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Interaction of *Medicago truncatula* Lysin Motif Receptor-Like Kinases, NFP and LYK3, Produced in *Nicotiana benthamiana* Induces Defence-Like Responses

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**Abstract**

Receptor-like kinases with Lysin Motif (LysM) domains in their extracellular region play crucial roles during plant interactions with microorganisms; e.g. *Arabidopsis thaliana* CERK1 activates innate immunity upon perception of fungal chitin/chitooligosaccharides, whereas *Medicago truncatula* NFP and LYK3 mediate signalling upon perception of bacterial lipo-chitooligosaccharides, termed Nod factors, during the establishment of mutualism with nitrogen-fixing rhizobia. However, little is still known about the exact activation and signalling mechanisms of MtNFP and MtLYK3. We aimed at investigating putative molecular interactions of MtNFP and MtLYK3 produced in *Nicotiana benthamiana*. Surprisingly, heterologous co-production of these proteins resulted in an induction of defence-like responses, which included defence-related gene expression, accumulation of phenolic compounds, and cell death. Similar defence-like responses were observed upon production of AtCERK1 in *N. benthamiana* leaves. Production of either MtNFP or MtLYK3 alone or their co-production with other unrelated receptor-like) kinases did not induce cell death in *N. benthamiana*, indicating that a functional interaction between these LysM receptor-like kinases is required for triggering this response. Importantly, structure-function studies revealed that the MtNFP intracellular region, specific features of the MtLYK3 intracellular region (including several putative phosphorylation sites), and MtLYK3 and AtCERK1 kinase activity were indispensable for cell death induction, thereby mimicking the structural requirements of nodule or chitin-induced signalling. The observed similarity of *N. benthamiana* response to MtNFP and MtLYK3 co-production and AtCERK1 production suggests the existence of parallels between Nod factor-induced and chitin-induced signalling mediated by the respective LysM receptor-like) kinases. Notably, the conserved structural requirements for MtNFP and MtLYK3 biological activity in *M. truncatula* (nutation) and in *N. benthamiana* (cell death induction) indicates the relevance of the latter system for studies on these, and potentially other symbiotic LysM receptor-like kinases.

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**Introduction**

Legumes can establish a mutualism with compatible rhizobia ultimately leading to nodulation, i.e. a formation of specialized symbiotic organs (nodules) in which atmospheric dinitrogen is converted into ammonia by the bacteria in exchange for plant carbohydrates. Nod factors (NFs) play a central role during most *Rhizobium*-legume (RL) symbioses [1]. They are secreted rhizobial signals whose perception by host legume roots is required for root nodule organogenesis, invasion of rhizobia toward a nodule primordium, and accommodation of bacteria inside nodule cells [2–4]. In two model legumes, NF-induced responses during the pre-infection step of RL interaction require *M. truncatula* (*Medicago*).

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Receptor(-like) kinases with Lysin Motif (LysM) domains in their extracellular region play crucial roles during plant interactions with microorganisms; e.g. *Arabidopsis thaliana* CERK1 activates innate immunity upon perception of fungal chitin/chitooligosaccharides, whereas *Medicago truncatula* NFP and LYK3 mediate signalling upon perception of bacterial lipo-chitooligosaccharides, termed Nod factors, during the establishment of mutualism with nitrogen-fixing rhizobia. However, little is still known about the exact activation and signalling mechanisms of MtNFP and MtLYK3. We aimed at investigating putative molecular interactions of MtNFP and MtLYK3 produced in *Nicotiana benthamiana*. Surprisingly, heterologous co-production of these proteins resulted in an induction of defence-like responses, which included defence-related gene expression, accumulation of phenolic compounds, and cell death. Similar defence-like responses were observed upon production of AtCERK1 in *N. benthamiana* leaves. Production of either MtNFP or MtLYK3 alone or their co-production with other unrelated receptor(-like) kinases did not induce cell death in *N. benthamiana*, indicating that a functional interaction between these LysM receptor-like kinases is required for triggering this response. Importantly, structure-function studies revealed that the MtNFP intracellular region, specific features of the MtLYK3 intracellular region (including several putative phosphorylation sites), and MtLYK3 and AtCERK1 kinase activity were indispensable for cell death induction, thereby mimicking the structural requirements of nodule or chitin-induced signalling. The observed similarity of *N. benthamiana* response to MtNFP and MtLYK3 co-production and AtCERK1 production suggests the existence of parallels between Nod factor-induced and chitin-induced signalling mediated by the respective LysM receptor(-like) kinases. Notably, the conserved structural requirements for MtNFP and MtLYK3 biological activity in *M. truncatula* (nutation) and in *N. benthamiana* (cell death induction) indicates the relevance of the latter system for studies on these, and potentially other symbiotic LysM receptor-like kinases.

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Legumes can establish a mutualism with compatible rhizobia ultimately leading to nodulation, i.e. a formation of specialized symbiotic organs (nodules) in which atmospheric dinitrogen is converted into ammonia by the bacteria in exchange for plant carbohydrates. Nod factors (NFs) play a central role during most *Rhizobium*-legume (RL) symbioses [1]. They are secreted rhizobial signals whose perception by host legume roots is required for root nodule organogenesis, invasion of rhizobia toward a nodule primordium, and accommodation of bacteria inside nodule cells [2–4]. In two model legumes, NF-induced responses during the pre-infection step of RL interaction require *M. truncatula* (*Medicago*).
All four genes encode receptor-like kinases (RLKs) with an extracellular region (ExR) predicted to contain three LysM domains, a transmembrane helix, and a protein kinase domain (KD) within the intracellular region (InR) [3,20–22]. Remarkably, in contrast to MtLYK3 and LjNFR1, which both display kinase activity, MtNFP and LjNFR5 seem to function as pseudokinases that neither show nor rely on the intrinsic kinase activity to signal [9,20–22]. LjNFR5 is hypothesized to form a receptor complex with LjNFR1; a notion consistent with their demonstrated co-functioning during the determination of RL specificity [23]. Similarly, a receptor complex composed of MtNFP and a yet-identified LysM-RLK or MtLYK3 is predicted to initiate the pre-infection responses and the infection process, respectively [12,15]. Since mutagenesis studies in *Medicago* have not identified alterations in genes other than MtNFP that lead to complete lack of responsiveness to NFs, a function of this additional LysM-RLK in the pre-infection stage is most likely redundant. In addition, MtNFP has been implicated in *Medicago* interactions with pathogens (*Aphanomyces euteiches* and *Colletotrichum trifolii*), and with beneficial arbuscular mycorrhiza (AM) fungi [24–27]. However, it remains to be shown whether MtNFP functions in these processes alone or in co-operation with (an)other RLK(s).

