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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* (nodulation) 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.

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*) nod factor receptor 1 and 5 (LjNFR1 and LjNFR5) [5–11]. At a later step, LjNFR1, LjNFR5, MtNFP, and an additional *Medicago* gene, LysM domain-containing Receptor-Like Kinase/Root Hair Curling (MtLYK3/MtHICL), are required for rhizobial infection via so-called infection threads through which the bacteria penetrate nodule primordia [9,12–15]. Additionally, MtNFP and MtLYK3 might co-function during nodule development and/or accommodation of rhizobia inside nodule cells [9,16–18]. Recently demonstrated binding of NF derivatives to LjNFR1 and LjNFR5 confirmed their role as NF receptors [19], whereas the exact mechanism of MtNFP and MtLYK3 activation by compatible NFs remains to be shown.
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) [5,20–22]. 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- unidentified 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 RLKs.

LysM-RLKs in non-legume species also govern plant-microbe interactions. An MtNFP/LjNFR5 homolog in Parasponia andersonii, PaNFP, is involved in interactions with Sinorhizobium sp. NGR234 and Rhizophagus irregularis (formerly Glnus intraradices), resulting in nitrogen-fixing and arbuscular mycorrhiza symbiosis, respectively [28]. Arabidopsis thaliana (Arabidopsis) LysM-RLK1/ CERK1 (Chitin Elicitor Receptor Kinase 1) and its ortholog from rice (Oryza sativa), OsCERK1, are essential for microbe-associated molecular pattern (MAMP)-triggered immunity. MAMPs are specific molecules conserved in various classes of microorganisms that activate receptor-mediated defence signalling [29–30]. CERK1-mediated innate immunity to fungal and bacterial pathogens is activated 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 LYM s [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 situ. 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 described essentially as described [42], except that Agrobacterium cultures were grown in LB medium supplemented with 25 µg/mL of rifampicin and 50 µg/mL of kanamycin. Resuspended cells were incubated at room temperature for at least 1 h before being infiltrated into fully expanded leaves of green house-grown plants using needleless syringes. Agrobacterium transformants carrying the respective construct were resuspended in the infiltration medium to desired OD600: all MtNFP and MtNFP[ΔInR]-CYP2 constructs - OD600 = 0.4; all MtLYK3 and AtCERK1 constructs - OD600 = 0.7; MtDMI2-AFP2 - OD600 = 1.0. Then, they were mixed 1:1 with GV3101::pMP90 transformants carrying: pCambia1390 vector with an empty CaMV 35S promoter: positive control for separate expression), a desired MtNFP construct or a desired MtLYK3 construct before being infiltrated into Nicotiana leaves. All experiments included mock infiltration with GV3101::pMP90 transformants carrying pCambia1390 vector with an empty CaMV 35S promoter: negative control (for separate expression), a desired MtNFP construct or a desired MtLYK3 construct before being infiltrated into Nicotiana leaves. All experiments included mock infiltration with GV3101::pMP90 transformants carrying pCambia1390 vector with an empty CaMV 35S promoter: negative control (for separate expression), a desired MtNFP construct or a desired MtLYK3 construct before being infiltrated into Nicotiana leaves. All experiments included mock infiltration with GV3101::pMP90 transformants carrying pCambia1390 vector with an empty CaMV 35S promoter: negative control (for separate expression), a desired MtNFP construct or a desired MtLYK3 construct before being infiltrated into Nicotiana leaves.

To confirm efficient accumulation of MtLLR11-CYP and MtBR11-CYP fusions in Nicotiana, Agrobacterium transformants carrying the respective constructs were co-infiltrated at high optical densities (final OD600 = 0.5) with Agrobacterium transformants carrying either MtNFP-CYPs or MtLYK3-CYP constructs (final OD600 = 0.5). The observed complementation of YFP
fluorescence reported on efficient accumulation, and even unspecific oligomerization of the respective encoded fusions.

