Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in *Arabidopsis*

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Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are secreted by a wide range of plant-associated microorganisms. They are best known for their cytotoxicity in dicot plants that leads to the induction of rapid tissue necrosis and plant immune responses. The biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* encodes 10 different noncytotoxic NLPs (HaNLPs) that do not cause necrosis. We discovered that these noncytotoxic NLPs, however, act as potent activators of the plant immune system in *Arabidopsis thaliana*. Ectopic expression of *HaNLP3* in *Arabidopsis* triggered resistance to *H. arabidopsidis*, activated the expression of a large set of defense-related genes, and caused a reduction of plant growth that is typically associated with strongly enhanced immunity. N- and C-terminal deletions of HaNLP3, as well as amino acid substitutions, pinpointed to a small central region of the protein that is required to trigger immunity, indicating the protein acts as a microbe-associated molecular pattern (MAMP). This was confirmed in experiments with a synthetic peptide of 24 aa, derived from the central part of HaNLP3 and corresponding to a conserved region in type 1 NLPs that induces ethylene production, a well-known MAMP response. Strikingly, corresponding 24-aa peptides of fungal and bacterial type 1 NLPs were also able to trigger immunity in *Arabidopsis*. The widespread phylogenetic distribution of type 1 NLPs makes this protein family (to our knowledge) the first proteinaceous MAMP identified in three different kingdoms of life.

plant immunity | microbe-associated molecular pattern | Nep1-like protein | plant-associated microbe | biotrophic pathogen

Immune responses in plants generally start by receptor-mediated detection of nonself molecules that are conserved among different classes of microbes, both beneficial and pathogenic (1). These molecules often have essential functions in microbial fitness (2) and are known as microbe-associated molecular patterns (MAMPs). Upon their perception by the plant, MAMPs trigger basal immune responses (3), e.g., ethylene biosynthesis, production of reactive oxygen species, release of antimicrobial compounds (4), and in certain cases programmed cell death (2). Collectively, these responses contribute to resistance against nonadapted pathogens [MAMP-triggered immunity (MTI)].

MAMPs of plant-infecting microbes have been described for bacteria, fungi, and oomycetes. Three characterized bacterial MAMPs are flagellin (5), EF-Tu (6), and peptidoglycan (7). Flagellin is the main protein of the bacterial flagellum, which is used by eubacteria for movement. A highly conserved fragment of 22 aa, named flg22 (5), is sufficient to activate MTI in *Arabidopsis* and other plant species. Elongation factor thermo unstable (EF-Tu) is an abundant and conserved bacterial protein that plays a central role in the elongation phase of protein synthesis. An 18-aa domain of EF-Tu, named elf18, is recognized as a MAMP in *Brassicaceae* species, but not in other tested plant families (6). Peptidoglycan (PGN), the third characterized bacterial MAMP, is a major structural component of most bacterial cell walls. PGN, consisting of strands of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, triggers immunity in *Arabidopsis* (7). An important fungal MAMP is chitin, a structural component of all fungal cell walls. Plants are able to recognize chitin, and fragments of 4–10 *N*-acetylglucosamine residues are the most potent inducers of defense (8). Recently, a second fungal MAMP was identified, a secreted polygalacturonase of *Botrytis cinerea* that triggers immunity in *Arabidopsis* (9).

Four oomycete-derived MAMPs have been identified to date (10): (*i*) heptaglucoside fragments, originating from branched β-glucans that are major cell wall polysaccharides, and that trigger defense responses in many Fabaceous plants (11); (*ii*) glycoprotein 42, a calcium-dependent transglutaminase that functions in irreversible protein cross-linking and is abundant in *Phytophthora* cell walls, and a 13-aa peptide fragment thereof that elicit MTI responses in parsley (12) and potato (13); (*iii*) elicitins, secreted proteins with sterol-binding activity (14), which provoke necrosis in *Nicotiana* plants through induction of cell death (15); and (*iv*) the *Phytophthora* cellulose-binding elicitor lectin, which is thought to cause perturbation of the cell wall cellulose status, thereby triggering necrosis and MTI in tobacco and *Arabidopsis* (16, 17). Other groups of cell death-inducing proteins may also qualify as MAMPs based on their widespread occurrence among different pathogens (2), e.g., the Crinklers and the cytotoxic necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) (10).

Two major NLP types are found in bacteria, fungi, and oomycetes (18, 19) and are known to cause rapid necrosis and ethylene production in many dicot, but not in monocot plant

Significance

Peptide fragments of Nep1-like proteins (NLPs), occurring in diverse microorganisms of three different kingdoms of life, were found to trigger immunity in the model plant *Arabidopsis*, indicating that they act as a microbe-associated molecular pattern (MAMP). A synthetic peptide of 24 aa from the central part of the downy mildew HaNLP3 protein was found to activate the plant immune system and trigger resistance to this pathogen. Strikingly, not only peptides of oomycete NLPs, but also those of bacteria and fungi were shown to act as a MAMP. This unprecedented broad taxonomic distribution demonstrates that the occurrence of a MAMP is not necessarily restricted to a class of microorganisms but can occur in a wide range of species from the tree of life.

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species (18, 20). Type 2 NLPs differ from type 1 by an additional conserved second cysteine bridge and putative calcium-binding domain (19). In *Arabidopsis*, cytotoxic NLPs were found to activate immunity-related gene expression, which strongly overlapped with that induced by flg22 (21, 22). However, it was suggested that immune responses resulted from cytotoxicity. Moreover, necrosis was only induced upon treatment with the complete NLP protein (23). In vitro, cytotoxic NLPs cause rapid leakage of dicot membrane-derived vesicles, suggesting a direct cytolytic activity (24). The immunogenic effect of NLPs was therefore suggested to result from direct cellular damage (24), or release of damage-associated molecular patterns (3).

Several plant-infecting oomycetes have large expansions of *NLP*s in their genomes (25–27), suggesting that these proteins play an important role in the pathogen's lifestyle. A clear virulence function was observed for NLP_{Pcc} of the rot bacterium *Pectobacterium carotovorum* (27). Also, individual deletion of two *NLP* genes in the fungus *Verticillium dahliae* resulted in reduced virulence on different host plants (28). Five other *NLP* genes in this fungus encode noncytotoxic proteins (29), a phenomenon that is also observed in oomycetes. When tested by transient expression in tobacco, necrosis was only induced by 1 out of 3 tested NLPs of *Phytophthora infestans* (30), 8 out of 33 NLPs of *Phytophthora sojae* (31), whereas not a single 1 of 10 NLPs of *Hyaloperonospora arabidopsidis* tested caused necrosis (26). In contrast to cytotoxic *NLPs* that are mainly expressed during necrotrophic stages of infection, noncytotoxic *NLPs* appear to be expressed early during infection (26, 30), suggesting they serve an, as-yet-unknown, function during penetration or initial colonization of the host.

