Antifungal symbiotic peptide NCR044 exhibits unique structure and multifaceted mechanisms of action that confer plant protection

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In the indeterminate nodules of a model legume Medicago truncatula, ~700 nodule-specific cysteine-rich (NCR) peptides with conserved cysteine signature are expressed. NCR peptides are highly diverse in sequence, and some of these cationic peptides exhibit antimicrobial activity in vitro and in vivo. However, there is a lack of knowledge regarding their structural architecture, antifungal activity, and modes of action against plant fungal pathogens. Here, the three-dimensional NMR structure of the 36-amino acid NCR044 peptide was solved. This unique structure was largely disordered and highly dynamic with one four-residue α-helix and one three-residue antiparallel β-sheet stabilized by two disulfide bonds. NCR044 peptide also exhibited potent fungicidal activity against multiple plant fungal pathogens, including Botrytis cinerea and three Fusarium spp. It inhibited germination in quiescent spores of B. cinerea. In germlings, it breached the fungal plasma membrane and induced reactive oxygen species. It bound to multiple bioactive phosphoinositides in vitro. Time-lapse confocal and superresolution microscopy revealed strong fungal cell wall binding, penetration of the cell membrane at discrete foci, followed by gradual loss of turgor, subsequent accumulation in the cytoplasm, and elevated levels in nucleoli of germlings. Spray-applied NCR044 significantly reduced gray mold disease symptoms caused by the fungal pathogen B. cinerea in tomato and tobacco plants, and postharvest products. Our work illustrates the antifungal activity of a structurally unique NCR peptide against plant fungal pathogens and paves the way for future development of this class of peptides as a spray-on fungistat/fungicide.

Significance

Several nodule-specific cysteine-rich (NCR) peptides expressed in a model legume Medicago truncatula exhibit potent antimicrobial activity. However, their structure–activity relationships and mechanisms of action against fungal pathogens of plants are still largely unknown. A small highly cationic peptide NCR044 with potent antifungal activity has been identified. This peptide has a unique highly dynamic structure and exhibits multifaceted mechanisms of action against a fungal pathogen Botrytis cinerea. Exogenous application of this peptide confers resistance to a gray mold disease caused by B. cinerea in tobacco and tomato plants as well as postharvest products. Our work paves the way for future development of NCR peptides as spray-on antifungal agents.

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Data deposition: The coordinates of the NMR structure have been deposited in the Protein Data Bank, www.rcsb.org (PDB code: 6U6G) and the NMR chemical shifts have been deposited in the BioMagResBank, www.bbrm.bsc.wisc.edu (accession no. 30660).

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In a recent study, 19 cationic NCR peptides were tested for their ability to inhibit the growth of a clinically relevant human fungal pathogen Candida albicans. Of these, nine with a net charge above +9 killed this fungal pathogen efficiently, showing fungicidal effects on both the yeast and hyphal forms at concentrations ranging from 1.5 to 10.5 μM, demonstrating their potential for development as antifungal drugs. The subcellular localization and intracellular targets of these peptides in fungal cells remain to be determined. There are several cationic NCR peptides with sequence heterogeneity expressed in the nodules of M. truncatula and other IRLC legumes, and it is likely that at least some of them have potent antifungal activity against plant fungal pathogens and are unique in structure and MOA.

In this study, we showed that NCR044, a 36-amino acid peptide with a net charge of +9 (8), exhibited potent antifungal activity against plant fungal pathogens. However, its three-dimensional (3D) NMR structure was determined to be very different from other well-characterized plant antifungal peptides such as defensins, thionins, lipid-transfer proteins, thaumatins, and heveins (14). This peptide localizes to the nucleolus in fungal cells, the site of ribosomal biogenesis. NCR044 conferred strong resistance to B. cinerea when applied topically on lettuce leaves and rose petals. Young tomato and Nicotiana benthamiana plants sprayed with this peptide were also protected from the gray mold disease caused by this pathogen, thus warranting its development as an antifungal peptide. Our work illustrates the antimicrobial activity of NCR peptides and paves the way for development of these peptides as a spray-on antifungal agent.