Nicotiana benthamiana their accumulation (even in the situation of an attempted have been unsuccessful, presumably due to stringent regulation of Agrobacterium tumefaciens However, our attempts to visualize these proteins in and MtLYK3, focusing on their postulated interaction in situ perception of the same MAMP, can differ between plant species. [37–38]. Therefore, modes of CERK1 activation, even upon perception of chitin/chitooligosaccharides (COs), or peptidoglycan (PGN), respectively [31–35]. In the latter case, both in rice and in *Arabidopsis* PGN binds not to OsCERK1/AtCERK1 but to extracellular LysM domain-containing proteins, termed LYPs or LYMs [35–36]. This in turn is postulated to induce a formation of AtCERK1/AtLYMs receptor complex, and subsequent signal transduction via the kinase activity of AtCERK1. A similar mechanism operates during COs-induced signalling in rice, involving OsCERK1 and (a) LYP protein(s) [34,36], whereas in *Arabidopsis* COs bind directly to AtCERK1 [37–38]. Therefore, modes of CERK1 activation, even upon perception of the same MAMP, can differ between plant species.

We are interested in NF-induced signalling mediated by MtNFP and MtLYK3, focusing on their postulated interaction in *Medicago* root However, our attempts to visualize these proteins in *Medicago* root have been unsuccessful, presumably due to stringent regulation of their accumulation (even in the situation of an attempted overproduction). *Nicotiana benthamiana* (*Nicotiana*) has proved to be a useful model for heterologous production and structure-function studies on multiple proteins, providing invaluable insights that guided their subsequent analyses in the respective homologous systems [39]. Therefore, we employed an *Agrobacterium tumefaciens* (*Agrobacterium*)-mediated transient transformation of *Nicotiana* leaves [40], which allowed us to produce both proteins to levels suitable for fluorescence microscopy. Remarkably, we found that heterologous co-production of MtNFP and MtLYK3 resulted in the induction of defence-like responses that are typically observed upon treatment with pathogen-derived molecules. As the apparent (functional) interaction of these LysM-RLKs in *Nicotiana* activated defence-like responses, similar to those mediated by AtCERK1,
fluorescence reported on efficient accumulation, and even unspecific oligomerization of the respective encoded fusions.

**Stereoscopic Analysis**

Blue light-excitible autofluorescence and far-red chlorophyll autofluorescence in intact *Nicotiana* leaves were imaged using 430/40 excitation and 485/50 emission BP filters, or 480/40 BP excitation and 510 LP emission filters, respectively. Images were captured using CMOS USB DCC1643C camera (THORLabs, Newton NJ, USA) implemented on a Leica MZ FLIII stereoscope. Evans blue staining was performed as described [42]. Leaves were cleared by boiling in acidic lactophenol/ethanol solution (10 g phenol in 10 ml lactic acid, mixed 2:1 with 96% ethanol) until the complete removal of chlorophyll (approximately 3 min per leaf). Ethanol-inextractable autofluorescence was excited with 312 nm wavelength. Images were captured using a Cool Snap CF camera (Photometrix, Tucson AZ, USA).

**qRT-PCR Analysis**

RNA extraction and qRT-PCR were performed as described [17] except that cDNA was prepared from 500 ng of total RNA (see Table S1 for primer sequences). Two technical replicates from two biological replicates were analyzed and results were collated.

**Medicago Transformations**

Complementation of *Mglyk3-1* mutant seedlings was performed as described [20] using *MtLYK3-3xFLAG, MtLYK3[K464A]-3xFLAG,* and *MtLYK3[T480A]-sYFP2* constructs driven by the CaMV 35S promoter. Results were scored as: (+ >75% of plants nodulated), reduced (<50% of plants nodulated) or - (0 plants nodulated).

**Results**

**Co-production of MtNFP and MtLYK3 in *Nicotiana* Leaves Induces Cell Death**

*MtNFP* and *MtLYK3* cDNA sequences were fused at their 3’ ends to the sequence encoding a fluorescent protein (FP); either a super yellow fluorescent protein 2 (sYFP2) or mCherry [43–44], and were expressed from a CaMV 35S promoter in *Nicotiana* leaves, where they were delivered by Agrobacterium-mediated transformation. These and similar *MtNFP* and *MtLYK3* constructs were shown to complement *Mglyp* and *Mglyk* mutants, respectively [17–18,22], and are therefore suitable for studying the encoded LysM-RLKs. Confocal laser-scanning microscopy analysis demonstrated co-localization of both MtNFP-sYFP2 and MtLYK3-sYFP2 fusions with a plasma membrane (PM) marker (the hypervariable region [HVR] of maize [*Zea mays*] ROP7 fused to the C-terminus of mCherry; [20,22]), hence indicating PM localization of MtNFP and MtLYK3 fusions in *Nicotiana* leaf epidermal cells. Surprisingly, co-infiltration of *Agrobacterium* transformants carrying *MtNFP-3xFLAG* or *MtLYK3-3xFLAG* constructs, leading to the co-production of the encoded fusions, resulted in collapsed and subsequent desiccation of the infiltrated region within 48 hours after infiltration (hai) (Fig. 1A), regardless of the *Agrobacterium* strain used (i.e. GV3101::pMP90 or LBA4404; unpublished data). This cell death (CD) response was not dependent on the tag attached to either protein, since an identical response was observed upon co-production of FP-tagged, 3xFLAG-tagged or untagged MtNFP and MtLYK3 (Fig. 1A, Table 1). Importantly, production of the separate MtNFP or MtLYK3 (fusions) did not induce CD as confirmed with an exclusion dye (Evans blue) staining (Fig. 1A), which reflects compromised membrane permeability attributed with cell death.

To investigate whether a similar CD response could be triggered by heterologous production of other plant RLKs, we analysed the *Nicotiana* response to expression of *Medicago Doesn’t Make Infection 2* (*DMI2*; [45]), *MtLR21* [41], and *Arabidopsis Bromosinotetra Inensitive 1* (*BTI1*; [46]), all driven by the CaMV 35S promoter. Notably, none of these RLKs, alone or in combination with either MtNFP or MtLYK3 fusions, induced CD (Fig. 1B, Table 1), despite being efficiently produced in *Nicotiana* leaves, as confirmed with fluorescence microscopy (see Materials & Methods). Thus, the *Nicotiana* CD response was not a general response to a heterologous production of RLKs but rather a specific response to MtNFP and MtLYK3 co-production.

**Production of AtCERK1 also Induces Cell Death in *Nicotiana* Leaves**

A rapid tissue collapse at the site of pathogen attack, termed the hypersensitive response (HR), is frequently observed in incompat-able plant-pathogen interactions where it is thought to contribute to pathogen restriction and to generate a signal that activates systemic plant defence mechanisms [47–48]. The apparent phenotypic similarity of the CD response to MtNFP and MtLYK3 co-production with the HR elicited by various pathogen-derived components (MAMPs and so-called effectors) [49–50], prompted us to investigate whether co-production of the symbiotic LysM-RLKs might activate defence signalling similar to that mediated by LysM-RLKs functioning in innate immunity. AtCERK1 mediates signalling upon the perception of COs or PGN, although, to our knowledge, CD induction in response to these MAMPs has not been reported so far in any plant species. We therefore investigated the *Nicotiana* response to heterologous production of wild-type (WT) AtCERK1 or its kinase-inactive variant carrying a substitution in the catalytic lysine (Lys 349 in a kinase subdomain [HVR]) of maize [*Zea mays*] ROP7 fused to the C-terminus of *MtNFP* and *MtLYK3* constructs driven by the CaMV 35S promoter. Notably, none of these RLKs, alone or in combination with either MtNFP or MtLYK3 fusions, induced CD (Fig. 1B, Table 1), despite being efficiently produced in *Nicotiana* leaves, as confirmed with fluorescence microscopy (see Materials & Methods). Thus, the *Nicotiana* CD response was not a general response to a heterologous production of RLKs but rather a specific response to MtNFP and MtLYK3 co-production.

