kinase activity for several ePKs previously classified as pseudokinases (Xu *et al.*, 2000; Mukherjee *et al.*, 2008; Eswaran *et al.*, 2009; Zegiraj *et al.*, 2009 and refs therein; Shi *et al.*, 2010) has revealed the existence of alternative ways of coordinating the ATP molecule. In the light of these findings, the observed impairment of MtNFP biological activity due to the K339A and K339A/K340A substitutions (B. Lefebvre, personal communication, Table 1) is highly interesting.
For instance, it might indicate that ATP binding, involving Lys 339, modulates the tertiary structure of the MtNFP KD, as demonstrated for other pseudokinases (Zegiraj & Aalten 2010). Alternatively, the conserved Lys 339, might be required for positioning of the α-helix C (through the conserved Lys 339 - Glu 348 salt bridge), and in result, for adopting an active conformation by the MtNFP KD. At the moment, MtNFP and LjNFR5 are proposed to signal via allosteric regulation of their putative protein interactors (Madsen et al., 2011; Lefebvre et al., 2012). Detailed analysis of ATP binding to MtNFP or any of its orthologs has not yet been investigated, and thereby cannot be excluded at this moment.

In conclusion, an extensive correlation between the effect of various substitutions on MtNFP function in nodulation (Lefebvre et al., 2012) and MtNFP role in CD induction (this study) implies that the heterologous system of Nicotiana leaf constitutes a relevant and convenient system for high-throughput structure-function studies on this, and potentially other symbiotic LysM-RLKs. As substitutions of the residues essential for correct folding of a KD are expected to abolish activity of both true ePKs and pseudokinases, we propose that the CD induction assay in Nicotiana leaf can be used for further high-throughput mutagenesis studies on the MtNFP KD.
EXPERIMENTAL PROCEDURES

Constructs for plant expression
pBin+ CaMV 35S::MtNFP-sYFP2 and pCambia1390 CAMV 35S::MtLYK3-mCherry constructs are described in Chapter 2. Point mutations were introduced in the pMon999 CaMV 35S::MtNFP-sYFP2 using the QuickChange™ site-directed mutagenesis kit (Stratagene) as described, and the HindIII-SmaI fragment (containing the CAMV 35S::MtNFP-sYFP2 sequence) was subsequently recloned into pBin+ vector. All truncated constructs were generated by PCR amplification (see Table 2 for primer sequences) and cloned into pBin+ 35S::sYFP2 vector. All pBin+ constructs were sequenced to verify the correct insert sequence.

Plant transformations
Transformation of Agrobacterium tumefaciens strain GV3101::pMP90 and Agrobacterium infiltration of N. benthamiana leaf is described in Chapter 2. Agrobacterium transformants carrying the respective constructs were resuspended in the infiltration medium to desired OD$_{600}$: WT MtNFP, all MtNFP truncated and mutated constructs (except for MtNFP [G474E]-sYFP2- OD$_{600}$=0.5; MtNFP [G447E]-sYFP2-OD$_{600}$=1; MtLYK3-mCherry-OD$_{600}$=0.7. Subsequently, they were mixed 1:1 with Agrobacterium transformants carrying an empty pCambia1390 vector (for separate expression) or MtLYK3-mCherry construct before being infiltrated into Nicotiana leaf. All experiments included mock control (GV3101::pMP90 transformants carrying empty pCambia1390 vector) and a positive control (co-expression of full length MtNFP-sYFP2 and MtLYK3-mCherry constructs). All CD assays were performed at least three times, every time using three different plants. Macroscopic observations were carried out between 24 and 72hai, and the results were collated. In case of lack or inefficient CD induction by the (co-)expression of (a) certain construct(s), three leaves were stained with Evans blue (as described in Chapter 2) 48hai to check for the presence of infrequent CD.

Microscopic analysis
Was carried as described in Klaus-Heisen et al. (2011).

Table 2. Primer and linker sequences.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtNFP fw</td>
<td>Cloning (NheI)</td>
<td>GGGGCCTAGGCATGCTGCTTCCTTTCTTC</td>
</tr>
<tr>
<td>MtNFP full JM rev</td>
<td>Cloning (EcoRI)</td>
<td>GGGGAATTCACCTCCATGATTGCATCAA</td>
</tr>
<tr>
<td>MtNFP ΔCtail rev</td>
<td>Cloning (EcoRI)</td>
<td>GGGGAATTCGTGTTGGTTGAGAAGAG</td>
</tr>
<tr>
<td>Linker to FP</td>
<td></td>
<td>GAATTC for all the constructs</td>
</tr>
</tbody>
</table>

Restriction sites are underlined.
Figure S1. Tertiary structure of human IRAK-4 kinase domain.

All protein kinases share a conserved KD characterized by a distinctive tertiary fold composed of an N-terminal and a C-terminal lobe. The α-helix F (in red) serves as an organizing element for the entire KD, i.e. provides a firm anchor for various structural elements located in the N- and C-lobe. The orientation of two regulatory elements, the α-helix C and the activation segment, governs the activity of many ePKs. Active conformation of the α-helix C is achieved mainly via two contacts made by its conserved Glu (E91 in PKA, E232 in IRAK-4) residue. One contact is made to the conserved Lys (K72 in PKA, K213 in IRAK-4) in the AxK motif (note the proximity of these two residues – highlighted in purple), and the other to the conserved Phe (F185 in PKA, F330 in IRAK-4) in the DFG motif. Orientation of the AS (green and lime) of many ePKs requires phosphorylation on a specific residue located in an activation loop within. Upon phosphorylation, this so-called primary phosphorylation site connects with two conserved basic residues: one in the catalytic loop (Arg165 in PKA, R310 in IRAK-4) and the other downstream from the DFG motif (K189 in PKA, R334 in IRAK-4).

In addition, the dynamic assembly/disassembly of two non-consecutive hydrophobic spines is postulated to regulate the activation of an ePK. The regulatory spine comprises of the following conserved residues (in blue): Leu (L95 in PKA, Met 237 in IRAK-4) in the α-helix C, Leu (L106 in PKA, L248 in IRAK-4) in a β-strand 4 (immediately proceeding the α-helix C), Phe (F185 in PKA, F330 in IRAK-4) in the DFG motif, and a residue at the position -2 from the conserved Asp in the catalytic loop (Y164 in PKA, His 309 in IRAK-4). The catalytic spine comprises of the following conserved residues (in orange): Val (V57 in PKA, V200 in IRAK-4) after the Gly-rich loop, Ala (A70 in PKA, A211 in IRAK-4) in the AxK motif,
and Leu (L173 in PKA, L318 in IRAK-4) in the catalytic loop, and is completed by the adenine ring of ATP.
The orientation of the α-helix C (via positioning of the L95/M237, the L106/L248 or the AxA motif) 
and the activation segment (via positioning of the catalytic loop and the DFG motif) governs the dynamic 
assembly/disassembly of both spines.
The structural elements that are either missing or highly modified in the MtNFP are highlighted: 
the Gly-rich loop (in cyan), and the AS (in green and lime).
Note for a putative assembly of the R spine in MtNFP: in kinase-active ePKs, the favorable orientation 
of the Phe 185/330 (F454 in MtNFP) and Tyr 164/His 309 (H433 in MtNFP) is dependent on the orientation 
of the activation segment. Due to the lack of a crystal structure of an ePK with a similarly altered sequence 
of the AS, homology modeling with respect to these residues and to the exact position of this element 
in the MtNFP KD is currently not possible.