**Stereoscopic Analysis**

Blue light-excitable 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 *Mtlyk-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 *Mtnfp* and *Mtlyk* 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-[K464A]-3xFLAG, and MtLYK3-[T480A]-sYFP2 constructs driven by the CaMV 35S promoter. Results were scored as + (CD response to MtNFP and MtLYK3 co-production. 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], *MtLRRII.1* [41], and *Arabidopsis Briosinosteroid Insensitive 1 (BRI1)* [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 incompatable 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 lyase (Lys 349 in a kinase subdomain II). Both AtCERK1 and AtCERK1[K349E] constructs were generated as fusions to the 5' end of the sYFP2 sequence, and their expression in *Nicotiana* leaves was driven by the CaMV 35S promoter.

Notably, heterologous production of AtCERK1-sYFP2 fusion resulted in tissue collapse and desiccation of (nearly) the entire infiltrated region in 20 out of 22 infiltrations 36 hai (Fig. 2A). This CD induction abolished our attempts of precisely characterizing the subcellular localization of AtCERK1 fusion in *Nicotiana* leaf epidermal cells, although we could detect sYFP2 fluorescence at the cell boundary (unpublished data). On the contrary, we observed clear co-localization of AtCERK1[K349E]-sYFP2 fusion with the PM marker using confocal laser-scanning microscopy analysis (Fig. S1). The PM localization of AtCERK1[K349E] fusion in *Nicotiana* leaf epidermal cells is in agreement with the reported subcellular localization of AtCERK1 fluorescence fusion in onion (*Allium cepa*) leaf epidermal cells [31]. Importantly, production of the kinase-inactive variant of AtCERK1 did not result in CD induction, as confirmed with Evans blue staining (Fig. 2B), indicating that biological activity of AtCERK1 in *Nicotiana* leaves was dependent on its kinase activity.

**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.
receptors [53] 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 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 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 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 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 COs and/or PGN [54–56]. We started by analysing the kinetics of CD development. To this end, *Agrobacterium* transformants carrying MtNFP-3xFLAG, MtLYK3-3xFLAG or AtCERK1-3xFLAG fusion were (co-)produced in adjacent circles in 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

### Table 1. Cell death induction upon (co-)expression of various RLK-encoding genes in Nicotiana leaves.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Cell death induction</th>
<th>Co-expression with MtNFP-FP #</th>
<th>Co-expression with MtLYK3-FP #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Separate expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MtNFP-sYFP2</td>
<td>0/12</td>
<td>Not applicable</td>
<td>20/22</td>
</tr>
<tr>
<td>MtNFP–3xFLAG</td>
<td>0/9</td>
<td>Not applicable</td>
<td>12/13*</td>
</tr>
<tr>
<td>MtNFP</td>
<td>0/9</td>
<td>Not applicable</td>
<td>8/9**</td>
</tr>
<tr>
<td>MtLYK3-sYFP2</td>
<td>0/12</td>
<td>20/22</td>
<td>Not applicable</td>
</tr>
<tr>
<td>MtLYK3–3xFLAG</td>
<td>0/9</td>
<td>12/13*</td>
<td>Not applicable</td>
</tr>
<tr>
<td>MtLYK3</td>
<td>0/9</td>
<td>8/9**</td>
<td>Not applicable</td>
</tr>
<tr>
<td>MtDMI2-sYFP2</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>MtLRRII.1-YFP</td>
<td>0/9</td>
<td>0/9***</td>
<td>0/9***</td>
</tr>
<tr>
<td>AtBRI1-YFPc</td>
<td>0/7</td>
<td>0/9***</td>
<td>0/9***</td>
</tr>
</tbody>
</table>

# unless stated differently: with -3xFLAG (*) untagged (***), or -3xFLAG (**) tagged construct. Indicated constructs were expressed alone or co-expressed with either MtNFP or MtLYK3 in 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, production of kinase-inactive AtCERK1[K349E]-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, NbACRE32*, 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-
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 programmes 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-3xFLAG 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 treatment, a microsymbiont of Medicago, on this CD response. To this end, Agrobacterium transforms carrying either MtNFP-3xFLAG 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 Ala (encoded by the Mtlyk3-2 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 L3FR1, respectively in Medicago and in Lotus ([22,21–22], 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 theMtLYK3 InR but differ in their effect on MtLYK3 autoprophosphorylation 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-FP or WT MtLYK3-FP 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-FP or WT MtLYK3-FP construct was done on every leaf. Development of CD was monitored between 36 and 72 hai, and in case of the occurrence of CD, 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 confluent 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[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.