**Results**

**Two NCR044 Homologs Are Expressed in the Nodules of M. truncatula.** The M. truncatula genome contains genes encoding two NCR044 homologs, NCR044 (MtrunA17_Chrg0216231) and NCR473 (MtrunA17_Chrg0216241). The gene for each peptide encodes a signal sequence and a bioactive mature peptide. The mature peptides are each 36 amino acids in length and share 83% sequence identity (Fig. 1A). NCR044 has a net charge of +9 and 38% hydrophobic residues, whereas NCR473 has a net charge of +8 and 36% hydrophobic residues (Fig. 1B). Only two amino acid substitutions (I17P and R36S) account for the difference in net charge and hydrophobicity. The sequence of each mature peptide has a remarkably high concentration of cationic residues in the carboxyl-terminal half of each molecule. The primary sequence of each peptide displays no significant homology with any of the peptide sequences in the Antimicrobial Peptide Database (aps.unmc.edu/AP/main.php) (15).

During development of indeterminate nodules, NCR044 and NCR473 transcripts are each highly expressed in the interzone II–III and the nitrogen-fixation zone ZIII and with relatively low expression in the distal and proximal parts of the infection or differentiation zone (FIId and FIlp) (Fig. 1C and D) (16). The biological function of each peptide during nodule development and nitrogen fixation remains to be determined. Since NCR044 has been previously demonstrated to inhibit the human fungal pathogen C. albicans (8) and is more cationic than NCR473, it was chosen for further studies aimed at determining its structure, antifungal activity against plant fungal pathogens in vitro and in planta, and modes of action as described below.

**Expression of Recombinant NCR044 in Pichia pastoris and Mass Spectral Analysis of the Purified Peptide.** In order to generate the NCR044 peptide with correctly formed disulfide bonds, the NCR044 gene was expressed in a heterologous Pichia pastoris expression system. The peptide secreted into the growth medium was successfully purified using cation exchange and C18 reverse-phase high-performance liquid chromatography. Mass spectral analysis revealed a molecular mass of 4311.28 Da for the purified product (SI Appendix, Fig. S1 A and B) as expected for disulfide-linked NCR044. The purity of the NCR044 peptide was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis (SI Appendix, Fig. S1C).

**Disulfide Bonding Pattern in NCR044.** NCR044 contains four cysteine residues (C9, C15, C25, and C31) with three possible patterns of intermolecular disulfide bond formation: (i) C9–C15, C25–C31, (ii) C9–C25, C15–C31, (iii) C9–C31, C15–C25. The disulfide bonding pattern in NCR044 was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis (SI Appendix, Fig. S1C).

Fig. 1. Amino acid sequences and properties of NCR044 and NCR473 and expression of their genes in root nodules of M. truncatula cv. Jemalong A17. (A) Primary amino acid sequences of the signal and mature peptide sequences of NCR044 and NCR473. Mature peptide sequences are each 36 residues in length. Conserved cysteine residues are highlighted in red and six nonconserved residues are highlighted in blue. (B) Net charge, hydrophobic amino acid content, and molecular weight of the mature NCR044 and NCR473 peptides. (C) Gene expression analysis of NCR044 and NCR473 in root nodules and roots at 10 d postinfection (dpi) by S. meliloti. (D) NCR044 and NCR473 transcript abundance in laser-dissected zones of mature nodules at 15 dpi. FI: nodule meristematic zone, fraction; FIl: infection zone fraction; FILp: infection-late differentiation zone, proximal fraction; IZ: late differentiation zone, interzone II–III; ZIII: nitrogen-fixation zone. Web-based RNA sequencing data are available at the Symbimics website (https://iant.toulouse.inra.fr/symbimics).
pattern of oxidation in NCR044 is homogeneous. NMR measurement of the peptide’s rotational correlation time ($\tau_c$), $3.6 \pm 0.5$ ns, was consistent for an $\sim4.0$-kDa peptide (17), indicating it was primarily monomeric in solution with no detectable intermolecular disulfide bond formation.