In our search for the biological function of noncytotoxic NLPs of *H. arabidopsidis*, transgenic *HaNLP*-expressing *Arabidopsis* plants were generated that were severely stunted. In this paper, we show that *Arabidopsis* responds to noncytotoxic HaNLPs and small peptide fragments thereof that are highly conserved in type 1 NLPs. The peptides activate ethylene production and other typical MAMP-triggered defense responses, but not tissue necrosis, indicating they act as a MAMP. NLPs are not restricted to a single class of microbes but present in a broad range of mostly plant-associated microbes (bacteria, fungi, and oomycetes) belonging to three kingdoms of life, making this a MAMP with an unprecedented broad taxonomic occurrence.

Results

HaNLP Expression in _Arabidopsis_ Leads to Severe Growth Reduction and Resistance to Downy Mildew. *H. arabidopsidis*, the downy mildew pathogen of *Arabidopsis*, has an expanded family of 10 different *NLP* genes that encode noncytotoxic secreted proteins (26). To determine whether the HaNLPs would facilitate the infection process and enhance disease susceptibility of *Arabidopsis*, transgenic *HaNLP* overexpression lines were created. Surprisingly, overexpression of 7 of the 10 *NLP* genes (*HaNLP2*, *3*, *4*, *5*, *6*, *9*, and *10*) resulted in transgenic plants showing severely reduced growth, compared with control plants transformed with *Yellow Fluorescent Protein* (*YFP*) (Fig. 1*A*). Plants expressing *HaNLP1*, *7*, and *8* showed no, or limited growth reduction, which was not significantly different from the *YFP*-expressing control. All other transgenic lines, except for *HaNLP5*-expressing plants, produced seeds and were tested in the next generation (T3) by weighing the aerial parts of 10 seedlings per *NLP*-expressing line. NLP-induced weight reduction confirmed the growth effects observed on individual T2 plants (Fig. 1*B*).

HaNLP-expressing plants showed strongly reduced susceptibility to the downy mildew *H. arabidopsidis* (Fig. 1*C*), and strikingly, these same lines also showed severe growth reduction. There was a strong correlation ($R^2 = 0.89$) between the level of susceptibility and the fresh weight of the transgenic lines expressing different *HaNLP* genes. In the literature, there is a multitude of examples of plant growth inhibition as a result of activation of plant immunity (32). The fact that the level of immunity of the *HaNLP*-expressing plants is well correlated to their growth inhibition, therefore, suggests that activation of plant immunity causes the observed growth reduction.

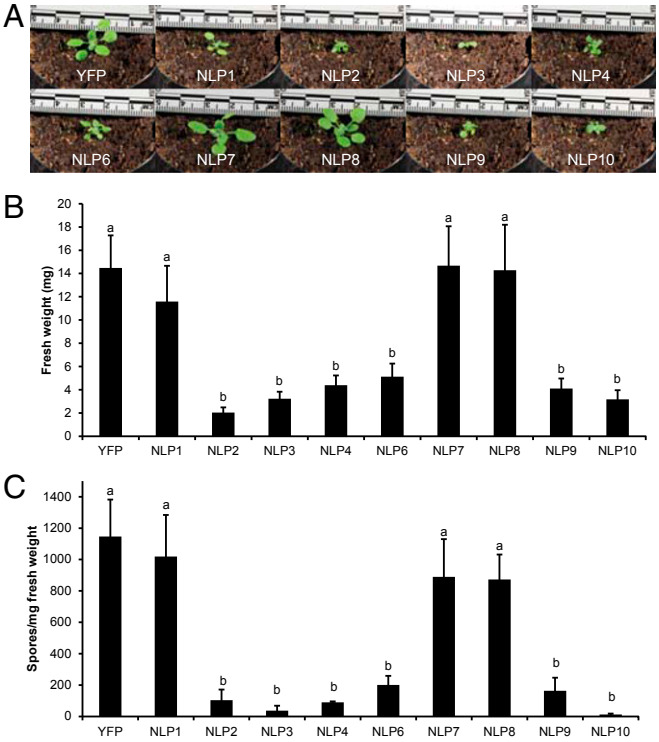

Fig. 1. *HaNLP* expression in *Arabidopsis* leads to growth reduction and enhanced resistance to downy mildew. (*A*) The result of reduced growth is visible as the smaller sizes of representative T3 transgenic *Arabidopsis* lines (21 d old) expressing *HaNLP2*, *3*, *4*, *6*, *9*, and *10*, but not of those expressing *HaNLP1*, *7*, *8*, and the *YFP* control. (*B*) The reduction in growth was quantified as fresh weight of the aerial parts of T3 seedlings [$n = 10$, with standard deviation (SD)]. *Arabidopsis* plants overexpressing *HaNLP5* died before day 21. (*C*) Transgenic T3 lines that showed growth reduction also showed enhanced resistance to the downy mildew *H. arabidopsidis* isolate Waco9, as measured by counting the number of spores per milligram of fresh-weight above-ground tissue (with SD). Plants were inoculated at 14 d after germination, and spores counted 6 d postinoculation ($n = 10$; the experiment was repeated three times with similar results). Significance of differences in the level of sporulation was assessed with the Tukey honestly significant difference (HSD) test and indicated with "a" and "b" ($\alpha = 0.05$).

HaNLP3 Is a Potent Activator of Plant Immunity. As the observed activation of plant immunity in 35S:*HaNLP*-expressing plants could be the result of continuous overexpression, we created an estradiol-inducible line (containing an XVE:*HaNLP3* construct). *HaNLP3* was chosen for this as we studied this protein in more detail previously (26). A transgenic line was selected that showed no detectable *HaNLP3* expression in untreated plants and a strong induction upon treatment with estradiol. When sprayed with estradiol every 2 d for a period of 2 wk, these plants showed strongly reduced growth, similar to that of the 35S:*HaNLP3* lines, whereas non–estradiol-treated plants developed normally (Fig. S1). A control estradiol-inducible *YFP* line (XVE:*YFP*) did not show any growth reduction upon estradiol treatment. These data clearly indicate that growth reduction indeed results from exposure of plants to HaNLP3. The same lines were next used to investigate the effect of *HaNLP3* expression on *H. arabidopsidis* infection. For this, the inducible XVE:*HaNLP3* and XVE:*YFP* lines, which were phenotypically identical, were sprayed with water or estradiol 24 h before inoculation. A very strong reduction in susceptibility was observed in the estradiol-induced *HaNLP3* line, but not in the *YFP* control line or water-treated *HaNLP3* line (Fig. 2*A*). These data strongly support the idea that