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

*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.

Figure 5. Cell death upon MtNFP and MtLYK3 co-production in Nicotiana leaves does not require SmNF. Agrobacterium transformants carrying either MtNFP-3xFLAG or MtLYK3-3xFLAG construct were co-infiltrated into Nicotiana leaves at a final concentration: OD₆₀₀ [MtNFP] = 0.25 and OD₆₀₀ [MtLYK3] = 0.4 (1); OD₆₀₀ [MtNFP] = 0.15 and OD₆₀₀ [MtLYK3] = 0.25 (2). Twelve hai parts of the transformed regions were syringe-infiltrated with 10⁻⁷ mM SmNF (circled in red) or DMSO diluted to the same concentration (circled in white). Macroscopic observation (left panel) and Evans blue staining (right panel) are depicted 33 hai. Bar is 1 cm.

[Figure 5](#)

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<tbody>
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<td>28/30</td>
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<tr>
<td>S67F (Mtnfp-2)</td>
<td>ER</td>
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<td></td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td>S67A</td>
<td>PM</td>
<td>+</td>
<td>6/9#</td>
<td>0/9</td>
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</tr>
<tr>
<td>ΔInR</td>
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<td>–</td>
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<td>0/9</td>
<td></td>
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<td>–</td>
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<td>0/9</td>
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</tr>
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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.

doi:10.1371/journal.pone.0065055.g005
Table 3. Cell death induction activity of MtLYK3-sYFP2 mutated variants in Nicotiana leaves.

<table>
<thead>
<tr>
<th>MtLYK3-sYFP2 construct</th>
<th>Auto-phosphorylation activity*</th>
<th>Nodulation activity**</th>
<th>Cell death induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>+</td>
<td>+</td>
<td>28/30</td>
</tr>
<tr>
<td>P87S (Mtlyk3-3)</td>
<td>Not applicable</td>
<td>-</td>
<td>15/15</td>
</tr>
<tr>
<td>T319A</td>
<td>-</td>
<td>-</td>
<td>0/11</td>
</tr>
<tr>
<td>G334E (Mtlyk3-1)</td>
<td>-</td>
<td>-</td>
<td>0/20</td>
</tr>
<tr>
<td>K349A</td>
<td>-</td>
<td>-</td>
<td>0/16</td>
</tr>
<tr>
<td>E362A</td>
<td>-</td>
<td>-</td>
<td>0/15</td>
</tr>
<tr>
<td>D441A</td>
<td>-</td>
<td>-</td>
<td>0/16</td>
</tr>
<tr>
<td>T475A</td>
<td>-</td>
<td>-</td>
<td>0/13</td>
</tr>
<tr>
<td>T480A</td>
<td>-</td>
<td>- (0/24)</td>
<td>0/18</td>
</tr>
<tr>
<td>T28A/S286A/T300A</td>
<td>+</td>
<td>Reduced with T300A</td>
<td>15/16</td>
</tr>
<tr>
<td>T433A</td>
<td>+</td>
<td>Reduced</td>
<td>Reduced 7/20</td>
</tr>
<tr>
<td>K464A</td>
<td>Reduced</td>
<td>Reduced 7/18</td>
<td>0/12*</td>
</tr>
<tr>
<td>S471A</td>
<td>+</td>
<td>Reduced</td>
<td>9/11</td>
</tr>
<tr>
<td>T472A</td>
<td>+</td>
<td>Reduced</td>
<td>Reduced 5/11</td>
</tr>
<tr>
<td>T512A</td>
<td>Reduced</td>
<td></td>
<td>Reduced 12/20</td>
</tr>
</tbody>
</table>