Fig. 2 A and B show the assigned $^1$H-$^{15}$N heteronuclear single quantum coherence (HSQC) spectra for the oxidized and reduced forms of NCR044, respectively. For oxidized NCR044 (Fig. 2A), the wide chemical shift dispersion of the amide resonances in both the proton and nitrogen dimensions are characteristic features of a structured protein (18). A single set of cross peaks that all could be assigned suggests one dominant species is present with a homogeneous intramolecular disulfide bond pattern. Disulfide bond formation in NCR044 was unambiguously verified from the NMR chemical shifts of the $\beta$-carbon of cysteine residues. Generally, the cysteine $^{13}$C$\beta$ chemical shift in the oxidized state is $>35$ ppm and decreases to $<32$ ppm in the reduced state (19). In the absence of Tris(2-carboxyethyl)phosphine (TCEP) or other reducing agents, the cysteine $^{13}$C$\beta$ chemical shifts for NCR044 were $38.0$ ppm or greater, as indicated in SI Appendix, Table S1, clearly indicating that cysteine residues were oxidized and incorporated in disulfide bonds. These $^{13}$C$\beta$ chemical shifts decreased to $28$ ppm or less in the presence of $5 \text{mM} \text{TCEP}$, a significant change indicating that disulfide bonds were ablated.

While the chemical shift data indicated disulfide bonds were present, it was not possible to use preliminary structure calculations to deduce unambiguously the disulfide bond pairs from $^1$H-$^1$H nuclear Overhauser effects (NOEs) due to the consequences of proximal $\gamma$-sulfur atoms. Instead, the disulfide bond pattern was deduced from the analysis of mass spectral data for trypsin-digested, unlabeled peptide in the reduced and oxidized states. These data showed that disulfide linkages were $C9$–$C30$ and $C15$–$C25$. Note that the disulfide bonds are essential for the stability of the peptide’s tertiary structure (20) as illustrated in the $^1$H-$^{15}$N HSQC spectrum for reduced NCR044 in Fig. 2B. The range of chemical shift dispersion of amide resonances was greatly compressed relative to the oxidized NCR044 (Fig. 2B), a characteristic feature of disordered proteins (18).

Solution NMR Structure of NCR044. Fig. 2C is a superposition of the final ensemble of structures calculated for NCR044 (PDB: 6U6G).
with a single cartoon representation of this ensemble shown in Fig. 2D. The most striking feature is the paucity of canonical elements of secondary structure in the peptide. NCR044 contains one short (A23 to R25, G28 to C30) antiparallel β-sheet and a “whiff” (S11 to E14) of an α-helix. The rest of the peptide was largely disordered and dynamic, even with the tethering afforded by the two disulfide bonds. A search for structures in the PDB similar to NCR044 with the DALI server (21) generated no hits, suggesting this structure was new to the PDB. The disorder in the structure was reflected in the paucity of 1H-1H NOEs observed in the nuclear Overhauser effect spectroscopy NMR data. As shown in SI Appendix, Table S2, most of the NOEs were intraresidue with only 12 long-range NOEs observed. The prevalent disorder was also reflected in the analysis of the 13Cα and 13Cβ chemical shifts. Deviations of 13Cα and 13Cβ chemical shifts from random coil values are predictive of canonical α-helical (positive Δδ13Cα, negative Δδ13Cβ) and β-strand (negative Δδ13Cα, positive Δδ13Cβ) secondary structure (20, 22). These deviations from random coil values were plotted for oxidized and reduced NCR044 in Fig. 2E and showed modest correlation for their respective short elements of the secondary structure identified in the NMR structure calculations for the oxidized peptide. Upon reducing the disulfide bonds in the presence of 5 mM TCEP, aside from the N-terminal region (A1 to I10) which was largely disordered in the oxidized state to begin with, these modest correlations disappear with most of the chemical shift values not significantly differing from random coil values. These observations suggest the disulfide bonds play a significant role in stabilizing the structure of the oxidized peptide and in their absence the peptide was largely disordered with little transient structure.

