Evidence for Functional Diversification Within a Fungal NEP1-Like Protein Family

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In this study, we functionally analyzed the gene family encoding necrosis- and ethylene-inducing-like proteins (NLP) of the vascular wilt pathogen *Verticillium dahliae*. We show that the composition of the NLP gene family varies little among *V. dahliae* isolates. The cytotoxic activity of NLP family members of a tomato-pathogenic *V. dahliae* strain was determined, demonstrating that only two of the seven NLP induced plant cell death. The genes encoding these cytotoxic NLP were found to be induced in *V. dahliae* upon colonization of tomato. Interestingly, targeted deletion of either of the two genes in *V. dahliae* significantly compromised virulence on tomato as well as on *Arabidopsis* plants, whereas deletion of only one of the two genes affected virulence on *Nicotiana benthamiana*. This could be attributed to differential induction of the two NLP genes in *V. dahliae* upon *N. benthamiana* colonization, revealing that the in planta induction of NLP genes varies between plant hosts. Intriguingly, one of the NLP genes appears to also affect vegetative growth and conidiospore production, because the corresponding deletion strain produced significantly fewer conidiospores and developed extensive aerial mycelium. In conclusion, we demonstrate that the expanded *V. dahliae* NLP family shows functional diversification, revealing not only differential cytotoxicity between family members but also that the cytotoxic NLP play a role in vegetative growth and asexual reproduction in addition to their contribution to virulence.

Microbial pathogens employ secreted effectors to modulate the physiology of their intended host, often through deregulation of host defenses, in order to establish disease (de Jonge et al. 2011). Whereas particular effectors remain extracellular, others are able to enter the host cell cytoplasm (Dodds and Rathjen 2010). Typically, effectors are species-specific molecules that are often small, cysteine-rich peptides with no homology to previously characterized proteins. However, some effectors seem to be conserved across species (Thomma et al. 2011). An example of such conserved effectors is provided by

the fungal LysM effectors, members of which enhance pathogen virulence by suppression of chitin-triggered immunity in a number of plant pathogens (Bolton and Thomma 2008; de Jonge et al. 2010; Kombrink et al. 2011; Marshall et al. 2011; Mentlak et al. 2012). Another group of conserved effectors are the necrosis- and ethylene-inducing-like proteins (NLP) (Gijzen and Nürnberger 2006).

The first member of the NLP family has been identified as NEP1, found in culture filtrates of the fungus *Fusarium oxysporum* (Bailey 1995). However, it is presently recognized that NLP are widely distributed, because they have been identified in many pathogenic bacteria, fungi, and oomycetes, and especially in this latter group of organisms, the NLP family is significantly expanded (Dong et al. 2012; Gijzen and Nürnberger 2006; Pemberton and Salmond 2004). NLP typically share a conserved NPP1 domain, containing a heptapeptide “GHRH DWE” motif (Fellbrich et al. 2002). NLP generally induce immune responses and cell death in dicotyledonous plants and, based on crystal structure analysis and mutagenesis, it has been proposed that NLP can function as cytolytic toxins that induce plasma membrane leakage, thus causing cytotoxicity (Ottmann et al. 2009; Qutob et al. 2006). Several reports have shown that NLP contribute to pathogen virulence (Mattinen et al. 2004; Pemberton et al. 2005), although targeted deletion of NLP genes in other species did not appear to affect pathogen virulence (Motteram et al. 2009; Staats et al. 2007). Thus, likely, NLP play different roles in different pathogens and on different hosts.

Verticillium dahliae is a soilborne vascular wilt fungus that is characterized by a broad host range of over 200 plant species (Fradin and Thomma 2006). Little is known about *V. dahliae* pathogenicity and, until recently, no effectors of this fungus had been characterized. Recently, the genome sequence of a *V. dahliae* strain has been determined (Klosterman et al. 2011) and, subsequently, the race 1-specific effector that is recognized by the Ve1 immune receptor of tomato was identified through comparative population genomics of race 1 and race 2 strains (de Jonge et al. 2012). This effector, named Ave1, is characterized as a small, secreted protein that is required for full virulence on tomato plants lacking the *Ve1* resistance gene (de Jonge et al. 2012). Interestingly, Ave1 is homologous to a widespread family of plant natriuretic peptides, mobile signaling molecules that are secreted in the apoplast, particularly under conditions of biotic and abiotic stress, and play an important role in the regulation of water and ion homeostasis and, consequently, can affect many downstream processes, including photosynthesis (Gehring and Irvin 2003; Ruzvidzo et al. 2011). Ave1 homologs are found in a

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handful of plant-pathogenic fungi but a role in virulence has not yet been demonstrated for these homologs (de Jonge et al. 2012).

Query of the *V. dahliae* genome revealed that 127 (conserved) hypothetical proteins can be designated as small, cysteine-rich candidate effector proteins. However, no orthologs were found of the well-characterized SIX effectors that have been identified in *F. oxysporum* f. sp. *lycopersici* which, similar to *V. dahliae*, is also a vascular wilt pathogen of tomato (Takken and Rep 2010). Orthologs were only found of effectors that have previously been assigned to large superfamilies, such as the Hce2 family (homologs of *Cladosporium fulvum* effector Ecp2), the LysM effector family (homologs of *C. fulvum* effector Ecp6), and the NLP family (de Jonge and Thomma 2009; de Jonge et al. 2010; Klosterman et al. 2011; Stergiopoulos et al. 2012).