**Cell Death Induction Upon MtNFP and MtLYK3 Co-production, and AtCERK1 Production in *Nicotiana* Leaves Requires an Influx of Extracellular Ca²⁺**

An influx of extracellular Ca²⁺ causes an increase in the cytosolic [Ca²⁺] that is required for MAMP (including COs)-induced activation of a MAPK cascade, ROS production, and gene expression. Thus, Ca²⁺ influx is postulated to occur very early in the plant defence signalling pathway [51–52], possibly immediately upon the activation of the PM-localised MAMP

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**Symbiotic LysM-RLKs Induce a Defence Response**
We wanted to know whether an influx of extracellular Ca\(^{2+}\) was similarly involved in CD induction upon MtNFP and MtLYK3 co-production or separate production of AtCERK1. To this end, MtNFP-3xFLAG and MtLYK3-3xFLAG fusions or AtCERK1-3xFLAG fusion were (co-)produced in adjacent regions in \textit{Nicotiana} leaves. Twelve hours later, parts of the infiltrated leaf regions were syringe-infiltrated with 5 mM lanthanum chloride (an established inhibitor of the PM-localized calcium channels) or water, and the CD development was monitored between 24 and 72 hours after the first infiltration (with \textit{Agrobacterium}). Notably, in 24 out of 30 leaf regions co-producing MtNFP and MtLYK3 fusions, compromised membrane permeability and tissue collapse were first (i.e. between 36 and 42 hai) localized only (or mostly) outside the lanthanum chloride-treated regions (Fig. 3A). Later on (i.e. 60 hai), 26 out of 30 parts of leaf regions treated with lanthanum chloride showed confluent death of the entire infiltrated region (unpublished data). Similar delay of the CD development was observed 33 hai in 11 out of 21 leaf regions producing AtCERK1 fusion and treated with lanthanum chloride (Fig. 3C). On the contrary, control treatment with water did not affect the development of confluent CD upon (co-)production of MtNFP and MtLYK3 fusions or AtCERK1 fusion (Fig. 3B, D).

Cell Death Upon MtNFP and MtLYK3 Co-production, and AtCERK1 Production in \textit{Nicotiana} Leaves is Associated with an Induction of Defence-like Responses

Subsequently, we investigated whether co-production of MtNFP and MtLYK3 or production of AtCERK1 in \textit{Nicotiana} leaves was associated with an accumulation of phenolic compounds and/or induction of defence-related gene expression, two established hallmarks of plant defence response, including that induced by C0s and/or PGN [54–56]. We started by analysing the kinetics of CD development. To this end, \textit{Agrobacterium} transformants carrying MtNFP-3xFLAG, MtLYK3-3xFLAG fusions or AtCERK1-3xFLAG fusion were (co-)produced in adjacent regions in \textit{Nicotiana} leaves, and CD development was monitored between 24 and 48 hai. In case of co-production of MtNFP and MtLYK3 fusions, macroscopic symptoms of CD were first observed around

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**Table 1.** Cell death induction upon (co-)expression of various RLK-encoding genes in \textit{Nicotiana} leaves.

| Construct | Separate expression | Co-expression with MtNFP-3xFLAG | Co-expression with MtLYK3-3xFLAG |
|-----------|------------------|-------------------------------|-------------------------------|
| MtNFP-sYFP2 | 0/12 | Not applicable | 20/22 |
| MtNFP-3xFLAG | 0/9 | Not applicable | 12/13* |
| MtNFP | 0/9 | Not applicable | 8/9** |
| MtLYK3-sYFP2 | 0/12 | 20/22 | Not applicable |
| MtLYK3-3xFLAG | 0/9 | 12/13* | Not applicable |
| MtLYK3 | 0/9 | 8/9** | Not applicable |
| MtDMI2-sYFP2 | 0/12 | 0/12 | 0/12 |
| MtBRI1.1-YFP | 0/9*** | 0/9*** | 0/9*** |
| ABR111-YFP | 0/7 | 0/9*** | 0/9*** |

# = unless stated differently: with -3xFLAG (*) untagged (**), or -YFPc (*** tagged construct.

Indicated constructs were expressed alone or co-expressed with either MtNFP or MtLYK3 in \textit{Nicotiana} leaves, and the infiltrated regions were marked. Macroscopic symptoms of cell death were scored 48 hai: only infiltrations that resulted in confluent death of (nearly) the entire infiltrated region were scored and are presented as a fraction of total infiltrations performed. 

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36 hai (Fig. 4A) as a type of flaccidity and the appearance of small patches of collapsed tissue (these were more pronounced on the abaxial side of the leaf). Forty-eight hai, 30 out of 31 infiltrations showed pronounced tissue desiccation of the entire infiltrated region (Fig. 1A). Compromised membrane permeability preceded tissue collapse and often occurred over the entire infiltrated region approximately 33 hai (Fig. 4A). Compromised membrane permeability of leaf regions producing AtCERK1 fusion was observed already approximately 27–30 hai, and pronounced macroscopic symptoms of CD developed 36 hai (Fig. 2A, Fig. 3 C, D).

In addition, co-production of MtNFP-3xFLAG and MtLYK3-3xFLAG fusions resulted in accumulation of blue light-excitable autofluorescence (Fig. 4B) approximately 36 hai. This was not observed upon separate production of either fusion, or upon co-production of MtNFP-3xFLAG and kinase-inactive MtLYK3[G334E]-3xFLAG fusions (unpublished data). Accumulation of ethanol/lactophenol-inextractable and UV-excitable autofluorescence, indicative of phenolic compounds, was detected approximately 36 hai and 30 hai in leaf regions (co-)producing MtNFP-3xFLAG and MtLYK3-3xFLAG fusions or AtCERK1-3xFLAG fusion, respectively (Fig. 4C). Mock infiltration, separate production of MtNFP-3xFLAG or MtLYK3-3xFLAG fusion or co-production of MtNFP-3xFLAG and kinase-inactive MtLYK3[G334E]-3xFLAG fusions did not result in the accumulation of similar autofluorescence (Fig. 4C).