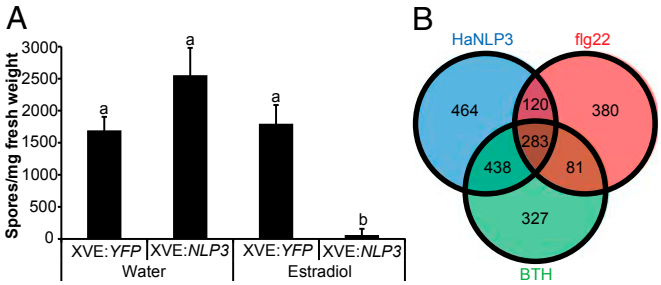

Fig. 2. Estradiol-induced expression of *HaNLP3* in *Arabidopsis* results in the activation of immunity to downy mildew and defense-associated gene expression. (*A*) Susceptibility of estradiol-inducible *HaNLP3* and *YFP* lines of *Arabidopsis* to *H. arabidopsidis* as measured by counting the number of spores per milligram of fresh-weight above-ground tissue (with SD). *Arabidopsis* seedlings were sprayed with either water or 100 μM estradiol 24 h before inoculation with *H. arabidopsidis* Waco9. Spore counts were performed 6 d after inoculation. Significance of differences in the level of sporulation was assessed with a Tukey HSD test ($n = 4$; the experiment was repeated three times with similar results). Significant differences between the lines is indicated with "a" and "b" ($\alpha = 0.05$). (*B*) Venn diagram showing the overlap in *Arabidopsis* genes that are activated in response to different inducers of plant defense responses with the 1,305 genes that are activated by HaNLP3 (blue). flg22-induced genes (864; red) are up-regulated 1 h and/or 4 h after treatment with flg22 peptide (22). BTH-induced genes (1,129; green) are activated at 24 h after treatment with BTH (33).

HaNLP3 triggers the plant immune system, resulting in resistance to *H. arabidopsidis*.

The question why transient expression of *HaNLP3* leads to immunity to *H. arabidopsidis* was addressed by analyzing gene expression changes at 24 h after induction of the *HaNLP3* transgene with estradiol. The expression of *HaNLP3* resulted in a strong transcriptional response (Dataset S1); 2,586 genes were significantly (q value, <0.05) differentially expressed (at least fourfold) between estradiol- and water-treated seedlings of XVE:*HaNLP3*, of which 1,305 genes showed enhanced expression (more than fourfold up) and 1,281 genes were down-regulated (more than fourfold down). Comparing the 1,305 HaNLP3-induced gene set to other publicly available data showed that there was a strong overlap with genes up-regulated in response to the flagellin-derived MAMP flg22 (22), and to BTH (33), a salicylic acid analog that activates plant immune responses (Fig. 2*B*). The fact that HaNLP3 activates immunity-related gene expression, as well as resistance to downy mildew, strongly suggests that the protein acts as a MAMP.

Defense Induction in *Arabidopsis* by Recombinant NLPs. The observed induction of defense could be caused by artificial *in planta* production of high levels of the secreted HaNLP3 protein. We therefore tested whether recombinant HaNLP3 protein, delivered extracellularly in the leaf intercellular space, would activate plant immune responses, e.g., the defense gene *PR-1*. For this, HaNLP3 was produced in *Pichia pastoris* and the purified protein infiltrated in leaves of an *Arabidopsis* promoter *PR-1*:*GUS* reporter line. Leaves infiltrated with HaNLP3 showed a high β-glucuronidase (GUS) activity, indicating that the *PR-1* promoter is strongly activated, similar to leaves infiltrated with the flg22 peptide that is a potent MAMP in *Arabidopsis* (Fig. 3). In contrast, the control sample, PIC3 (*P. pastoris* empty vector control, purified in the same way as HaNLP3), as well as the buffer control, showed very little GUS activity. This experiment clearly shows that extracellular exposure of plant cells to HaNLP3 activates plant immune responses.

A Fragment of HaNLP3 Is Sufficient to Induce Plant Growth Reduction. Proteinaceous MAMPs, e.g., flagellin or EF-Tu, are often recognized through smaller protein epitopes. To test whether smaller NLP fragments can still act as MAMPs, we made N- and C-terminal deletions and substitutions in HaNLP3 and expressed them in transgenic *Arabidopsis* lines, measuring plant growth reduction as a proxy for activation of immune responses (Fig. 4*A*). Disruption of the disulfide bridge, which is essential for toxicity of cytolytic NLPs (23), by substitution of the first cysteine residue by serine (C79S), did not reduce the growth-inhibiting effect of HaNLP3. Deletion of a 26-aa region between the two conserved cysteine residues also did not affect HaNLP3-induced growth reduction. Transgenic expression of successive C-terminal deletions of HaNLP3 resulted in a reduced growth phenotype for fragments 1–4, whereas further C-terminal deletions did no longer have a negative effect on plant growth (fragments 5–8). This suggested that sequences N-terminal of the heptapeptide motif are important for HaNLP3-induced growth reduction. Fragment 4, which ends with the heptapeptide motif, was further reduced in size by successive N-terminal deletions while leaving the signal peptide intact. Expression of fragments 9–12 in *Arabidopsis* showed that fragments 9 and 10, but not 11 and 12, reduced growth when expressed in transgenic plants. A 28-aa fragment of HaNLP3 (fragment 10) is thus sufficient to cause the growth effect. This fragment contains two regions that are highly conserved in type 1 NLPs (Fig. 4*B*): conserved region I starting with the AIMY amino acid sequence, and conserved region II matching the heptapeptide motif. The corresponding conserved region I in the structure of NLP_{Pya} is fully located inside of the protein, whereas the heptapeptide motif (conserved region II) is partly surface exposed (Fig. S2). Nevertheless, native recombinant HaNLP3 protein induces ethylene production in *Arabidopsis*, a well-known MAMP response (Fig. S3). Interestingly, heat-denatured HaNLP3 (boiled for 1 h) was an approximately three times more potent inducer of ethylene production (EC_{50} = 0.2 μM) than native recombinant protein (EC_{50} = 0.5 μM), suggesting the immunogenic epitope is not fully exposed in the native protein.

Synthetic NLP Peptides Trigger Immunity. A synthetic peptide of 24 residues (nlp24) was made that contains both conserved region I and II, but lacks the first 4 aa of the 28-aa peptide that are not conserved in type 1 NLPs (Fig. 4*B*). nlp24 appeared to be a strong inducer of ethylene production in *Arabidopsis* (Fig. 5*A*), confirming that this HaNLP3 peptide is sufficient to trigger an immune response. To investigate whether peptide fragments of other microbial NLPs also act as MAMPs in *Arabidopsis*, the corresponding nlp24 peptides of BcNEP2 (of the fungus *B. cinerea*) and BsNPP1 (of the bacterium *Bacillus subtilis*) were tested and found to induce ethylene production in *Arabidopsis* (Fig. 5*A*). In contrast, the corresponding 26-aa peptide of the type 2 NLP of *P. carotovorum* (NLP_{Pcc}) did not induce ethylene production. Similarly, the nlp24 peptide of HaNLP3, but not the nlp26 peptide of NLP_{Pcc}, was a strong inducer of GUS expression in the *Arabidopsis* promoter *PR-1*:*GUS* reporter line (Fig. S4). It is striking to see that nlp24 is the most conserved part (containing both conserved region I and II) in type 1 NLPs of bacteria, fungi, and oomycetes, as illustrated by alignment of HaNLP3, BcNEP2, and BsNPP1 (Fig. S5). In contrast, conserved

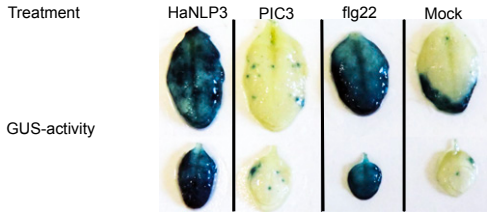

Fig. 3. Recombinant HaNLP3 protein activates *PR-1* expression. Induction of defense in *Arabidopsis* leaves was measured by staining for GUS expression in leaves of *pPR-1:GUS Arabidopsis* plants infiltrated with recombinant HaNLP3 protein (0.5 μM), a control sample (PIC3), flg22 peptide (0.5 μM), and water (Mock). GUS staining was performed at 24 h after infiltration.