Years ago, the first *V. dahliae* NLP, named VdNEP, was already identified based on sequencing of expressed sequence tags (ESTs) from cultured mycelium of a cotton-pathogenic *V. dahliae* strain (Wang et al. 2004). The protein was found to induce typical defense responses, including cell death, in various plant species. Although a contribution to fungal aggressiveness was not demonstrated, it was suggested that VdNEP acts a wilt-inducing virulence factor (Wang et al. 2004). Interestingly, compared with other ascomycete plant pathogens, the NLP gene family is expanded in the *V. dahliae* genome (Klosterman et al. 2011). Whereas most fungal genomes contain up to three NLP genes (Dallal et al. 2010; Garcia et al. 2007; Motteram et al. 2009; Schouten et al. 2008; Staats et al. 2007), *V. dahliae* was found to have eight NLP gene homologs (Klosterman et al. 2011). It has been speculated that the expansion of the NLP family, similarly reported for the genome of *F. oxysporum*, may contribute to the broad host range among dicotyledonous plant hosts (Klosterman et al. 2011; Ma et al. 2010). In this article, we describe our study of the NLP family of *V. dahliae*. We show that not all of the *V. dahliae* NLP display cytotoxic activity, and investigate the role of the cytotoxic

NLP in fungal virulence. Finally, we provide evidence for functional diversification not only with the *V. dahliae* NLP family but even between the cytotoxic NLP.

RESULTS

Identification of the NLP family in tomato-pathogenic strain JR2.

Recently, the NLP family of the defoliating cotton-pathogenic isolate V592 was reported to contain nine members (Zhou et al. 2012), while it was previously reported that the genome of the lettuce-pathogenic *V. dahliae* VdLs.17 contains eight NLP gene homologs (Klosterman et al. 2011). The difference in NLP gene family size is due to the fact that Zhou and associates (2012) annotate gene VDAG_02984 as an NLP family member, whereas this member was not annotated as an NLP gene by Klosterman and associates (2011). To reevaluate the composition of the NLP family, an HMMER alignment search was performed on the proteome of the *V. dahliae* VdLs.17 with the hidden Markov model (HMM) profile of the NLP-specific NPP1 domain (PF05630). Manual selection resulted in the identification of eight NLP family members, NLP1 to NLP7, and NLP9 (Table 1; Supplementary Figs. S1 and S2). A negative bit score for NLP8 disqualified this protein as a bona fide NLP family member due to insufficient support for the validity of the NPP1 domain, although the *E* value suggests that the sequence is significantly related (Table 1). The degree of homology between the eight NLP members of *V. dahliae* VdLs.17 is rather low (maximum 46.6% identity), and four of the NLP (NLP1, NLP2, NLP3, and NLP6) belong to type I containing two conserved Cys residues, whereas the remaining four (NLP4, NLP5, NLP7, and NLP9) belong to type II that contains four conserved Cys residues. Only NLP1 to NLP5 have an intact heptapeptide “GHRHDWE” motif whereas, in the remaining NLP, the first amino acid of this motif is changed from Gly to Ala (NLP6 and NLP7) or Asn (NLP9), and the sixth amino acid is changed from Trp into Phe (NLP6).

Table 1. Composition of the necrosis- and ethylene-inducing-like protein (NLP) gene family in *Verticillium dahliae*

Gene number ^a	Alias ^b	Bit score ^c	<i>E</i> value ^c	<i>V. dahliae</i>											<i>V. albo-atrum</i>	<i>V. tricorpus</i>
				VdLs.17 ^d	V592 ^e	JR2 ^f	CBS381.66 ^g	DVD-3 ^h	St14.01 ⁱ	DVD-S94 ^j	DVD-S26 ^j	DVD-S29 ^j	DVD-31 ^k	DVD-161 ^l	VaMs.102 ^m	Muc9792 ⁿ
VDAG_04701	NLP1	23.9	9.70E ⁻¹¹	+	+	+	+	+	+	+	+	+	+	+	+	-
VDAG_01995	NLP2	338.9	3.80E ⁻⁹⁸	+	+	+	+	+	+	+	+	+	+	+	+	-
VDAG_06993	NLP3	382.4	2.90E ⁻¹¹¹	+	+	+	+	+	+	+	+	+	+	+	+	+
VDAG_04550	NLP4	161.4	9.80E ⁻⁴⁵	+	+	+	+	+	+	+	+	+	+	+	+	+
VDAG_09117	NLP5	107.1	2.30E ⁻²⁸	+	+	+	+	+	+	+	+	+	+	+	+	-
VDAG_04834	NLP6	52.7	1.20E ⁻¹²	+	+	-	-	-	-	-	-	-	-	-	-	-
VDAG_03497	NLP7	113.1	3.30E ⁻³⁰	+	+	+	+	+	+	+	+	+	+	+	+	+
VDAG_02984	NLP8	-9	1.40E ⁻⁰⁸	+	+	+	+	+	+	+	+	+	+	+	-	+
VDAG_08022	NLP9	64.9	1.10E ⁻¹⁵	+	+	+	+	+	+	+	+	+	+	+	+	-

^a Gene numbers according to the reference sequence of *V. dahliae* VdLs.17 (Klosterman et al. 2011).