NCR044 Exhibits Potent Antifungal Activity Against Plant Fungal Pathogens. Chemically synthesized NCR044 was previously reported to exhibit fungicidal activity against the human fungal pathogen C. albicans at low micromolar concentrations (8). Here, we determined the antifungal activity for recombinant NCR044 against a panel of closely related plant fungal pathogens, Fusarium spp. and B. cinerea, using a spectrophotometric assay. NCR044 inhibited the growth of B. cinerea at low micromolar concentration with a half-maximal inhibitory concentration (IC50) value of 1.55 ± 0.21 μM (Fig. 3A). NCR044 also inhibited the growth of Fusarium graminearum and Fusarium virguliforme with IC50 values of 1.93 ± 0.23 and 1.68 ± 0.20 μM, respectively. Fusarium oxysporum was most sensitive to NCR044 with an IC50 value of 0.52 ± 0.01 μM (Fig. 3A). The resazurin cell viability assay revealed that F. oxysporum spores lose their cellular metabolic activity at a concentration of 1.5 μM, whereas other fungi, including B. cinerea, lose their metabolic activity at a concentration of 3 μM (Fig. 3B and C). We also determined the antifungal activity of the chemically synthesized reduced form of the NCR044 peptide against B. cinerea. The reduced form of the NCR044 peptide inhibited the growth of B. cinerea with IC50 value of 4.16 ± 0.18 μM as compared with 1.55 ± 0.21 μM for the native oxidized form of the peptide. The resazurin cell viability assay revealed that B. cinerea spores lose their cellular metabolic activity at a concentration of 6 μM as compared with 3 μM for the native oxidized form of the peptide (SI Appendix, Fig. S2A and B).

NCR044 Disrupts the Plasma Membrane of B. cinerea. Antifungal peptides are known to interact with phospholipid bilayers and disrupt the plasma membrane (14). We postulated that the antifungal activity of NCR044 occurred via membrane permeabilization. Using a fluorescent SYTOX Green (SG) nucleic acid staining dye that permeates fungal cells when their plasma membrane integrity is compromised, we tested the ability of NCR044 to permeabilize the plasma membrane of B. cinerea.

The uptake of SG in B. cinerea spores and germlings treated with 3 μM NCR044 for 15 min was first monitored using confocal microscopy. The nuclei of both spores and germlings were stained with SG, suggesting altered cellular membrane integrity induced by NCR044 (Fig. 4 A–H). The kinetics of membrane permeabilization was determined in B. cinerea germlings treated with various concentrations of NCR044 every 30 min for up to 3 h. In NCR044-treated fungal germlings, permeabilization of the plasma membrane was observed within 30 min and reached its maximal level at 120 min. The rate of membrane permeabilization increased with increasing concentrations of NCR044 as shown in Fig. 4I. To test if membrane disruption was sufficient for antifungal activity of NCR044 in ungerminated B. cinerea spores, we incubated the spores in 3 μM NCR044 for 1 h, washed off the free peptide, and then allowed the spores to germinate for 24 h at room temperature in peptide-free growth medium. Our data showed that B. cinerea spores were able to resume their growth (Fig. 4 J–L) in a peptide-free growth medium suggesting that, while increased membrane permeability is insufficient to induce death, the peptide served as a potent inhibitor of germination.

NCR044 Elicits Rapid Production of Reactive Oxygen Species (ROS) in B. cinerea Germlings, but Much Reduced ROS in Spores of B. cinerea. Antimicrobial peptides with a different mode of action are
known to induce ROS and cause cell death putatively through apoptosis or necrosis-like processes (23, 24). To determine if NCR044 elicited production of ROS, we challenged the spores and germlings of *B. cinerea* with 3 μM of NCR044 for 2 h in the presence of the dye 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). Upon intracellular oxidation by ROS, H$_2$DCFDA is converted into a highly fluorescent 2′,7′-dichlorofluorescein (DCF) that can be monitored using confocal microscopy. ROS production was observed in germlings, but not in spores (SI Appendix, Fig. S3A–F and M–R). Indeed, relative to germlings, only very weak ROS fluorescence signal was detected in spores even after overnight incubation in peptide and histogram rescaling to enhance the very-low-level ROS signals (SI Appendix, Fig. S3 G–L). Real-time quantification of ROS production was measured spectrophotometrically every 30 min for up to 2 h with untreated germlings and germlings treated with 3 μM NCR044. Rapid induction of ROS fluorescence within 30 min was observed and the fluorescence intensity increased in a time- and dose-dependent manner. The highest fluorescence intensity was observed at 3 μM, suggesting the elicitation of oxidative stress by ROS in germlings following NCR044 treatment (SI Appendix, Fig. S35).