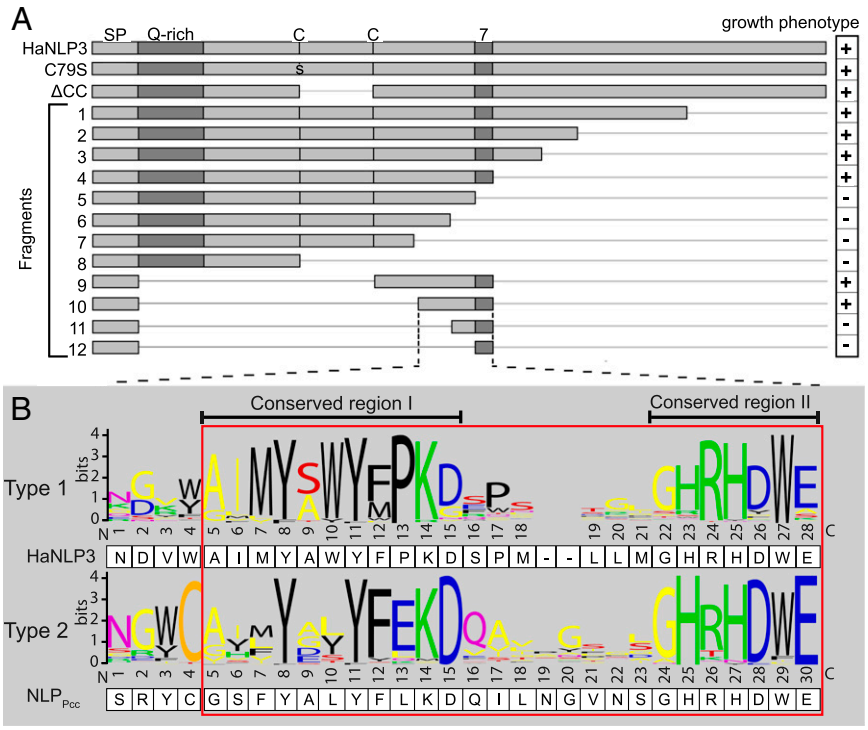

Fig. 4. A conserved region from the central part of HaNLP3 is sufficient for MAMP-associated growth reduction. (*A*) Schematic representation of substituted and deleted versions of the HaNLP3 protein (showing the signal peptide, "SP"; glutamine-rich region, "Q-RICH"; cysteine residues, "C"; and heptapeptide motif, "7") and their effect on growth when overexpressed in transgenic *Arabidopsis* seedlings. Multiple T1 lines per construct were scored for growth reduction (with "+" indicating strong growth reduction and "−" indicating no growth reduction) following transformation of the different 35S: *HaNLP3* variants. Fragment 10 contains the minimal region of 28 aa that is still able to induce MAMP-associated growth reduction. (*B*) A 24-aa peptide is conserved in type 1 NLPs from oomycetes, fungi, and bacteria (red-lined box). The 11-residue conserved region I is less conserved in type 2 NLPs. The second conserved region in the 28-aa fragment is the GHRHDWE heptapeptide that is characteristic for the NLP family, and that is conserved in both type 1 and type 2 NLPs. The weblogo is based on 378 type 1 NLP sequences and 122 type 2 NLP sequences (19).

region I of type 2 NLPs differs at several amino acid positions from that of type 1 NLPs, whereas the heptapeptide motif is highly conserved in the two NLP types (Fig. 4*B*).

To study whether nlp24 peptides also trigger immunity in *Arabidopsis*, we pretreated leaves with 100 nM peptide 1 d before inoculation with the downy mildew *H. arabidopsidis* Noco2. The nlp24 peptides corresponding to HaNLP3, BcNEP2, and BsNPP1 induced a strong immune response, resulting in resistance to Noco2. In contrast, treatment with the peptide of NLP$_{Pcc}$ did not reduce susceptibility to downy mildew, but resulted in sporulation levels similar to that of mock-treated leaves (Fig. 5*B*). Our data show that peptides derived from type 1 NLPs of microbes occurring in three kingdoms of life are recognized as MAMPs by *Arabidopsis* and trigger effective immune responses.

nlp24 Peptides Are Diverse and Tolerant to Substitutions. The *HaNLPs*, when transgenically expressed in *Arabidopsis*, trigger different levels of immunity. To test whether this is caused by differences in affinity, the EC_{50} values for ethylene production were determined (Table 1). For NLP2, 4, 5, 6, and 10, the EC_{50} values were in the range of 0.1–0.2 μM, similar to that obtained for nlp24 of HaNLP3, and of the heat-denatured HaNLP3 protein (0.2 μM). However, the EC_{50} values were higher for HaNLP1, 7, 8, and 9 (range of 0.4–0.6 μM). Their reduced activity could explain the lower effect on growth in transgenic plants transformed with HaNLP1, 7, and 8, but not for HaNLP9 (Fig. 1).

We next tested the minimal peptide length and composition by measuring ethylene production in response to truncated peptides and alanine-substituted versions of nlp24 (HaNLP3) (Table 1). Activity was not affected when the first 2 aa (AI) were not included (nlp22), but was strongly reduced when the first 4 aa (AIMY) were absent (nlp20). Deletion of the C-terminal heptapeptide motif from nlp24 (in nlp17) did not increase the EC_{50} value. Further C-terminal truncated forms were still active, including an 11-aa peptide with the sequence AIMYAWYFPKD (nlp11) that corresponds to conserved region I (Fig. 5*B*) and even had a slightly lower EC_{50} of 0.1 μM. Removal of the first 2 aa of nlp11 resulted in a peptide (nlp9) that was 40 times less active. Ethylene-inducing activity of alanine substitutions in the conserved region I of nlp24 showed that methionine at position 3 of the peptide is required for full activity. Two other substitutions, of tyrosine 7 and aspartic acid 11, resulted in peptides with slightly reduced activity. Substitution of histidine 19, which is highly conserved in NLPs and is required for necrosis induction by cytolytic NLPs (24), did not result in a decreased EC_{50} value, confirming that conserved region I, but not II, is required for MAMP activity of NLPs.