^b Naming according to Zhou and associates (2012).

^c Based on HMMER alignment search on *V. dahliae* VdLs.17 proteome with NLP-specific NPP1 domain (PF05630).

^d Lettuce-pathogenic isolate (Bhat and Subbarao, 1996).

^e Defoliating cotton-pathogenic isolate (Zhou et al. 2012).

^f Tomato-pathogenic isolate (Fradin et al. 2009).

^g Tomato-pathogenic isolate (Center for Biological Sequence Analysis, Utrecht, The Netherlands).

^h Potato-pathogenic isolate.

ⁱ Pistachio-pathogenic isolate.

^j Soil isolate.

^k Tomato-pathogenic isolate.

^l Potato-pathogenic isolate.

^m Alfalfa-pathogenic isolate (Bhat and Subbarao 1999).

ⁿ Tomato-pathogenic isolate (Lievens et al. 2003).

We subsequently queried the genome sequence of the tomato-pathogenic strain JR2 (de Jonge et al. 2012) for its *NLP* gene homologs. This analysis revealed seven *NLP* genes, designated *NLP1* to *NLP5*, *NLP7*, and *NLP9* (Table 1), whereas a homolog of *NLP6* that is identified in strain VdLs.17 is not present in strain JR2. To further investigate the distribution of *NLP* gene family members in the *Verticillium* genus, we queried the genomes of nine additional *V. dahliae* strains (de Jonge et al. 2012), as well as a *V. tricorpus* strain and a *V. albo-atrum* VaMs.102 (Klosterman et al. 2011). To this end, the DNA sequences of the *V. dahliae* strains were mapped to the *V. dahliae* VdLs.17 reference genome. The mapping analysis showed that the genomic regions harboring *NLP1* to *NLP5* and *NLP7* to *NLP9* are covered by DNA sequences from the other *V. dahliae* strains, indicating the presence of these genes. However, similar to the JR2 strain, the genomic region harboring *NLP6* was not found in any of the other *V. dahliae* strains. *NLP6* was similarly lacking in *V. albo-atrum* VaMs.102 (Klosterman et al. 2011), while *NLP8* is also lacking in this strain (Table 1). Interestingly, the *V. tricorpus* genome contains homologs of *NLP3*, *NLP4*, *NLP7*, and *NLP8* only.

Only *NLP1* and *NLP2* display cytotoxic activity.

Various reports have shown that *NLP* trigger necrosis upon infiltration into leaves of dicotyledonous plants. To test the necrosis-inducing activity of the *NLP* encoded by the tomato-pathogenic JR2 strain, expression of the corresponding cDNAs in *N. benthamiana* by agroinfiltration was pursued. To this end, the coding sequences of the *NLP* genes were cloned into the Gateway-compatible vector *pFAST-R02* (Shimada et al. 2010) to generate expression constructs driven by the CaMV 35S promoter, and transformed into *Agrobacterium tumefaciens* GV3101. Subsequently, the transgenic *A. tumefaciens* strains were infiltrated into the leaves of *Nicotiana benthamiana* plants. Leaf tissue started to collapse at 1 to 2 days post agroinfiltration of *NLP1* and *NLP2*, and the infiltrated leaves developed clear necrosis by 3 days post agroinfiltration (Fig. 1A). In contrast, no tissue collapse or necrosis could be observed upon agroinfiltration of any of the other *NLP* genes. Western analysis performed on total protein extracts of the

Fig. 1. Only expression of *Verticillium dahliae* necrosis- and ethylene-inducing-like protein (*NLP1*) and *NLP2* induces cell death in *Nicotiana benthamiana*. **A**, Agroinfiltration of constructs for constitutive expression of *V. dahliae* *NLP* genes reveals necrosis at the injection sites for *NLP1* and *NLP2* but none of the other *NLP* genes. Pictures were taken at 3 days after agroinfiltration. **B**, Western blot analysis on total protein extracts of *N. benthamiana* upon agroinfiltration of constructs for constitutive expression of *V. dahliae* *NLP* genes that did not induce necrosis in A.

agroinfiltrated leaf area confirmed the presence of the proteins that did not induce necrosis in planta (Fig. 1B).

To confirm the differential cytotoxic activity, the *NLP* were also produced in the yeast *Pichia pastoris* and purified. Whereas production of *NLP6* and *NLP9* failed, sufficient amounts of protein were obtained from the other *NLP* and infiltrated abaxially into leaves of *N. tabacum* plants. Also in this experiment, only infiltration of *NLP1* and *NLP2* resulted in tissue necrosis, whereas infiltration of *NLP3* to *NLP5* and *NLP7* did not trigger necrosis (Supplementary Fig. S3). Collectively, these data show that only *NLP1* and *NLP2* display cytotoxic activity, whereas the remaining *V. dahliae* *NLP* do not.

NLP1 and *NLP2* are expressed in planta but not under control of VdSge1.