Subsequently, we investigated induction of defence-related genes expression in Nicotiana leaves in response to: separate production and co-production of MtNFP-3xFLAG, MtLYK3-3xFLAG, MtLYK3[G334E]-3xFLAG, and AtCERK1-3xFLAG fusion(s). Induction of NbHIN1 – a postulated marker gene for HR [50]; two PR1 genes, i.e. NbPR1a acidic and NbPR1 basic [57]; and NbACRE31, NbACRE132, and NbCYP71D20 – postulated marker genes for MAMP-triggered immunity [49] was analyzed 24 hai
Figure 4. MtNFP and MtLYK3, or AtCERK1 (co-)production in *Nicotiana* leaves induces defence-like responses. A, Kinetics of cell death development in *Nicotiana*. *Agrobacterium* transformants carrying either MtNFP-3xFLAG or MtLYK3-3xFLAG construct were co-infiltrated into *Nicotiana* leaves at five different time points (1–5). Macroscopic observation (left panel) and subsequent Evans blue staining (right panel) are depicted 42 hai (region 1), 39 hai (region 2), 36 hai (region 3), 33 hai (region 4) and 30 hai (region 5). Mock infiltration (region 6) was done concomitantly with the infiltration of region 1. Bar is 1 cm. B, Changes in leaf autofluorescence upon MtNFP and MtLYK3 co-production. Leaf regions co-producing MtNFP-3xFLAG and MtLYK3-3xFLAG fusions were analyzed between 24 and 48 hai (here depicted 36 hai) using a stereoscope. Note the decrease in chlorophyll content, as indicated by the decrease of far-red autofluorescence of chlorophyll (left panel), and enhanced accumulation of blue light-
using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Co-production of MtNFP and MtLYK3 fusions, and separate production of AtCERK1 fusion resulted in an induction of NbHIN1, NbPR1 basic, NbACRE11, and NbACRE132 gene expression that was significantly higher than that following co-production of MtNFP and MtLYK3(G334E) fusions or separate production of MtNFP, MtLYK3, and MtLYK3(G334E) fusions (Fig. 4D). The NbPR1a acidic and NbCYP71D290 genes did not display significant induction upon (co-) production of any of the protein(s) tested (unpublished data).

Taken together, the indication of a localized accumulation of phenolic compounds and induction of defence-related gene expression suggested that the Nicotiana response to MtNFP and MtLYK3 co-production triggered responses that were qualitatively similar to the responses to heterologous production of AtCERK1. In addition, the fact that in both cases the impairment of Ca2+ influx delayed the CD development suggested that the apparent functional interaction of two symbiotic LysM-RLKs in Nicotiana leaves mimics the action of the MAMP receptor, AtCERK1, and triggers defence-like responses.

Cell Death Induced in Nicotiana Leaves Upon MtNFP and MtLYK3 Co-production is a NF-independent Response

Perception of NFs results in triggering host symbiotic program mediated by MtNFP and/or MtLYK3 [2]. In contrast, co-production of these LysM-RLKs in Nicotiana leaves apparently triggered some signalling cascade in the absence of NFs. Therefore, we investigated the effect of NF produced by Sinorhizobium meliloti, a microsymbiont of Medicago, on this CD response. To this end, Agrobacterium transforms carrying either MtNFP-sYFP2 or MtLYK3-mCherry construct were co-infiltrated into Nicotiana leaves at varying concentrations (as measured with OD600). Then, purified SmNF at 10−7 M (in diluted DMSO) or diluted DMSO alone was applied between 9 and 24 hai to parts of the leaf regions co-producing MtNFP and MtLYK3 fusions, and CD development was monitored between 24 and 72 hours after the first infiltration (with Agrobacterium) using Evans blue staining. For all bacterial concentrations and time-points of SmNF/DMSO application tested, compromised membrane permeability in leaf regions co-producing MtNFP and MtLYK3 fusions was observed at similar time irrespective of the SmNF or DMSO treatment (Fig. 5), indicating similar kinetics of CD development. Therefore, we did not obtain evidence for any stimulatory or inhibitory effect of the SmNF on the CD development upon MtNFP and MtLYK3 co-production.

The Intracellular Region of MtNFP and Kinase Activity of MtLYK3 are Required for Cell Death Induction in Nicotiana Leaves

The independence of CD induction upon MtNFP and MtLYK3 co-production from the SmNF perception prompted us to compare structural requirements of CD induction and nodulation with regard to these LysM-RLKs. In case of MtNFP, a recent structure-function study in Medicago [22] showed that loss-of-function mutations located in the ExR could be attributed to retention of the mutated protein in the endoplasmic reticulum (ER), whereas most substitutions located in the InR were found not to have an effect on the MtNFP function in nodulation. Therefore, we decided to limit our analysis of MtNFP to three point-mutated variants carrying: Ser 67 Phe (encoded by the Mtlyk3-3 allele), Ser 67 Ala, and Gly 474 Glu substitution; and a truncated variant with almost the entire InR deleted, termed MtNFP[ΔInR] (amino acids: 1–283) (see Table 2). Based on structure-function studies on MtLYK3 and LjNFR1, respectively in Medicago and in Lotus ([12,20–21], Table 3 in this study), we decided to test the effect of 16 point mutations (listed in Table 3) on MtLYK3 ability to induce CD in Nicotiana leaves in the presence of MtNFP. These included: a Pro 87 Ser (encoded by the Mtlyk3-3 allele) and a Gly 334 Glu (encoded by the Mtlyk3-1 allele) mutations, and Ala substitutions of Thr 285, Ser 286, Thr 300, Thr 319, Lys 349, Glu 362, Thr 433, Asp 441, Lys 464, Ser 471, Thr 472, Thr 475, Thr 480, and Thr 512. With the exception of the P87S substitution located in the first LysM domain of MtLYK3, all the above mutations are located in the MtLYK3 InR but differ in their effect on MtLYK3 autophosphorylation activity in vitro ([20] and Fig. S2; see Table 3). All truncated/mutated variants were prepared as fusions to the N-terminus of sYFP2, and their production and correct PM localization in Nicotiana leaf epidermal cells was confirmed, except for two MtNFP variants: MtNFP[S67F]-sYFP2 fusion was retained in the ER, and MtNFP[G474E]-sYFP2 fusion showed a partial PM localization, ([20,22] and Fig. S1). Additionally, we ruled out a possibility that the presence of WT MtNFP-PP or WT MtLYK3-PP fusion might affect stability/localization of the truncated/mutated fusions by confirming their efficient production and PM localization in Nicotiana leaf epidermal cells also upon co-production with MtLYK3 or MtNFP fusions (unpublished data).