The bacterial BsNPP1 and fungal BcNEP2 peptides are 5–10 times more potent triggers of ethylene production than nlp24 of HaNLP3. In contrast, the 26-aa peptide of the type 2 NLP$_{Pcc}$ had a very high EC_{50} value (>10 μM) and is clearly not acting as a MAMP in *Arabidopsis*. This was confirmed for nlp11 peptides that show slightly lower EC_{50} values than HaNLP3 for BcNEP2,

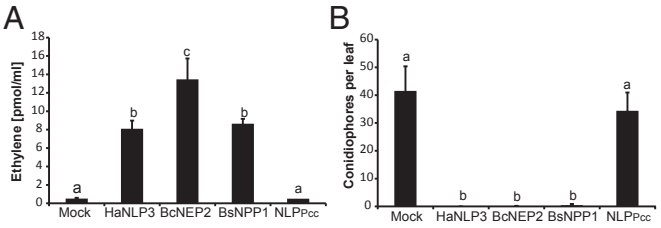

Fig. 5. Synthetic 24-aa NLP peptides (nlp24) induce MAMP responses and trigger immunity to downy mildew. (*A*) Ethylene production in *Arabidopsis* is induced in response to nlp24 peptides (1 μM) of HaNLP3, BcNEP2, and BsNPP1, but not to the nlp26 fragment of the type 2 NLP$_{Pcc}$. Leaf pieces were incubated for 4 h in buffered peptide solution before ethylene concentrations were determined (n = 3; SD is indicated and the experiment was performed three times with similar results). (*B*) Resistance to *H. arabidopsidis* in *Arabidopsis* is induced by nlp24 (100 nM) of HaNLP3, BcNEP2, and BsNPP1, but not of the type 2 NLP$_{Pcc}$. The numbers of conidiophores per leaf is a measure of susceptibility. Leaves of 4.5-wk–old *Arabidopsis* plant were infiltrated with nlp24 peptides 1 d before inoculation with downy mildew isolate Noco2. Conidiophore counts were performed 10 d after inoculation. Significance of differences in the level of sporulation (with SE) was assessed with a Tukey HSD test (n = 44) and significant differences between lines are indicated with "a" and "b" (α = 0.01).

Table 1. Half-maximum effective concentration (EC_{50}) of different nlp24-based peptides for the induction of ethylene biosynthesis in *Arabidopsis*

Name	Size, aa	Amino acid sequence	EC_{50}, μM
nlp24(HaNLP1)	24	AIMFAYYFPKSQPRRSVSVRHSWE	0.3
nlp24(HaNLP2)	24	GIVYAWFFPKDSVRHGIGHRYDWE	0.2
nlp24(HaNLP4)	24	GIIFAWYFPKDSVRDGVGHRHDWE	0.1
nlp24(HaNLP5)	24	AIMFSWYFPKGFHDRKASRRHDWA	0.2
nlp24(HaNLP6)	24	GIVYAWYFPKDSVRDGIGHRYDWE	0.1
nlp24(HaNLP7)	24	AIAYAYYSPKAHPHQRVWIRHVWN	0.6
nlp24(HaNLP8)	24	AIMYALYFPKDMKVLNRGYRHAFE	0.6
nlp21(HaNLP9)	21	AIMYVWYFPKD---NRDDDRHDWE	0.4
nlp24(HaNLP10)	24	AIMYAWYFPKDAPDEESGQRHDWE	0.1
nlp24(HaNLP3)	24	AIMYAWYFPKDSPMLLMGHRHDWE	0.2
nlp22	22	--MYAWYFPKDSPMLLMGHRHDWE	0.1
nlp20	20	----AWYFPKDSPMLLMGHRHDWE	2.0
nlp17	17	AIMYAWYFPKDSPMLLM-------	0.2
nlp13	13	AIMYAWYFPKDSP-----------	0.3
nlp11	11	AIMYAWYFPKD-------------	0.1
nlp9	9	--MYAWYFPKD-------------	4.0
nlp24_M3A	24	AIAYAWYFPKDSPMLLMGHRHDWE	1.5
nlp24_Y4A	24	AIMAAWYFPKDSPMLLMGHRHDWE	0.2
nlp24_W6A	24	AIMYAAYFPKDSPMLLMGHRHDWE	0.2
nlp24_Y7A	24	AIMYAWAFPKDSPMLLMGHRHDWE	0.4
nlp24_F8A	24	AIMYAWYAPKDSPMLLMGHRHDWE	0.2
nlp24_P9A	24	AIMYAWYFAKDSPMLLMGHRHDWE	0.2
nlp24_K10A	24	AIMYAWYFPADSPMLLMGHRHDWE	0.1
nlp24_D11A	24	AIMYAWYFPKASPMLLMGHRHDWE	0.3
nlp24_H19A	24	AIMYAWYFPKDSPMLLMGARHDWE	0.2
nlp24 (BcNEP2)	24	AIMYSWYMPKDEPSTGIGHRHDWE	0.03
nlp24 (BsNPP1)	24	AIMYSWYFPKDEPSPGLGHRHDWE	0.02
nlp26 (NLP_{Pcc})	26	GSFYALYFLKDQILNGVNSGHRHDWE	>10
nlp11 (BcNEP2)	11	AIMYSWYMPKD	0.07
nlp11 (BsNPP1)	11	AIMYSWYFPKD	0.09
nlp11 (NLP_{Pcc})	11	GSFYALYFLKD	>10
nlp11 (NLP_{Pya})	11	AIMYSWYMPKD	0.07

Values were determined for nlp24 peptides of 10 different HaNLPs, for truncated versions and alanine substitutions of HaNLP3, as well as for nlp24- and nlp11-based peptides for BcNEP2, BsNPP1, NLP_{Pcc}, and NLP_{Pya}. EC_{50} data were based on three measurements for each of six peptide concentration tested, repeated three times with similar results.

BsNPP1, and NLP_{Pya}, but again a very high EC_{50} for NLP_{Pcc}. The data presented demonstrate that microbial NLPs, occurring in three kingdoms of life, act as MAMPs, making this an immunity-triggering protein family of unprecedented broad taxonomic distribution.

Discussion

NLPs Act as MAMPs. The discovery that noncytotoxic NLPs activate immunity in *Arabidopsis* was made while searching for a virulence function of these proteins in the downy mildew *H. arabidopsidis* (26). When transgenically expressed in *Arabidopsis*, 7 of the 10 *HaNLPs* induced severe growth reduction that resembled that of documented *Arabidopsis* autoimmune mutants, e.g., *cpr1* and *cpr5* (34, 35), suggesting that the secreted proteins activate plant immunity. Inducible expression of *HaNLP3* resulted in the activation of many well-known immunity-related genes, which are also activated by the flg22 MAMP and the defense hormone salicylic acid (or its analog BTH). By creating C- and N-terminal truncations of HaNLP3, a 28-aa fragment was pinpointed as sufficient for MAMP-associated growth reduction. This fragment could be further reduced to a synthetic peptide of 24 aa that was sufficient to induce MAMP responses, e.g., ethylene production, and immunity to *H. arabidopsidis*.