It has previously been shown for one of the cytotoxic *NLP* from *V. dahliae* that it has the potential to induce necrosis and wilting in planta; however, a role in virulence on plant hosts has not been demonstrated thus far for any of its cytotoxic *NLP* (Wang et al. 2004). To address such a role, it was assessed whether both cytotoxic *NLP* are expressed during host colonization. To this end, *V. dahliae* JR2 was root inoculated on susceptible plants of 'MoneyMaker' tomato, and complete plants were harvested at regular intervals up to 16 days postinoculation (dpi). After RNA isolation and cDNA synthesis, real-time polymerase chain reaction (PCR) was performed to assess *NLP1* and *NLP2* transcript levels that were calibrated using transcript levels of the elongation factor 1- α gene as a refer-

Fig. 2. Necrosis- and ethylene-inducing-like protein (*NLP1*) and *NLP2* expression during infection of *Verticillium dahliae* on tomato and *Nicotiana benthamiana*. Plants of 10-day-old tomato (*Solanum lycopersicum*) 'MoneyMaker' (top graph) or 15-day-old *N. benthamiana* (bottom graph), were root inoculated with wild-type *V. dahliae* and harvested at regular intervals from 4 to 16 days postinoculation (dpi). After RNA isolation and cDNA synthesis, real-time polymerase chain reaction was performed to determine the relative expression levels of *NLP1* (white bars) and *NLP2* (gray bars) using the *V. dahliae* elongation factor 1- α gene as a reference, and compared with expression upon *V. dahliae* growth in vitro in Murashige-Skoog medium. *NLP1* and *NLP2* expression levels are compared with expression of *NLP1* in vitro, which is set to 1. No *NLP2* expression was detected in *N. benthamiana*.

ence. This analysis demonstrated that both *NLP1* and *NLP2* are induced in planta, with a similar expression pattern that peaks approximately 12 dpi (Fig. 2). By this point in time, major symptoms of disease, including the onset of necrosis and significant stunting, become apparent in susceptible tomato plants.

We recently demonstrated that *V. dahliae* *Sge1* (*VdSge1*) is required for growth and development as well as for pathogenicity on tomato (Santhanam and Thomma 2013). In contrast to *F. oxysporum* *Sge1*, we found that *VdSge1* is not a general regulator of effector gene expression, because some candidate effector genes were negatively regulated by *VdSge1*, some positively, and others not (Santhanam and Thomma 2013). Because the *VdSge1* deletion mutant is not pathogenic on tomato, assessment of *NLP1* and *NLP2* gene expression was assessed in a tomato cell suspension culture (Santhanam and Thomma 2013). To this end, a suspension of MSK8 tomato cells was inoculated with conidia of a *VdSge1* deletion strain and the cor-

responding wild type and, after 96 h, the cells were harvested and examined for *NLP1* and *NLP2* expression with real-time PCR. Expression of *NLP1* and *NLP2* was not abolished in the *VdSge1* deletion strain; therefore, we conclude that the expression of the genes encoding cytotoxic NLP is not governed by *VdSge1* (Supplementary Fig. S4).

NLP1 affects vegetative growth and conidiospore production.

Because both *NLP1* and *NLP2* were found to be expressed in *V. dahliae* during colonization of tomato plants, targeted deletion was pursued by replacement of the coding sequence of each of the genes by a hygromycin resistance cassette through homologous recombination. Deletion of the coding sequences was subsequently verified by PCR. Several independent deletion mutants were obtained for each of the genes, of which two were used for further analysis in this study (Supplementary Fig. S5). To assess the role of the cytotoxic NLP on growth and development, various growth characteristics were assessed. Radial growth of the *NLP1* and *NLP2* deletion strains was similar to that of the wild-type strain and of the ectopic transformants (Fig. 3A). However, when conidiospores were isolated from 1-week-old cultures, *NLP1* deletion strains appeared to have developed approximately 40% less conidiospores than *NLP2* deletion strains, the wild-type strain, and ectopic transformants (Fig. 3B). Nevertheless, germination of the conidiospores was similar for all strains, including the *NLP1* deletion strains (Fig. 3C). The reduced conidiospore production of the *NLP1* deletion strains

Fig. 3. Targeted deletion of necrosis- and ethylene-inducing-like protein (*NLP1*) affects conidiospore production. **A**, Radial growth and colony morphology of wild-type *Verticillium dahliae* (WT), two *NLP1* deletion strains (*ΔNLP1-1* and *ΔNLP1-2*), two *NLP2* deletion strains (*ΔNLP2-1* and *ΔNLP2-2*), and ectopic transformants (EC1 and EC2) after 7 days of incubation on PDA medium at 22°C. **B**, Average number of conidia produced after 7 days of growth on potato dextrose agar medium based on two independent experiments. Different letters indicate significant differences at $P < 0.05$ as calculated with Student's *t* test. **C**, Microscopic observation of the germination of conidia at 16 h after incubation in Czapek-Dox medium.