Subsequently, we analyzed the ability of truncated/mutated MtNFP and MtLYK3 variants to induce CD upon either their separate production or co-production with WT MtLYK3-mCherry or WT MtNFP-mCherry fusion, respectively. In order to compare the CD induction ability of truncated/mutated variants with WT proteins, concomitant co-infiltration with Agrobacterium transforms carrying either WT MtNFP-PP or WT MtLYK3-PP construct was done on every leaf. Development of CD was monitored between 36 and 72 hai, and in case of the absence of or weakly pronounced macroscopic symptoms, the occurrence of CD was further scrutinized with Evans blue staining. None of the truncated/mutated variants was able to induce CD in Nicotiana leaves on its own (Table 2, 3). Co-production of MtNFP[S67A]-sYFP2 and MtLYK3 fusions resulted in a confluently death of (nearly) the entire infiltrated region in 6 out of 9 infiltrations, and compromised membrane permeability that could be observed in the entire infiltrated region (Table 2). In contrast, co-production of MtLYK3 fusion with MtNFP[S67F]-sYFP2,
MtNFP\([G474E]\)-sYFP2, or MtNFP\([ΔInR]\)-sYFP2 fusion did not induce CD in *Nicotiana* leaves (Table 2). In case of MtLYK3 mutated variants, co-production of MtLYK3\[P87S\]-sYFP2 and MtNFP fusions induced confluent CD in all infiltrated regions (Table 3). In contrast, co-production of MtNFP fusion with all seven MtLYK3-sYFP2 mutated variants affected for their autophosphorylation activity *in vitro* did not induce CD in *Nicotiana* leaves (Table 3). In case of mutations that do not affect autophosphorylation activity of MtLYK3 kinase, we found that MtLYK3\[T285A S286A T300A\]-sYFP2 and MtLYK3\[S471A\]-sYFP2 fusions were as active as WT MtLYK3-sYFP2 fusion for CD induction upon their co-production with MtNFP fusion, whereas MtLYK3\[K464A\]-sYFP2 fusion induced compromised membrane permeability (but no macroscopic symptoms of cell death) upon co-production with MtNFP fusion (Fig. S3, Table 3). Co-production of MtNFP fusion with MtLYK3\[T433A\]-sYFP2, MtLYK3\[T472A\]-sYFP2 or MtLYK3\[T512A\]-sYFP2 fusion resulted in a confluent death of (nearly) the entire infiltrated region in, respectively, 7 out of 20, 5 out of 11, and 12 out of 20 infiltrations, whereas the remaining leaf regions displayed only (a) small patch(es) of dead tissue (Fig. S3, Table 3).

Taken together, most of the structural requirements regarding the MtNFP and MtLYK3 InR, and the autophosphorylation activity of the MtLYK3 KD, appeared to be identical for biological activity of these LysM-RLKs in both *Medicago* and *Nicotiana*. More specifically, we found out that both nodulation and CD induction displayed the same requirements for 11 out of 15 residues located in the MtLYK3 InR. On the contrary, a single mutation in the MtLYK3 ExR tested (that does not affect the PM localization of the fusion) was found to be crucial for MtLYK3 function in nodulation but not in CD induction. In case of MtNFP, the substitution of Ser 67 similarly abolished (S67F) or did not have an effect (S67A) on MtNFP function in nodulation and CD induction, which seemed to correlate with, respectively, the absence or presence of MtNFP fusion at the PM.

### Table 2. Cell death induction activity of MtNFP-sYFP2 truncated/mutated variants in *Nicotiana* leaves.

| MtNFP-sYFP2 construct | Subcellular localization* | Nodulation activity* | Cell death induction |
|-----------------------|---------------------------|----------------------|----------------------|
|                       |                           | Co-expression with MtLYK3-mCherry | Separate expression |
| WT                   | PM                        | +                    | 28/30                | 0/12          |
| S67F (Mtnfp-2)       | ER                        | –                    | 0/9                  | 0/9           |
| S67A                 | PM                        | +                    | 6/9#                 | 0/9           |
| ΔInR                 | PM                        | –                    | 0/20                 | 0/9           |
| G474E                | partial PM                | –                    | 0/15                 | 0/9           |

*see [22]. PM-plasma membrane, ER-endoplasmic reticulum.

The designated constructs were expressed alone or co-expressed with MtLYK3-mCherry construct in *Nicotiana* leaves. Macroscopic symptoms of cell death were scored 48 hai: only infiltrations that resulted in confluent death of (nearly) the entire infiltrated region were scored and are presented as a fraction of total infiltrations performed. # - in the 3 remaining leaf regions, co-expression of MtNFP\[S67A\]-sYFP2 and MtLYK3-mCherry constructs resulted in increased staining with Evans blue in the entire infiltrated region.

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Table 3. Cell death induction activity of MtLYK3-sYFP2 mutated variants in Nicotiana leaves.

| MtLYK3-sYFP2 construct | Auto-phosphorylation activity* | Nodulation activity** | Cell death induction |
|------------------------|-------------------------------|-----------------------|----------------------|
|                        |                               |                       | Co-expression with MtNFP-mCherry | Separate expression |
| WT                     | +                             | +                     | 28/30                | 0/12                |
| P87S (Mtlyk3-3)        | Not applicable                | -                     | 15/15                | 0/9                 |
| T319A                  | -                             | -                     | 0/11                 | 0/9                 |
| G334E (Mtlyk3-1)       | -                             | -                     | 0/20                 | 0/9                 |
| K349A                  | -                             | -                     | 0/16                 | 0/9                 |
| E362A                  | -                             | -                     | 0/15                 | 0/9                 |
| D441A                  | -                             | -                     | 0/16                 | 0/9                 |
| T475A                  | -                             | -                     | 0/13                 | 0/9                 |
| T480A                  | -                             | - (0/24)              | 0/18                 | 0/9                 |
| T285A/S286A/T300A      | +                             | Reduced with T300A    | 15/16                | 0/9                 |
| T433A                  | +                             | Reduced               | Reduced 7/20          | 0/10                |
| K464A                  | Reduced                       | Reduced               | Reduced 7/18          | 0/12                |
| S471A                  | +                             | Reduced              | Reduced 9/11          | 0/9                 |
| T472A                  | +                             | Reduced              | Reduced 5/11          | 0/9                 |
| T512A                  | Reduced                       |                       | Reduced 12/20         | 0/9                 |

* - see [20], except for the T480A (Fig. 52), ** - see [20], except for the P87S [12], K464A and T480A (this study; number of plants nodulated/number of plants tested).

Discussion

Co-production of MtNFP and MtLYK3 in Nicotiana Induces Defence-like Responses that Resemble Nicotiana Responses to AtCERK1 Production

Efficient production of both MtNFP-FP and MtLYK3-FP fusions in Nicotiana leaves facilitated characterization of their subcellular localization [20,22] and oligomerization status in vivo (manuscript in preparation), and led to the surprising observation of a CD induction (Fig. 1A). This response phenotypically and kinetically (Fig. 4A) resembled the HR elicited in leaves facilitated characterization of their subcellular localization [20,22] and oligomerization status in vivo (manuscript in preparation), and led to the surprising observation of a CD induction (Fig. 1A). This response phenotypically and kinetically (Fig. 4A) resembled the HR elicited in

Nicotiana leaves [21] and Arabidopsis [19] response to LjNFR1 and LjNFR5 co-production. However, in these studies the associated induction of putative defence-related responses has not been investigated. We here showed that both MtNFP and MtLYK3 co-production and AtCERK1 production in Nicotiana leaves triggered local accumulation of phenolic compounds, and a similar induction of expression of 4 out of 6 tested defence-related genes (Fig. 4C, D). We speculate that the two other genes might display different kinetics of induced expression (here analyzed only 24 hai) or might undergo suppression by Agrobacterium [62]. Importantly, COs- and/or PGN-induced expression of PRI, ACRE31, ACRE132 and a member of the HIN1 gene family was reported previously [32,52,54], linking these genes to MAMP-induced gene regulation mediated by AtCERK1 in Arabidopsis and/or NcERK1 in Nicotiana. In addition, we found that the lanthanum chloride-induced impairment of a Ca²⁺ influx similarly delayed the CD development upon (co-)production of the LysM-RLKs in our study (Fig. 3A, C). Therefore, we speculate that the signalling triggered upon MtNFP and MtLYK3 co-production in Nicotiana mimics AtCERK1-mediated signalling and thereby results in an induction of defence-like responses.