**NCR044 Binds to Multiple Membrane Phospholipids.** Several antifungal defensins have been shown to recruit plasma membrane-resident phospholipids as part of their MOA (24–26). Using the protein–lipid overlay assay, we assessed the ability of NCR044 to bind different bioactive membrane phospholipids. NCR044 strongly bound to phosphatidylinositol 3,5-bisphosphate [PI(3,5)P$_2$]. It also bound weakly to multiple other phosphoinositides and to phosphatidic acid (PA) (Fig. 5A and B). To further investigate the phospholipid-binding specificity of NCR044, a polyPIPosome assay was performed with polymerized liposomes. As shown in Fig. 5C, NCR044 bound strongly to liposomes that contained PC:PE:PI(4)P and PC:PE:PI(3,5)P$_2$ and weakly to PC:PE containing liposomes.

**NMR Spectroscopy Reveals Interaction of NCR044 with PI3P.** On the basis of the above protein–phospholipid overlay assay, we conducted an NMR chemical shift perturbation study with
15N-labeled oxidized NCR044 and PI(3)P to further corroborate phospholipid binding, and ideally, identify the lipid binding surface of NCR044. This experiment is based on the premise that backbone amide protons are sensitive to their molecular environment, and consequently, it is possible to identify the surface of ligand binding to a polypeptide by following amide chemical shift (or intensity) perturbations in 1H-15N HSQC spectra following the titration of ligand into the sample (27, 28). For NCR044, no chemical shift perturbations were observed over the titration range, but the intensity of the amide cross peaks was observed to decrease starting at a PI(3)P:NCR044 molar ratio of 9.4:1. As shown in SI Appendix, Fig. S4A, there were remnants of only 12 amide cross peaks at a PI(3)P:NCR044 molar ratio of 18.8:1. These intensity perturbations in the 1H-15N HSQC spectra of NCR044 showed that the peptide was interacting with PI(3)P (29). This was corroborated by overall rotational correlation time ($\tau_c$) measurements of the peptide at each titration point that increased from 3.6 ± 0.5 ns in the absence of PI(3)P to 8.7 ± 2.5 ns at a PI(3)P:NCR044 molar ratio of 18.8:1. The latter $\tau_c$ value corresponded to a molecular weight in the 15-kDa range (17). Because amide cross peaks should be readily detectable in the 1H-15N HSQC spectrum of an ~15-kDa species, potential explanations for the disappearing amide resonances include 1) heterogeneous binding to the phospholipid that generates multiple different chemical environments for the peptide with populations that cannot be detected and 2) dynamic interactions with a large molecular complex formed by PI(3)P in solution. To the best of our knowledge, the solution properties of PI(3)P have not been characterized at high concentrations. However, given the aliphatic tails on one end of the molecule and the hydrophilic inositol head group on the other end, it is possible that PI(3)P forms high molecular weight micellar structures at high lipid concentrations. Interactions between PI(3)P and NCR044 are likely electrostatic in nature. The hydrophilic head group of PI(3)P contains two negatively charged phosphate groups. As shown in SI Appendix, Fig. S4B, the surface of NCR044 is dominated by a positively charged surface that covers the majority of the peptide, providing multiple sites for binding to the hydrophilic head group of PI(3)P.