The nlp24 peptide is strongly conserved in both cytotoxic and noncytotoxic type 1 NLPs. Conserved region I (of 11 aa) contains the immunogenic part of nlp24. In the fungal VdNLP2 protein, this region was analyzed in more detail by Zhou et al. (29), who observed that alanine substitution of six different amino acid residues resulted in loss or reduction of necrosis induction by this cytotoxic type 1 NLP. The fact that region I is also strongly conserved in noncytolytic NLPs, in particular those of the *Arabidopsis* pathogen *H. arabidopsidis*, suggest that this region is also important for a, so-far-unknown, noncytotoxic function related to virulence. A synthetic peptide (nlp26) of the type 2 NLP_{Pcc} does not induce ethylene production in *Arabidopsis*, nor does it trigger immunity to downy mildew. This suggests that perception of NLPs is specific for type 1 NLPs (although we do not rule out that other type 2 NLPs can trigger immunity in *Arabidopsis*). The cause for this may be that conserved region I of type 2 NLPs differs from that of type 1 NLPs (Fig. 4*B*).

Activation of immune responses by cytolytic NLPs has always been causally linked to their toxic activity (22, 23). Ottmann et al. (24) demonstrated that the purified NLP_{Pp} protein caused membrane leakage in vesicles from dicots, indicating the protein has a cytolytic activity. The immune response was suggested to result from cellular damage, or the release of damage-associated molecular patterns. The finding that *Arabidopsis* mounts an effective immune response to only a small, highly conserved, peptide of noncytotoxic and cytotoxic type 1 NLPs demonstrates that cellular damage is not required for NLP-triggered immunity in *Arabidopsis*. However, the fact that the type 2 NLP_{Pcc} induces immune responses in *Arabidopsis*, but its internal peptide fragment is not recognized as a MAMP, suggests that cytotoxic NLPs also activate immunity through a different mechanism.

NLP Recognition in *Arabidopsis*. In their natural environment, *Arabidopsis* plants are exposed to a wide range of microbial organisms, a few of which are known to cause disease under field conditions (36). Of these natural pathogens, the downy mildew *H. arabidopsidis* is the only one, known so far, that contains *NLP* genes. As pathogens are known to be important in shaping the evolution of host species, it is tempting to speculate that *Arabidopsis* has evolved the capability to detect NLPs as a mechanism to protect itself from downy mildew infection. The NLP-triggered immune response is clearly effective as pretreatment of plants with NLP proteins or peptides provide protection against downy mildew infection. Nevertheless, in untreated plants, *H. arabidopsidis* can overcome these defenses, as it is able to cause disease. We envision that, during its coevolution with *Arabidopsis*, the downy mildew has evolved effectors that suppress NLP-triggered immunity, a specific form of MTI. Candidate effectors of *H. arabidopsidis* for this suppression are the well-known host-translocated RXLR proteins that are encoded by an estimated 130–150 genes in this oomycete (37). A large number of these RXLRs have been identified as effective suppressors of defense responses and MTI (38–43), and could suppress the early responses induced by NLPs.

MAMPs are generally recognized by pattern recognition receptors (PRRs) that are either receptor-like kinases (RLKs) and/or receptor like-proteins (RLPs) (44). A peptide fragment of 10–25 aa is, in most cases, sufficient for triggering immunity, e.g., flg22 (5), Pep13 (13), and elf18 (6). The specificity of the ligand is determined by the receptor, but often a coreceptor, e.g., BAK1 (45) or SOBIR1 (9, 46), is required for signal transduction. Other host factors could be required for the recognition of nlp24, as the peptide fragment is predicted not to be surface exposed, but located on the inside of the protein (Fig. S2), based on the structure of the type 1 NLP_{Pya} protein (24). This suggests that it cannot directly be recognized by a cognate receptor, but requires (partial) degradation of the protein, likely by host proteases.

NLP MAMPs Occur in Microorganisms of Three Kingdoms of Life. MAMPs have been defined as "highly conserved molecules within a class of microbes and to contribute to general microbial fitness" (2). Some MAMPs are so important to microbes that they cannot thrive without the associated molecules. In *Phytophthora* and downy mildew species, belonging to the oomycetes,

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NLP genes have considerably expanded in number, suggesting they contribute to the lifestyle of these pathogens. It is striking to see that the NLP immunogenic region of 24 aa (nlp24) is highly conserved in type 1 NLPs. Substitutions in the nlp24 region of the fungal VdNLP2 protein in most cases led to loss of cytotoxicity, indicating the region has an important function (29). The observed conservation of the recognized NLP peptide is important for the efficiency and durability of MTI and makes the application of NLP-triggered immunity to generate resistance to nonadapted phytopathogens promising.

NLPs are unique in their extremely wide taxonomic occurrence, suggesting they are advantageous to many different microbial species. Our finding of NLPs acting as proteinaceous MAMPs in *Arabidopsis* clearly shows that these recognized molecules are not confined to a single class of microbes; they are found in oomycetes, bacteria, and fungi. Therefore, the definition of MAMPs could be broadened to "highly conserved molecules found in microbes." The widespread occurrence of this class of secreted proteins, in particular in plant-associated microorganisms, makes their role as MAMPs highly relevant.

Materials and Methods

Generation of Transgenic Lines. The *HaNLP* coding sequences were amplified from *H. arabidopsidis* genomic DNA (primers listed in Table S1), cloned in plant transformation vectors, and used for stable transformation of *Arabidopsis thaliana* Col-0 plants as described in *SI Materials and Methods*.

Microarray Analysis. RNA, of 10-d–old *Arabidopsis* seedlings containing either *XVE:HaNLP3* or *XVE:YFP* and induced by spraying with estradiol solution, was used for microarray analysis as described in *SI Materials and Methods*.

All other methods are described in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Generation of Transgenic Lines. The coding sequences of the *HaNLP*s were amplified from *H. arabidopsidis* (isolate Emoy2) genomic DNA using the gene-specific primers (Table S1), cloned into a pENTRY/D-TOPO vector using Gateway cloning (Invitrogen), and verified by PCR and Sanger sequencing. For HaNLP3, fusion 4 was used (1), which has the PsojNIP signal peptide instead of the *HaNLP3* signal peptide to secrete the protein more efficiently when expressed *in planta*. All *HaNLP*s cloned into a pENTRY/D-TOPO were subsequently recombined into the binary vectors pB7WG2 (2), pFAST (3), or a Gateway-compatible version of XVE (4) that was kindly provided by Dr. A. P. Mähönen, University of Helsinki, Helsinki. Binary vectors were transformed into *Agrobacterium tumefaciens* strain C58C1 (pGV2260) by electroporation. *Arabidopsis* Col-0 plants were transformed using the floral dip method (5). Transformants were selected for BASTA resistance (pB7WG2 and XVE) or for fluorescence of the seed coat (pFAST). Multiple independent T1 lines showing expression of the transgenes, as analyzed by RT-PCR, were selected for further studies. An estradiol-inducible line with proper induction and no measurable leakage was selected by RT-PCR analysis of *HaNLP3* expression.

Plant Growth Conditions. All plants were grown on potting soil (Primasta) at 22 °C, 75% relative humidity. *NLP* (inducible)-overexpressing plants (both full-length and truncated proteins) were grown with 16 h of light per day. Plants used for ethylene measurements had 8 h of light per day at an age of 5–6 wk. Finally, plant used for pathogenicity assays after peptide treatment were grown under 10 h light per day and were 4.5 wk old when used.