Similarities and Differences between Symbiotic and Defence Signalling Mediated by LysM-RLKs

The similarity between Nicotiana response to MtNFP and MtLYK3 co-production and AtCERK1 production suggests a possible overlap in signalling mediated by these LysM-RLKs. Several NF-induced processes, such as: a transient increase of reactive oxygen species (ROS) production; activation of phospholipase C (PLC) and PLD; and prolonged oscillations of perinuclear [Ca²⁺] were implicated in switching on the symbiotic programme [63–69], whereas a Ca²⁺ influx is postulated to act as a signal for infection thread formation [70]. Interestingly, (CERK1-mediated) COs- and/or PGN-induced responses also
involve a Ca\(^{2+}\) influx, an elevated ROS production, and PLC activation [33,33,37–38,52–53,71–73]. We speculate that these similar processes might be activated/regulated by related molecular components, hence allowing two *Medicago* LysM-RLKs to activate signalling components present in *Nicotiana* leaf. Remarkably, Nakagawa and associates [74] demonstrated that swapping of the *AtCERK1* ExR and a certain part of the *AtCERK1* InR for the corresponding regions from *LjNFR1* conferred on *AtCERK1* a competence, albeit inefficient, for symbiotic signalling during *Lotus-Mesorhizobium loti* interaction. Conversely, our results demonstrate that MnNP and MtLYK3, when co-produced in *N. benthamiana*, are capable of signalling in a similar manner to *AtCERK1*. We hypothesize that due to the absence of symbiosis-specific “decoders” or “modulators” in *N. benthamiana*, MnNP- and MtLYK3-mediated signalling might be differentially interpreted in this species, resulting in the induction of defence-like responses.

Importantly, NF-induced host responses are partially contradictory. On one hand, NFPs are postulated to suppress the production in legume roots of salicylic acid and ROS, two potent dictory. On one hand, NFs are postulated to suppress the defence-like response comes from defence-related proteins in the initial stage of symbiosis. On the other hand, even perception of compatible NFs leads to the induction of Pathogenesis-related genes 1 (initial) host’s defence responses via LysM-RLKs that mediate NF-mediated signalling [76]. On the other hand, even perception of compatible NFs leads to the induction of defence-related gene expression and phosphorylation of defence-related proteins in the initial stage of symbiosis [7,11,60,74]. An even more striking example of a NF-induced defence-like response comes from *Solanum rostratum*. NF produced by its microsymbiont, *Azoarcus loti* 

**Possible Co-functioning of MtNFP and MtLYK3 in Nicotiana and Medicago**

To explain a possible signalling mechanism employed by the kinase-inactive MtNFP, it has been proposed to form a receptor complex with MtLYK3 and another LYK protein during, respectively, the infection thread growth and pre-infection stage of symbiosis [12,15]. In contrast, it is not known whether MnNP functions alone during arbuscular mycorrhiza symbiosis [24–26] or resistance towards fungal and oomycete pathogens [27] or requires a similar co-functioning with another RLK(s). We here demonstrated that MnNP required MtLYK3 to induce CD in *N. benthamiana*, and that neither of these LysM-RLKs could be substituted by an unrelated RLK (Fig. 1B, Table 1). We propose that this *N. benthamiana* response reflects a functional interaction between these LysM-RLKs. A rather limited heteromerization of MnNP and a kinase-inactive MtLYK3 variant observed in the PM of *N. benthamiana* epidermal cells (manuscript in preparation) does not exclude either hypothesized mechanism: a direct, phosphorylation-dependent physical interaction between MnNP and WT MtLYK3; or indirect (functional) interaction between MnNP and MtLYK3 that requires independent activation of different molecular components by either LysM-RLK, and a later convergence of such putative signalling pathways. Interestingly, MtLYK3, but not the homologous [78] *AtCERK1*, required the presence of MnNP for CD induction in *N. benthamiana* (Fig. 2A), indicating the specific requirement for MnNP to potentiate the MtLYK3-mediated signalling. This observation agrees with the hypothesized specialization of the LysM-RLKs mediating NF-induced signalling during the co-evolution of legumes with rhizobia [28].

**Cell Death Induction in Nicotiana and Nodulation in Medicago Share Certain Structural Requirements Regarding MtNFP and MtLYK3**

Curiously, CD induction in *N. benthamiana* was independent from the NF perception (Fig. 5), and the presence of Pro 87 in the MtLYK3 ExR (Table 3), in contrast to MnNP and MtLYK3 function in nodulation [12,15]. Further mapping of crucial amino acid residues, and detailed characterization of their exact role in signalling would be required to clarify in the future whether or not nodulation and CD induction indeed hold different structural requirements with regard to the ExRs of these LysM-RLKs. On the contrary, the biological activity of MnNP in *N. benthamiana* was dependent on its PM localization, the presence of its InR, and the Gly 474 (Table 2), thus mimicking the structural requirements of RL symbiosis regarding MtLYK3 [22], and proteins encoded by *MtNFP* orthologs in *Lotus* [79] and pea (*Pisum sativum*) [5]. The overlap between structural requirements of nodulation and CD induction was even more pronounced with respect to the MtLYK3 InR. Out of 16 residues whose role in nodulation was identified, 9 residues (Thr 319, Gly 334, Lys 349, Glu 362, Thr 433, Asp 441, Thr 472, Thr 475, and Thr 480) were found to be equally important, and 2 residues (Thr 205, Ser 296) were equally dispensable for MtLYK3 biological activity both in *Medicago* and in *N. benthamiana* (Table 3). In addition, the K464A and T512A substitutions had a negative effect of MtLYK3 biological activity in both *N. benthamiana* and *Medicago*, although this effect was more (K464A) or less (T512A) severely pronounced during CD induction assays than during nodulation (Table 3). Various mutations abolishing MtLYK3 autophosphorylation activity [20] and Fig. S2) similarly abolished its biological activity in *Medicago* [20] and in *N. benthamiana* (Table 3), supporting the hypothesis that autophosphorylation of MtLYK3 is crucial for its signalling function. Importantly, as the role of Thr 480 in nodulation has not been described so far, our results revealed its importance for MtLYK3 function in *vivo*. Notably, the shared structural requirements of nodulation and CD induction were also confirmed with regard to several (putative) phosphorylation sites that do not abolish MtLYK3 autophosphorylation activity *in vitro* (Table 3). Phosphorylation within the InR of a RLK is often required for activation and regulation of its catalytic activity, and for generation of docking sites for (downstream) signalling components [80–82]. The shared importance of three (Thr 433, Thr 472, and Thr 512) out of five such phosphorylation sites for MtLYK3 biological activity in both plant species suggests that some of these phosphorylation-dependent functions required for MtLYK3-mediated signalling are conserved in *N. benthamiana* leaf.