Fig. 4. Targeted deletion of necrosis- and ethylene-inducing-like protein (*NLP1*) induces formation of aerial hyphae. **A**, Morphological appearance of wild-type *Verticillium dahliae* (WT) and an *NLP1* deletion strain (*ΔNLP1-1*) after 14 days of incubation on potato dextrose agar (PDA) medium at 22°C. **B**, WT *V. dahliae* and an *NLP1* deletion strain (*ΔNLP1-1*) after 21 days of incubation on PDA medium at 22°C. Top pictures show side views of petri dishes with fungal strains on PDA medium, showing aerial hyphae for the *NLP1* deletion strain. Middle and lower pictures show magnification of fungal colonies.

was overcome by complementation with a genomic construct containing the wild-type *NLP1* gene, demonstrating that the reduced conidiospore production is a genuine phenotype of *NLP1* deletion (Supplementary Fig. S6B). When the fungal cultures got older, *NLP1* deletion strains became morphologically distinct. Whereas the color of the wild-type strain, *NLP2* deletion strains, and ectopic transformants became gray, *NLP1* deletion strains were white (Fig. 4A). Closer inspection revealed that the white appearance was due to extensive formation of aerial hyphae that covered the fungal colonies of the *NLP1* deletion strains on the potato dextrose agar (PDA) plates (Fig. 4B). Also, this phenotype was overcome by complementation of the deletion strain with a genomic construct encoding the wild-type *NLP1* gene. All other fungal strains did not show the extensive formation of these aerial hyphae (Supplementary Fig. S7). In support for a role in absence of host colonization during growth, we found that NLP are expressed in vitro, because 228 reads belonging to this *NLP* gene family member were retrieved in the RNAseq data of *V. dahliae* grown in Czapek-Dox medium (de Jonge et al. 2012). Collectively, these data support a role for *NLP1* in vegetative growth and asexual reproduction.

NLP1 and *NLP2* are required for virulence.

To assess the role of *NLP1* and *NLP2* in pathogenicity on tomato plants, the deletion strains were root inoculated on sus-

ceptible MoneyMaker tomato plants along with the wild-type strain and ectopic transformants. Interestingly, whereas ectopic transformants were found to be as virulent as the wild-type strain, *NLP1* as well as *NLP2* deletion strains were found to be significantly less pathogenic (Fig. 5). The plants that were inoculated with the *NLP1* and *NLP2* deletion strains still developed Verticillium wilt symptoms but were less stunted and showed less typical disease symptoms (e.g., darkening of the leaves) than plants that were inoculated with ectopic transformants or the wild-type strain (Fig. 5). Intriguingly, deletion of *NLP1* seemed to have a stronger effect on virulence than deletion of *NLP2*, because plants inoculated with *NLP1* deletion strains remained taller and showed fewer symptoms of infection than plants inoculated with *NLP2* deletion strains (Fig. 5). This was confirmed upon measuring the surface area of the foliage of the plants inoculated with the various fungal genotypes, because plants that were inoculated with *NLP1* deletion strains developed as much foliage as mock-inoculated plants, whereas the amount of foliage developed by plants that were inoculated with *NLP2* deletion strains was significantly reduced (Fig. 6A). Nevertheless, plants that were inoculated with *NLP2* deletion strains developed significantly more foliage than those inoculated with either the wild-type strain or ectopic transformants (Fig. 6A). Fungal outgrowth assays upon plating of stem sections harvested from the hypocotyls of the inoculated plants demonstrated that *NLP1* and *NLP2* deletion strains were still able to colonize tomato plants (Fig. 5). However, real-time PCR quantification of fungal biomass demonstrated

Fig. 5. Necrosis- and ethylene-inducing-like protein (*NLP1* and *NLP2*) are required for virulence of *Verticillium dahliae* on tomato. Pathogenicity assay to investigate the role of *NLP1* (top panel) and *NLP2* (bottom panel) in *V. dahliae* virulence. Side and top view of 'MoneyMaker' tomato plants that were mock inoculated (mock) or inoculated with wild-type *V. dahliae* (WT), two *NLP1* deletion strains ($\Delta NLP1-1$ and $\Delta NLP1-2$), two *NLP2* deletion strains ($\Delta NLP2-1$ and $\Delta NLP2-2$), and corresponding ectopic transformants (EC1 and EC2) at 14 days postinoculation are shown. Fungal outgrowth at 7 days after plating of stem sections harvested at 14 days postinoculation are shown at the bottom of each panel.

Fig. 6. Necrosis- and ethylene-inducing-like protein (*NLP1* and *NLP2*) are required for virulence of *Verticillium dahliae* on tomato. **A**, Average canopy area of 6 'MoneyMaker' tomato plants at 14 days after mock inoculation (mock) or inoculation with wild-type *V. dahliae* (WT), two *NLP1* deletion strains ($\Delta NLP1-1$ and $\Delta NLP1-2$), two *NLP2* deletion strains ($\Delta NLP2-1$ and $\Delta NLP2-2$), and corresponding ectopic transformants (EC1 and EC2). **B**, Real-time polymerase chain reaction quantification of fungal biomass. Different letter labels indicate significant differences ($P < 0.05$).

that *NLP1* and *NLP* deletion strains developed significantly less fungal biomass in planta than the ectopic transformants and the wild-type strain (Fig. 6B).