Pathogenicity Assays. Infection assays on seedlings were performed with *H. arabidopsidis* isolate Waco9 (50 spores per μL) and on adult plant (4.5 wk) with isolate Noco2 (100 spores per μL). After inoculation, plants were left to dry for ∼30 min and were subsequently incubated at 100% humidity at 16 °C with 10 h of light per day. Five to 10 days after inoculation, the disease severity was quantified. For seedlings, the shoots were cut and suspended in a known volume of water and the number of spores per milligram of plant tissue (fresh weight of aerial parts) was determined. For adult plants, the number of conidiophores per leaf was counted.

Ethylene Measurements. Leaves of 5-wk old *Arabidopsis* plants (Col-0) were cut into 3-mm squares and left in demiwater overnight at room temperature. The next day, three leaf pieces were transferred to 5-mL glass tubes containing 400 μL of 20 mM Mes, pH 5.7, and the appropriate amount of synthetic peptide. Vials were sealed with rubber septa, and after 4 h, ethylene accumulation was measured by taking a 1-mL sample from the headspace for analysis by gas chromatography (6).

GUS Staining. Expression of β-glucuronidase (GUS) in promoter *PR-1:GUS Arabidopsis* lines was assessed by vacuum infiltrating leaves with GUS-staining solution [1 mM X-Gluc, 100 mM NaPi-buffer, pH 7.0, 10 mM EDTA, and 0.1% (vol/vol) Triton X-100]. Leaves were incubated for 24 h at 37 °C in the GUS-staining solution, and subsequently chlorophyll was removed by repeated washes in 70% ethanol.

Microarray Analysis. Twenty-four hours before harvesting, 10-d-old *Arabidopsis* seedlings containing either *XVE:HaNLP3* or *XVE:YFP* were induced by spraying with estradiol solution (100 μM estradiol in 0.02% Silwet) or 0.02% Silwet as control. RNA was extracted from three biological replicates each using an RNeasy kit (Qiagen) following the manufacturer's instructions. RNA quality was assessed by NanoDrop, and three samples from estradiol-sprayed as well as three samples from control *XVE:HaNLP3* plants were analyzed using ATH1 Affymetrix chips (ServiceXS B.V.). Microarray data were normalized using RMA (7), compared with data of estradiol-sprayed and control *XVE:YFP* plants, and differentially expressed genes were selected using the R package Limma (8).

Protein Production and Peptide Synthesis. HaNLP3 was produced as described by Cabral et al. (1). Peptides were ordered at Genscript and prepared as 10 mM stock solutions in 100% DMSO before use.

Creation of Weblogos. The weblogos (9) were generated on a total of 378 type 1 NLP sequences (231 oomycete, 135 fungal, and 12 NLPs of bacterial origin), and 122 type 2 NLP sequences (61 fungal and 61 bacterial NLPs) (10).

1. Cabral A, et al. (2012) Nontoxic Nep1-like proteins of the downy mildew pathogen *Hyaloperonospora arabidopsidis*: Repression of necrosis-inducing activity by a surface-exposed region. *Mol Plant Microbe Interact* 25(5):697–708.
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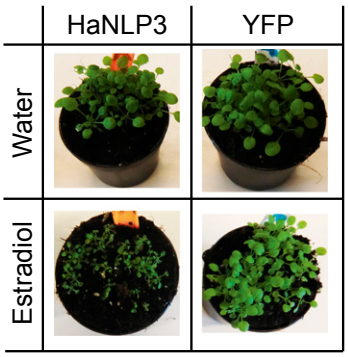

Fig. S1. The inducible *XVE:HaNLP3* line shows severe growth reduction when treated with estradiol. From 1 d after germination, *XVE:HaNLP3* and *XVE:YFP* transgenic lines were sprayed every 2 d with either water or 100 μM estradiol. The pictures were taken 14 d after germination, showing only growth reduction of the estradiol-treated *XVE:HaNLP3* line but not of the control *XVE:YFP* line.

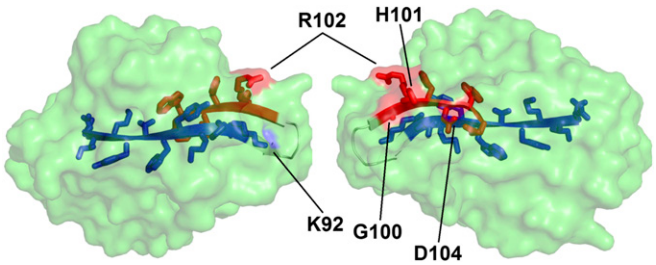

Fig. S2. Three-dimensional model of the 24-aa immunogenic peptide (nlp24) visualized in NLP_{Pya} (Protein Data Bank ID code 3GNU). The left- and right-hand figure are views of the opposing sites of the protein. The model shows both the individual residues of conserved region I (blue) and conserved region II (red), as well as the surface of the complete protein (green). The less conserved 6-aa region connecting the two conserved regions is also in green, and its side chains are not displayed. Of the conserved region I, only the side chain of K92 partly reaches the protein surface (as shown by the paler blue surface), whereas the rest is completely located on the inside of the protein. Of conserved region II, 4 of the 7 aa (G100, H101, R102, and D104) are on the surface of the protein (shown by the pink surface). The image was generated with Polyview 3D.

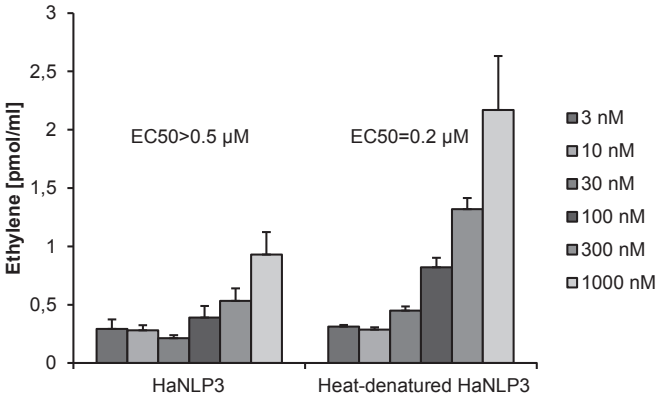

Fig. S3. Heat-denatured HaNLP3 protein is a stronger inducer of ethylene production in *Arabidopsis* than native recombinant HaNLP3. Ethylene accumulation was tested at different protein concentrations of native and heat-denatured (boiled for 1 h) recombinant NLP protein. The EC_{50} value for the heat-denatured protein was 0.2 μM, similar to that of the nlp24 peptide, and approximately threefold lower than that of the native recombinant HaNLP3 protein.