Demonstrated significant overlap between structural requirements of nodulation and CD induction regarding the MnNP and MtLYK3 InRs supports our notion of the relevance of the *N. benthamiana* system for studies on these, and potentially other (symbiotic) LysM-RLKs. This system presents certain practical advantages over the legume root system, in terms of rapidity and ease of expression of multiple constructs. In view of hypothesized similarities between NF (i.e. lipo-chitooligosaccharide)-induced and COs-induced signalling, analyzing known molecular components/processes involved in the CERK1-mediated signalling [35–38,83] might provide information on the yet-identified players implicated in the perception and/or transduction of the NF signal. This would be especially important as still very little is known about the identity of interactors of these symbiotic LysM-RLKs [17–18,21,84]. Possible candidate signalling molecule(s) function-
ing in co-operation with, or downstream from the LysM-RLKs, and identified in this heterologous system should then be evaluated in legume root in order to confirm their involvement in symbiosis.

Supporting Information

Figure S1 Subcellular localization of various protein fusions in Nicotiana leaf epidermal cells. The plasma membrane marker, mCherry-HVR, was co-produced with the designated fusions in Nicotiana leaf epidermal cells, and the fluorescence (viewed from abaxial side) was imaged 24 h after transfection using confocal laser scanning microscopy. From left to right: green fluorescence of sYFP2; orange fluorescence of mCherry; superimposition of green, orange, and far-red (chlorophyll) fluorescence with the differential interference contrast (DIC) image. Bars are 20 μm. Note 1: in case of subcellular localization of MtNFP[G474E]-sYFP2 fusion, strong fluorescent puncta (indicated with an arrowhead) at the cell boundary of many cells (sometimes in association with nuclei), and pronounced ER localization (indicated with an arrow) of the fusion were still visible at 48 hai. Nevertheless, some cells showed a more uniform pattern of fluorescence at the cell boundary, and this observation, together with a partial insensitivity of this mutated variant to the PNGaseF treatment [22], indicated that at least some MtNFP[G474E] fusion had reached the PM. Note 2: as all kinase-inactive MtLYK3 variants were produced and correctly localized to the plasma membrane in Nicotiana leaf epidermal cells, their lack of biological activity can be attributed to the general abolishment of kinase activity rather than to an individual effect of a particular mutation. (TIF)

Figure S2 Effect of the Thr 480 Ala substitution on MtLYK3 autophosphorylation activity in vitro. The purified intracellular regions of WT MtLYK3, MtLYK3[G334E], and MtLYK3[T480A], fused to the C terminus of GST, were analyzed for their autophosphorylation activity in vitro using radiolaabeled ATP (γ-32P ATP) and phosphorimaging (PI). The coomassie blue staining (CB) shows the protein loading. (TIF)

Figure S3 Various (putative) phosphorylation sites are differentially required for MtLYK3 biological activity in Nicotiana. MtLYK3-sYFP2 mutated variants were co-produced with MtNFP-mCherry fusion in Nicotiana leaves: MtLYK3[T285A S286A T300A]+MtNFP (1); MtLYK3[T433A]+MtNFP (2); MtLYK3[T512A]+MtNFP (3); MtLYK3[T480A]+MtNFP (4). Macroscopic observation (left panel) and Evans blue staining (right panel) are depicted 48 hai. Bar is 1 cm. (TIF)

Table S1 Primer and linker sequences. (DOC)

Materials and Methods S1. (DOC)

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Author Contributions

Conceived and designed the experiments: AP-B BL RG JVC TWJG. Performed the experiments: AP-B BL MAK DK-H JVC. Analyzed the data: AP-B BL FLWT RG JVC TWJG. Contributed reagents/materials/analysis tools: AP-B BL MAK DK-H FLWT RG JVC TWJG. Wrote the paper: AP-B BL MAK FLWT RG JVC TWJG.

References

1. Masson-Boitie C, Giraud E, Perret X, Batut J (2009) Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipies? Trends Microbiol 17: 458–466.
2. Downie JA (2010) The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEBS Microbiol Rev 34: 150–170.
3. Gust AA, Willmann R, Desaki Y, Grabherr HM, Nürnberger T (2012) Plant LysM proteins: modules mediating symbiosis and immunity. Trends Plant Sci 17: 495–502.
4. Oldroyd GED (2013) Speak friend, and enter: signalling systems that promote in cooperation with, or downstream from the LysM-RLKs, beneficial symbiotic associations in plants. Nature 11: 252–263.
5. Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, et al. (2003) A LysM receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 637–640.
6. Radutoiu S, Madsen LH, Madsen EB, Felle HH, Unemura Y, et al. (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 585–592.
7. El Yahyaoui FE, Kuster H, Ben Amor B, Hohnjec N, Puñer A, et al. (2004) Identification of more than 750 genes extend the symbiotic host range. EMBO J 23: 2861–2871.
8. Høgslund N, Radutoiu S, Krusell L, Voroshilova V, Hannah MA, et al. (2009) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nature doi: 10.1038/ncomms10809.
9. Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, et al. (2010) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nature doi: 10.1038/ncomms10809.
10. Miwa H, Sun J, Oldroyd GED, Downie JA (2010) The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEBS Microbiol Rev 34: 150–170.
11. Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, et al. (2010) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nature doi: 10.1038/ncomms10809.
12. Bensmihen S, de Billy F, Gough C (2011) Formation of organelle–N-fixing symbiosomes in legume root nodules is controlled by DMI2. Proc Natl Acad Sci U S A 108: 10375–10380.
13. Hayaishi T, Banba M, Shimoda Y, Koschi H, Hayashi M, et al. (2010) A dominant function of CCaMK in intracellular accommodation of bacterial and fungal endosymbionts. Plant J 63: 141–154.
14. Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, et al. (2010) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nature doi: 10.1038/ncomms10809.
15. Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, et al. (2010) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nature doi: 10.1038/ncomms10809.
16. Radutoiu S, Madsen LH, Madsen EB, Felle HH, Unemura Y, et al. (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 637–640.
17. El Yahyaoui FE, Kuster H, Ben Amor B, Hohnjec N, Puñer A, et al. (2004) Identification of more than 750 genes extend the symbiotic host range. EMBO J 23: 2861–2871.
18. Haney CH, Riely BK, Tricoli DM, Cook DR, Ehrhardt DW, et al. (2011) Symbiotic rhizobia bacteria trigger a change in localization and dynamics of the Medicago truncatula receptor kinase LYYK3. Plant Cell 23: 2774–2787.
19. Broughammer A, Kruell L, Blaise M, Sauer J, Sullivan JT, et al. (2012) Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proc Natl Acad Sci U S A 109: 13059–13064.
20. Klaus-Heisen D, Nurisso A, Pietraszewska-Bogiel A, Mbengue M, Camut S, et al. (2011) Structure-function similarities between a plant receptor-like kinase and the human Interleukin-1 Receptor/Associated Kinase-4. J Biol Chem 286: 11202–11210.
21. Madsen EB, Antolin-Llovera M, Grossmann C, Ye J, Vieweg S, et al. (2011) Autophosphorylation is essential for in vivo function of the Lotus japonicus Nod Factor Receptor 1 and receptor mediated signalling in cooperation with Nod Factor Receptor 3. Plant J 65: 404–417.
22. Lefebvre B, Klaus-Heisen D, Pietraszewska-Bogiel A, Mbengue M, Camut S, et al. (2011) Structure-function similarities between a plant receptor-like kinase and the human Interleukin-1 Receptor/Associated Kinase-4. J Biol Chem 286: 11202–11210.
24. Ohlah B, Bežíć G, Bécard G, Dénerie J, Gough C (2005) nod factors and a diffusible factor from arbuscular mycorrhizal fungus stimulate lateral root formation in Medicago truncatula via the DM1/DM2 signalling pathway. Plant J 44: 195–207.