* - see [20], except for the T480A (Fig. 52). ** - see [20], except for the P875 [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 Nicotiana spp. by pathogen-derived components [49], or CD induced by (co-)production of certain defence-related proteins, e.g. Pto [58]. Notably, a similar CD response was observed in Nicotiana leaves upon production of AtCERK1 (Fig. 2A), a MAMP receptor. Although CD induction was not demonstrated in any plant species in response to COs or PGN [54–56], it has been observed upon deregulation of various stress/defence-related signalling components [59–60], including a mitogen-activated protein kinase kinase (OsMKK4) implicated in COs-induced signalling in rice [61]. In addition, expression of AtCERK1 from the CaMV 35S promoter (used also in our studies) in Arabidopsis was shown to result in a ligand-independent dimerization of AtCERK1 [38]. We hypothesize that overproduction of this LysM-RLK in Nicotiana analogously leads to its dimerization and enhanced (deregulated) kinase activity, which in turn is required and sufficient for triggering CD; a response not observed upon ligand-induced activation of AtCERK1 in Arabidopsis.

Cell death induction upon MtNFP and MtLYK3 co-production in Nicotiana is in agreement with the Nicotiana [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 a HIN1 gene family was reported previously [32,52,54], linking these genes to MAMP-induced gene regulation mediated by AtCERK1 in Arabidopsis and/or NicCERK1 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⁺] are 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 [33,35,37–39,52–53,71–73]. 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 luteum* interaction. Conversely, our results demonstrate that MtNFP and MtLYK3, when co-produced in *Nicotiana*, are capable of signalling in a similar manner to AtCERK1. We hypothesize that due to the absence of symbiotic-specific “decoders” or “modulators” in *Nicotiana*, MtNFP- and MtLYK3-mediated signalling might be differently interpreted in this species, resulting in the induction of defence-like responses.

Importantly, NF-induced host responses are partially contradictory. On one hand, NFs are postulated to suppress the production in legume roots of salicylic acid and ROS, two potent signals implicated in defence signalling, upon rhizobia perception [63,75]. This differential induction of the symbiotic competence or signals implicated in defence signalling, upon rhizobia production in legume roots of salicylic acid and ROS, two potent\footnote{This differential induction of the symbiotic competence or signals implicated in defence signalling, upon rhizobia perception [63,75].}.

The cell death (CD) induced by NFs in *Medicago* and *Nicotiana* is an important mechanism required for the formation of cortical infection pockets [77]. Interestingly, 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 *Nicotiana* was independent from the NF perception [Fig. 5], and the presence of Pro 87 in the MtLYK3 ExR (Table 3), in contrast to MtNFP 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 MtNFP in *Nicotiana* 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 MtNFP [22], and proteins encoded by MtNFP orthologs in *Lutea* [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 206) were equally dispensable for MtLYK3 biological activity both in *Medicago* and in *Nicotiana* (Table 3). In addition, the K464A and T512A substitutions had a negative effect of MtLYK3 biological activity in both *Nicotiana* 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 *Nicotiana* (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 *Nicotiana* leaf.

Demonstrated significant overlap between structural requirements of nodulation and CD induction regarding the MtNFP and MtLYK3 InRs supports our notion of the relevance of the *Nicotiana* 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, analysing known molecular components/processes involved in the CERK1-mediated signalling [35–38,83] might provide information on the yet- unidentified 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-
Symbiotic LysM-RLKs Induce a Defence Response

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 hai using confocal laser scanning microscopy. From left to right: green fluorescence of sYFP2; orange fluorescence of mCherry; superposition 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.

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 radiolabeled ATP (γ-32P ATP) and phosphorimaging (PI). The coomassie blue staining (CB) shows the protein loading.

Table S1 Primer and linker sequences. (DOC)

Materials and Methods S1. (DOC)

Acknowledgments

We thank Dr. G. Moirieri for providing us with purified Smnod Factor, Dr. A. Streng for providing us with AtCRE1I sequence and for critical reading of the manuscript, and Dr. H. van den Burg for helpful discussions.

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.

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