To investigate whether the observed role of *NLP1* and *NLP2* in pathogenicity is confined only to tomato or also concerns other host species, we tested the pathogenicity of the *NLP1* and *NLP2* deletion strains on the Brassicaceous and Solanaceous model plants *Arabidopsis thaliana* and *N. benthamiana*, respectively. Similar to tomato, targeted deletion of *NLP1* as well as of *NLP2* resulted in markedly compromised virulence on *Arabidopsis* plants, because they were clearly less stunted (Fig. 7A). In contrast, a differential contribution of *NLP1* and *NLP2* to *V. dahliae* virulence was monitored on *N. benthamiana*. Whereas targeted deletion of *NLP1* resulted in markedly compromised virulence on *N. benthamiana*, targeted deletion of *NLP2* did not result in significantly compromised virulence on this host plant (Fig. 7B). This was confirmed not only by measurement of the surface area of the foliage of the plants inoculated with the various fungal genotypes but also by real-time PCR quantification of fungal biomass (Fig. 8). Considering the observation that *NLP2* displays cytotoxic activity also on *N. benthamiana* (Fig. 1), the observation that *NLP2* deletion did not result in significantly compromised virulence on this host plant prompted the question of whether this gene is expressed during *N. benthamiana* colonization. Surprisingly, whereas *NLP1* is expressed during colonization of *N. benthamiana* by *V. dahliae* in a pattern similar to that observed on tomato (Fig. 2), no expression of *NLP2* could be detected at all

Fig. 7. Requirement of necrosis- and ethylene-inducing-like protein (*NLP*)1 and *NLP2* for virulence of *Verticillium dahliae* on *Arabidopsis* and *Nicotiana benthamiana*. **A**, Top view of *Arabidopsis* plants of the Col-0 ecotype and **B**, top and side view of *N. benthamiana* plants that were mock inoculated (mock) or inoculated with wild-type *V. dahliae* (WT), two *NLP1* deletion strains ($\Delta NLP1-1$ and $\Delta NLP1-2$), two *NLP2* deletion strains ($\Delta NLP2-1$ and $\Delta NLP2-2$), and corresponding ectopic transformants (EC1 and EC2) at 21 days postinoculation (dpi) (*Arabidopsis*) and 14 dpi (*N. benthamiana*) are shown.

in this plant species. Nevertheless, overall, it can be concluded that *NLP1* and *NLP2* are required for virulence on various host plants.

DISCUSSION

In this study, we provide evidence for significant functional diversification within the NLP family of a fungal pathogen. We have shown that targeted deletion from the *V. dahliae* genome of *NLP1*, encoding a cytotoxic NLP that contributes to virulence on various host plants, directly affects growth and conidiospore production in vitro. Because this finding was highly unexpected, we carefully checked the veracity of our findings. To generate the *NLP1* deletion construct, genomic sequences flanking the *NLP1* coding sequence were used; more specifically, a 1.4-kb upstream and 250-bp downstream sequence. Approximately 2.2 kb upstream of *NLP1* lies a gene encoding a vegetative cell wall protein (VDAG_04700). Although this gene lies well outside the range of sequence that was used to generate the construct for targeted deletion of *NLP1* by homologous recombination, we confirmed that expression of this gene was not affected in the *NLP1* deletion strains when compared with the wild-type *V. dahliae* upon growth in vitro. No genes other than *NLP1* were annotated in the region that was used for homologous recombination. Furthermore, mapping of sequence reads obtained with RNAseq from a time course of *V. dahliae*-infected *N. benthamiana* resulted in the identification of reads for over 8,000 *V. dahliae* genes (de Jonge et al. 2012) but no reads were mapped in the range of sequence that was used to generate the construct for homologous recombination other than those belonging to *NLP1*. Furthermore, the in vitro

Fig. 8. Necrosis- and ethylene-inducing-like protein (*NLP*)1 but not *NLP2* is required for virulence of *Verticillium dahliae* on *Nicotiana benthamiana*. **A**, Average canopy area of six *N. benthamiana* plants at 14 days after mock inoculation (mock) or inoculation with wild-type *V. dahliae* (WT), two *NLP1* deletion strains ($\Delta NLP1-1$ and $\Delta NLP1-2$), two *NLP2* deletion strains ($\Delta NLP2-1$ and $\Delta NLP2-2$), and corresponding ectopic transformants (EC1 and EC2). **B**, Real-time polymerase chain reaction quantification of fungal biomass. Different letter labels indicate significant differences ($P < 0.05$).

growth and conidiation phenotype was overcome by complementation of the *NLP1* deletion strain with a genomic construct containing the wild-type *NLP1* gene. Finally, coinciding with a role in vitro in the absence of the host, our data show that *NLP1* is expressed in *V. dahliae* during growth in vitro. Thus, we conclude that *NLP1* unambiguously affects in vitro growth and conidiation and, with this observation, a novel function for a cytotoxic NLP outside the host plant is revealed.

The observed role for a cytotoxic NLP outside the plant and pathogen virulence was unexpected, and obviously leads to the question of how such a secreted effector protein affects growth and conidiation. In this respect, it is interesting to note that analysis of the crystal structure of a cytotoxic NLP from the phytopathogenic oomycete *Pythium aphanidermatum* revealed structural homology not only to actinoporins, a family of pore-forming toxins produced by sea anemones, but also to particular fungal lectins (Ottmann et al. 2009). All these proteins are small polypeptides containing a central β -sandwich architecture surrounded by helices, and both the actinoporins and lectins are soluble proteins that target cellular surfaces via a surface-exposed cavity (Birck et al. 2004; Ottmann et al. 2009). One of these lectins, XCL from *Xerocomus chrysenteron*, is an insecticidal protein that binds to *N*-acetyl galactosamine at the cell surface of target cells, after which it is internalized and changes the actin cytoskeleton (Birck et al. 2004). It is tempting to speculate that *NLP1* will bind to particular fungal cell wall carbohydrates, after which internalization occurs to influence developmental processes.