25. Muller F, Poizot V, André O, Peach-Pagés V, Haouy A, et al. (2011) Fungal lipopolysaccharide and symbiotic signals in arbuscular mycorrhiza. Nature 469: 58–64.

26. Czaja LF, Hodgkamp C, Lammin P, Maillet F, Andres Martínez E, et al. (2012) Transcriptional responses of plants to diffusible signals from symbiotic microbes reveal MAMP- and RIM-dependent reprogramming of host gene expression by arbuscular mycorrhizal fungal lipopolysaccharides. Plant Physiol 159: 1671–1685.

27. Riquier T, Nevo A, Bonhomme M, Bouton A, Huguet S, et al. (2013) NFP, a LysM receptor protein controlling nod Factor perception, also intervenes in Medicago truncatula resistance to pathogens. New Phytol 198: 875–886.

28. Strégé A, Op den Camp R, Binselting T, Geurts R (2011) Evolutionary origin of the Rhizobium Nod factor signalling. Plant Signal Behav 6: 1319–1324.

29. Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe- or damage-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60: 379–406.

30. Thomma BPHJ, Nürnberg T, Josten MHAJ (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell 23: 4–15.

31. Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, et al. (2007) CERK1, a receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in Arabidopsis. Cell Death Differ 18: 1247–1256.

32. Lefebvre B, Timmers T, Mbengue M, Moreau S, Hervé C, et al. (2010) A rice fungal MAMP-responsive cascade regulates metabolic flow to antimicrobial metabolism. Plant J 63: 599–612.

33. Boller T, Koch C, Delay J, Kuffel M (2009) Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced responses in rice. Plant Physiol 150: 1088–1104.

34. Lohar DP, Sharopova N, Endre G, Penuela S, Samac D, et al. (2006) Transcript analysis of lipoxygenase, antioxidant enzyme activities and H2O2 accumulation during the early stages of Rhizobium-legume symbiosis. New Phytol 151: 91–96.

35. Willmann R, Lajunen HM, Erbs G, Newman MA, Kolb D, et al. (2011) Arabidopsis lysin-motif proteins LYM1 LYM3 and CERK1 mediate bacterial peptidoglycan and chitin perception in rice. Plant Cell 23: 423–439.

36. Liu B, Li JF, Ao Y, Qu J, Li Z, et al. (2012) Lysin motif-containing proteins LYP4 and LYP6 play dual roles in peptidoglycan and chitin perception in rice innate immunity. Plant Cell 24: 3408–3419.

37. Meskiene I, Erbs G, Newman MA, Kolb D, et al. (2011) Arabidopsis lysin-motif proteins LYM1 LYM3 and CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proc Natl Acad Sci U S A 108: 12347–12352.

38. Segonzac C, Friele D, Gimenez-Ibanez S, Hanf DR, Züpfel C, et al. (2011) Hierarchy and roles of pathogen-associated molecular pattern-induced responses in Nicotiana benthamiana. Plant Physiol 156: 687–699.

39. Garcia-Berthou D, Al-Qurainy N, de la Hoz P, de la Hoz E, et al. (2011) Bacterial-elicited polyphenylipids constitute pathogen-associated molecular patterns inducing innate immune responses in Arabidopsis. J Biol Chem 286: 32338–32348.

40. Segonzac C, Friele D, Gimenez-Ibanez S, Hanf DR, Züpfel C, et al. (2011) Bacterial-elicited polyphenylipids constitute pathogen-associated molecular patterns inducing innate immune responses in Arabidopsis. J Biol Chem 286: 32338–32348.

41. Caponcelli B, Horevitz J, van Kan JA, Goldberg RB, Bel JF (1987) Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucl Acid Res 15: 6799–6811.

42. Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

43. Caponcelli B, Horevitz J, van Kan JA, Goldberg RB, Bel JF (1987) Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucl Acid Res 15: 6799–6811.

44. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

45. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

46. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

47. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

48. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

49. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

50. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

51. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

52. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.

53. McKee T, Ohlstein E, Sencar J, Vassilchuk V, Sykes A, Balskus E, et al. (2006) Plant microbe interactions regulate O6-methylguanine-DNA methyltransferase activity. Proc Natl Acad Sci U S A 103: 19823–19828.
79. Murray J, Karas B, Ross I, Brachmann A, Wang C, et al. (2006) Genetic suppressors of the *Lotus japonicus har1–1* hypernodulation phenotype. Mol Plant Microbe Interact 19: 1082–1091.

80. Wang X, Kota U, He K, Blackburn K, Li J, et al. (2008) Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signalling. Cell 15: 220–235.

81. Lemmon MA, Schlessinger J (2010) Cell signalling by receptor tyrosine kinases. Cell 141: 1117–1134.

82. Oh MH, Wang X, Clouse SD, Huber SC (2012) Deactivation of the *Arabidopsis* Brassinosteroid Insensitive 1 (BRI1) receptor kinase by autophosphorylation within the glycine-rich loop. Proc Natl Acad Sci U S A 109: 327–332.

83. Chen L, Hamada S, Fujiwara M, Zhu T, Thao NP, et al. (2010) The Hop/Sti1-Hsp90 chaperone complex facilitates the maturation and transport of a PAMP receptor in rice innate immunity. Cell Host Microbe 7: 185–196.

84. Ke D, Fang Q, Chen C, Zhu H, Chen T, et al. (2012) The small GTPase ROP6 interacts with NFR5 and is involved in nodule formation in *Lotus japonicus*. Plant Physiol 159: 131–143.