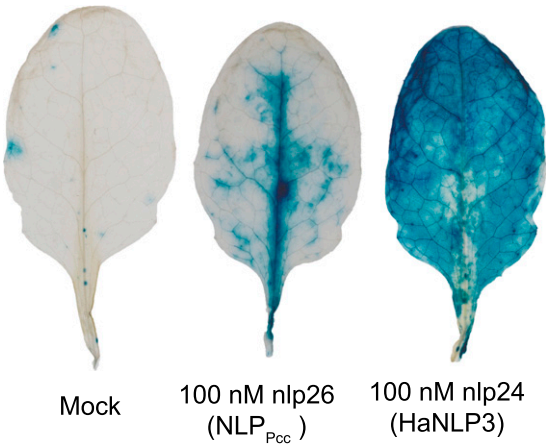

Fig. S4. A synthetic nlp24 peptide corresponding to HaNLP3 activates *PR-1* expression. Induction of defense in *Arabidopsis* leaves was measured by staining for GUS expression in leaves of *pPR-1:GUS Arabidopsis* plants infiltrated with the negative control 0.01% DMSO (Mock), 100 nM nlp26 of NLP$_{Pcc}$, and 100 nM nlp24 of HaNLP3. GUS staining was performed at 24 h after infiltration. The *PR-1* promoter was strongly activated by nlp24, whereas low signals were observed in mock-treated leaves and in response to nlp26 of NLP$_{Pcc}$.

```
HaNLP3   MKLDGFITTAILAHIPVYARNDYVQEEKQQQLQEPLDGQWKPTTTGHDAIVPFSEPKPVT 60
BcNEP2   --MVAFSKSLQLS-LSVLAS-TVIAIPTPSQLES-------RAVIDSDAVVGFAETVPSG 49
BsNPP1   --------MRKIA-LAVLMS-FFAFISLVPTVN--------AAVIGHDKVVGFDEVVPTT 42
                   :: :.*                ::          :. . * :* * *  *

HaNLP3   ISEKAGVKFKPLLDVNTGCAPYAAVNAEGETSGGLQTSGDPESGCRGSKYGSQVYGRSTW 120
BcNEP2   TVGTVYEAYKPFLKVVNGCVPFPAVDASGNTGGGLSPTGSSNGGC--SSSTGQVYVRGGQ 107
BsNPP1   IAQKAEKKFQPYLKVYSGCVPFPAVDAQGNTSGGLQPTGAPEGGC--SKHTGQVYSRSTW 100
            ..   ::* *.* .**.*:.**:*.*:*.***..:* .:.**  *.  .*** *.

HaNLP3   YNDVWAIMYAWYFPKDSPMLLMGHRHDWENVVVFINDPDEVEPT-ILGCSTSWHSGYIKY 179
BcNEP2   SGSNYAIMYSWYMPKDEPSTGIGHRHDWEGVIVWLSSATATTADNILAVCPSAHGGWD-C 166
BsNPP1   YNGVWAIMYSWYFPKDEPSPGLGHRHDWEGIVVWVDNPSIQNAK-VLSIAYSGHGKFTNV 159
          .. :****:**:***.*   :*******.::*:...    .  :*. . * *. :

HaNLP3   APCPTDSINGSSVMIKYEHSFPLNHALNITKDAGAYQDLIMWHQMPDLARRALNDTDFGK 239
BcNEP2   STDGY-SLSGTSPLIKYESIWPVDHSMGLTSTVGGKQPMIAWESLPTAAQTALENTDFGA 225
BsNPP1   QPNEK-NMKDTHPLIAYNSTWPLNHELHISDQVGGTQPLIGWEDLTPEARNALNITDFGK 218
          .    .:..:  :* *:  :*::* : ::. .*. * :* *..:.  *: **: ****

HaNLP3   AITPMNDLNFMEKIEAAWPFKTKKDGA 266
BcNEP2   ANVPFIPAVFTDNLAKAT-F------- 244
BsNPP1   ANVPFNDPNFTNHLEKAW-FR------ 238
         * .*:    * :::  *  *
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Fig. S5. Conservation of the nlp24 peptide in NLPs of microorganisms from three kingdoms of life. A multiple alignment was generated of HaNLP3 of the oomycete *H. arabidopsidis*, BcNEP2 of the fungus *B. cinerea*, and BsNPP1 of the bacterium *B. subtilis*. Signal peptides are indicated in yellow, and the 24-aa regions tested for ethylene induction in *Arabidopsis* are indicated in black.

Table S1. Primers used in this study

Primer	Forward	Reverse
HaNLP1	CACCATGAGGACTGGCGCCTTC	CTCATTAAAAAGGCCAAGAAGCG
HaNLP2	CACCATGAAGACCAGTGCCTTC	TCAATAGTCATTGTCCTCGAC
HaNLP3	CACCATGAACCTCCGCCCTGCA	TCATGCTCCATCTTTTTTCGTTTTAAACGG
HaNLP4	CACCATGAAGGCCAGCGCATTCCTG	TTAACTGTCGTAGCTATCTTGGC
HaNLP5	CACCATGAGCTTCCGGGCTCTAGTC	TCAGAATGCCATGCCCAGGC
HaNLP6	CACCATGAAGGCCAGCGCATTC	TCAATCTTGCCTCGCTTAACCT
HaNLP7	CACCATGAGGATAGGTAAGTCCTTGTGC	CTATCCAGCCATTTCGTAAGG
HaNLP8	CACCATGAAGACTTTGTCTTGCTTGTAT	TCACTTCAGCGGTGCAAAAG
HaNLP9	CACCATGAAGACCGGTCTCTTCTTGTA	TCAGCCTTCAACAAAGTCGTA
HaNLP10	CACCATGAAGGCCGTCGCCTTGTTG	CTAGCTAGCTGCGCTCACAT
HaNLP3 C79S	AGTGCACCGTACGCGGCT	GCCCGTATTAACATCGAGCA
HaNLP3 ΔCC	CGTGGATCGAAGTACGGGT	GCCCGTATTAACATCGAGCA
Fragment 1	TGAAAGGGTGGGCGCG	CACCATGATAAGGTCCTGGTAAGCT
Fragment 2	TGAAAGGGTGGGCGCG	ATACTTGATGTAGCCACTGTGCCA
Fragment 3	TGAAAGGGTGGGCGCG	ACCCAAGATCGTCGGCT
Fragment 4	TGAAAGGGTGGGCGCG	CTCCCAGTCATGCCGATGA
Fragment 5	TGAAAGGGTGGGCGCG	CATCAGTAGCATCGGCGAGT
Fragment 6	TGAAAGGGTGGGCGCG	GAAGTACCACGCGTACATAATAGC
Fragment 7	TGAAAGGGTGGGCGCG	GGTGGAGCGCCCATAAACT
Fragment 8	TGAAAGGGTGGGCGCG	GCCCGTATTAACATCGAGCAA
Fragment 9	CGTGGATCGAAGTACGGGT	GGCGCTCACGTACGCG
Fragment 10	AATGACGTCTGGGCTATTATGTACG	GGCGCTCACGTACGCG
Fragment 11	AACCCCAAGGACTCGCCGAT	GGCGCTCACGTACGCG
Fragment 12	GGTCATCGGCATGACTGG	GGCGCTCACGTACGCG

Other Supporting Information Files

Dataset S1 (XLS)