Further evidence for functional diversification within the *V. dahliae* NLP family comes from the identification of non-cytotoxic family members. Most fungal genomes contain up to three *NLP* genes (Dallal et al. 2010; Garcia et al. 2007; Motteram et al. 2009; Schouten et al. 2008; Staats et al. 2007). Nevertheless, although the *V. dahliae* NLP family is significantly expanded, this fungus also contains only two genes that encode cytotoxic NLP. Possibly, the noncytotoxic NLP contribute to pathogen virulence through mechanisms other than inducing cellular leakage. However, our data for *NLP1* suggest that diversification toward functions other than virulence may be likely as well. Thus far, noncytotoxic NLP have only been described for oomycete species that are generally characterized by significantly expanded NLP families, containing up to 70 potential *NLP* genes (Dong et al. 2012). Whereas some oomycetes contain cytotoxic and noncytotoxic NLP (Dong et al. 2012; Kanneganti et al. 2006), others contain only noncytotoxic NLP, such as the obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (Cabral et al. 2012). The finding that the noncytotoxic *H. arabidopsidis* NLP are expressed during early stages of infection has led to the hypothesis that these NLP still exert functions related to host colonization (Cabral et al. 2012). However, also for *H. arabidopsidis* NLP, alternative roles that are not related to virulence may occur as well. A role in growth and development outside the host plant may, at the same time, explain why *NLP* genes also occur in nonpathogenic microorganisms.

Our study confirms the findings of recent study on a virulent cotton-infecting *V. dahliae* isolate, for which nine potential NLP were identified, of which two displayed cytotoxic activity (Zhou et al. 2012). However, in contrast to our findings, targeted deletion of the genes encoding the cytotoxic NLP did not affect *V. dahliae* virulence on cotton (Zhou et al. 2012). In our study, we found that targeted deletion of *NLP1* as well as of *NLP2* significantly compromised *V. dahliae* virulence on tomato and *Arabidopsis*, whereas deletion of *NLP1* but not of *NLP2* compromised virulence on *N. benthamiana*. However, the differential role of *NLP2* we observed in virulence on different hosts could be attributed to the observation that *NLP2* is

not expressed during infection on *N. benthamiana*. This is not the case for the study on cotton because, despite the absence of a virulence phenotype for the corresponding deletion strains, *NLP1* as well as *NLP2* were found to be expressed during infection (Zhou et al. 2012). Moreover, targeted deletion of both genes simultaneously did not affect fungal virulence, suggesting that the role of the cytotoxic NLP in infection of cotton is dispensable and further supporting the differential contribution of cytotoxic NLP to virulence of *V. dahliae*. It is not surprising that a broad-host-range pathogen such as *V. dahliae* that is able to infect hundreds of hosts will express effectors that do not contribute to virulence on all of its hosts.

In conclusion, our data demonstrate extensive functional diversification within the expanded NLP family of the fungal plant pathogen *V. dahliae*. We have demonstrated differential cytotoxicity among the family members, a differential contribution to virulence of the cytotoxic family members, and, finally, also a differential effect of the cytotoxic family members outside the host plant on growth and conidiospore production. Future efforts will be devoted to investigate further functional diversification with the group of noncytotoxic *V. dahliae* NLP.

MATERIALS AND METHODS

Identification of *Verticillium* NLP family members.

The HMM profile of the NPP1 domain (PF05630) was retrieved from the Pfam database and an HMMER alignment search was performed against the *V. dahliae* VdLs.17 proteome using default parameters. The resulting hits were manually selected for positive bit scores and *E* values < 1e-10. Presence of signal peptides was confirmed using SignalP3.0 with default parameters. Homologous *NLP* genes were identified in other *V. dahliae* strains by mapping the Illumina DNA sequence reads of these strains (de Jonge et al. 2012) with the gsnap/gmap software to the reference genome of *V. dahliae* VdLs.17 using default settings (Wu and Nacu 2010; Wu and Watanabe 2005). Similarly, *NLP* homologs were identified using Illumina DNA sequence reads of *V. tricorpus* MUCL9792 (L. Faino and B. P. H. J. Thomma, unpublished data), and reciprocal BLAST searches to the *V. dahliae* VdLs.17 genome were used to confirm the identities of the *NLP* homologs.

Phylogenetic analyses.

Phylogenetic analyses of the *V. dahliae* NLP family was conducted using the Phylogeny.fr web-service. Sequences were aligned with MUSCLE and curated with Gblocks. The phylogenetic tree was reconstructed using the PhyML program (maximum likelihood method) and statistical reliability was tested using bootstrap with 500 replications.

Fungal transformations.

To generate *NLP1* and *NLP2* deletion constructs, flanking sequences of the *NLP1* and *NLP2* coding sequences were amplified from genomic DNA of *V. dahliae* JR2 using the primers mentioned in Supplementary Table S1. The amplified products were cloned into pRF-HU2 as described previously (Frandsen et al. 2008).

To generate the *NLP1* complementation construct, a 1,871-bp *EcoRI*/*PacI* fragment containing the *NLP1* coding sequence with a 998-bp upstream and 170-bp downstream sequence was amplified from *V. dahliae* JR2 genomic DNA, and cloned into binary vector pBT081 (Houterman et al. 2008).

Agrobacterium tumefaciens-mediated transformation of *V. dahliae* was performed as described previously (Santhanam 2012), and transformants were selected on PDA supplemented with cefotaxime (Duchefa, Haarlem, The Netherlands) at 200

µg/ml and hygromycin (Duchefa) at 50 µg/ml. Homologous recombination was verified by PCR. Complementation transformants were selected on PDA supplemented with cefotaxime at 200 µg/ml and phleomycin at 100 µg/ml (InvivoGen, San Diego, CA, U.S.A.).

Pathogenicity assays.

Pathogenicity assays were performed on 10-day-old tomato seedlings (MoneyMaker), 2-week-old *Arabidopsis thaliana* (Col-0), and 2-week-old *N. benthamiana* plants using root-dip inoculation as previously described (Fradin et al. 2009). Disease symptoms were scored up to 14 dpi (tomato and *N. benthamiana*) or 21 dpi (*Arabidopsis*), pictures were taken, and ImageJ was used to determine the canopy area. To determine in planta colonization, stem sections at the height of the first internode were taken, surface sterilized, sliced, placed on PDA supplemented with chloramphenicol at 50 µg/ml, and incubated at 22°C. Fungal outgrowth was monitored after 7 days. For in planta biomass quantification, roots of three inoculated plants were harvested at 14 dpi. The samples were ground to powder and genomic DNA was isolated. Real-time PCR on genomic DNA was carried out using an ABI7300 PCR machine (Applied Biosystems, Foster City, CA, U.S.A.) in combination with quantitative PCR core kit for SYBR Green I (Eurogentec, Seraing, Belgium) and analyzed using the 7300 SDS software (Applied Biosystems). *Verticillium* elongation factor 1-α was used to quantify fungal colonization. Tomato, *Arabidopsis*, and *N. benthamiana* actin primers were used as endogenous plant control.

Growth, conidiogenesis, and germination assays.

Radial growth was monitored by placing a 2-µl droplet of a conidial suspension of 10⁶ conidia/ml in the center of a PDA plate and incubating it at 22°C. For quantification of conidia production, 5 ml of water was added to the culture and conidial suspensions were prepared. Ten-times diluted conidial suspensions were counted in a hemocytometer. To determine the germination of conidia, 10⁶ conidia/ml were inoculated in Czapek-Dox medium at 22°C for 16 h and observed under a microscope.

Cytotoxic activity determination.

For transient expression of *NLP* genes in *N. benthamiana*, the full-length coding sequences containing a 3' FLAG tag were amplified and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, U.S.A.). After sequence verification, the inserts were subcloned in the destination vector pFAST-R02 (Shimada et al 2010) and transformed to *Agrobacterium tumefaciens* GV3101 by electroporation. *A. tumefaciens* strains were subsequently grown in yeast extract broth at 28°C overnight. The overnight cultures were diluted to an optical density at 600 nm of 0.5 with MMA (10 mM MES, 10 mM MgCl₂, 100 µM acetosyringone, pH 5.7) containing acetosyringone at 1 ml/liter and incubated at room temperature for 3 to 4 h. The cultures were infiltrated on the abaxial side of the youngest, fully expanded leaves of 4-week-old *N. benthamiana*.

To verify protein production, leaves without midveins were harvested at 3 dpi and flash frozen in liquid nitrogen. Total proteins were extracted using P-PER Plant Protein Extraction Kit (Pierce Biotechnology, Rockford, IL, U.S.A.) following the manufacturer's instructions. The proteins were separated using 15% sodium dodecyl sulfate polyacrylamide electrophoresis gels. Immunoblotting was performed using ANTI-FLAG M2 antibody and the proteins were visualized by enhanced chemiluminescence (Pierce Biotechnology).

NLP production in *P. pastoris* was performed essentially as described previously (Ottmann et al. 2009).

Gene expression analysis.

To determine the in planta expression of *NLP1* and *NLP2*, 10-day-old tomato seedlings and 2-week-old *N. benthamiana* plantlets were root inoculated with *V. dahliae*. Whole plants were harvested at 4, 8, 12, and 16 dpi and flash frozen in liquid nitrogen. After grinding, 100 mg of ground material was used for total RNA extraction (Qiagen, Venlo, The Netherlands) and cDNA synthesis (Invitrogen). Real-time PCR was performed to determine *NLP1* and *NLP2* expression. *Verticillium* elongation factor 1-α was used as an endogenous control and reactions were performed in triplicate. Real-time PCR conditions consisted of an initial 95°C denaturation step for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute *Verticillium* group database:
www.broadinstitute.org/annotation/genome/verticillium_dahliae
 Center for Biological Sequence Analysis SignalP3.0 database:
www.cbs.dtu.dk/services/SignalP-3.0
 HMMER alignment search database: hmmer.janelia.org
 Sanger Wellcome Trust Pfam database: pfam.sanger.ac.uk