**Exogenous NCR044 Is Translocated into the Cells of B. cinerea.** We used DyLight550-labeled NCR044 to examine its intracellular translocation into the B. cinerea spores and germlings. In spores incubated with 3 μM DyLight550-NCR044, the peptide first accumulated on the cell surface within 1 h (SI Appendix, Fig. S5 A–F), while only 7.7 ± 2.2% of spores internalized peptide. However, B. cinerea spores increased peptide uptake following overnight incubation with diffuse localization in the cytoplasm. Specifically, we quantified the percentage of spores that internalized DyLight550-labeled NCR044 following overnight incubation and observed a significant increase ($P = 0.0057, t = -5.4$, degrees of freedom [df] = 4, unpaired Student’s $t$ test) (Fig. 6 A–C) with internalization in 18.8 ± 2.2% in 1.5 μM and 47.5 ± 4.8% in 3-μM treated spores. We also treated spores overnight with 3 μM DyLight550-labeled NCR044, washed off the unbound peptide, and then allowed the spores to germinate in peptide-free growth medium. Our data showed that B. cinerea spores were able to resume their growth (SI Appendix, Fig. S6 A–F) and broad spore death had not occurred, suggesting that the continuous presence of peptide in solution was required to block germination. The continuous presence of DyLight550-labeled NCR044 blocked germination of B. cinerea spores (SI Appendix, Fig. S6 G–L). The membrane selective dye FM4-64 was subsequently used to determine if DyLight550-labeled NCR044 colocalized with cellular membranes in B. cinerea germlings. In spore heads and germlings, NCR044 first bound to the cell wall (Fig. 6 D–F, arrows) and cell membrane (Fig. 6 D–F, arrowheads). NCR044 colocalized with FM4-64 at bright foci at the germling tip and small foci intermittently along the cell periphery of B. cinerea (Fig. 6 G–I, asterisks). This suggested that this peptide likely interacted with plasma membrane resident phospholipids which invaginated and/or endocytosed to enter fungal cells. Additionally, we observed that NCR044 was distributed diffusely in the cytoplasm and concentrated within the nucleus (Fig. 6 D–I and N), whereas FM4-64 showed clear cytoplasmic membrane profiles and was excluded from the nucleus, suggesting that NCR044 localized at other nonmembrane intracellular structures.

Next, we applied time-lapse confocal microscopy to monitor the uptake of 3 μM DyLight550-labeled NCR044 in germlings. The peptide initially was observed associated with the germling cell wall (Fig. 6 J, T = 0 and 3:55, arrowheads) and then migrated toward foci at the germling tip (Fig. 6 J, T = 0, asterisks) or the germling and/or spore cell walls (Fig. 6 J, T = 0 through T = 9:51, asterisks). These foci appeared to be the sites of cytoplasmic entry and coincided with a gradual loss of turgor (compare Fig. 6 J, T = 0 with T = 36:41). Within 15 min, the peptide, in...
general, was diffusely localized in the cytoplasm of the germlings and tended to associate with bright spherical structures consistent with nuclei (Fig. 6f, T = 6:55 through 36:41, arrows). Often, early entry into the cytoplasm was observed as a gradient across the cell dependent on the origin of plasma membrane breach.

**Superresolution Microscopy Reveals Nucleolar Localization of NCR044 in B. cinerea.** We applied lattice structured illumination microscopy to clearly identify the subcellular localization of DyLight550-labeled NCR044 at high resolution. Since we determined that DyLight550-labeled NCR044 was preferentially localized to the nucleus of B. cinerea (Fig. 7A and B), we applied the DNA staining dye DAPI to see if the peptide bound to the nuclear DNA. As shown in Fig. 7C, DyLight550-NCR044 did not colocalize with DAPI in the nuclei of the germlings, and indeed, appeared to be explicitly excluded from nuclear DNA. We then used the nucleolus-specific RNA staining dye, Nucleolus Bright Green (NBG), to monitor the colocalization with the peptide. We observed that DyLight550-NCR044 strongly colocalized with NBG (Fig. 7D) at the site of ribosomal biogenesis.

**Fluorescence Recovery after Photobleaching Experiments Reveal that NCR044 Has Strong Binding Affinity to B. cinerea Spore Cell Walls and Increased Affinity to the Nucleolus in Germlings.** In order to understand the mobility of DyLight550-NCR044 to specific subcellular domains in B. cinerea, we performed a series of fluorescence recovery after photobleaching (FRAP) experiments.
NCR044 was also tested for its ability to confer resistance to B. cinerea in a rose petal infection assay. It significantly reduced virulence of the pathogen on rose petals as compared with control petals without peptide treatment ($K_{W_{\text{statistic}}} = 76.36$, df = 3, $P < 0.0001$). Almost complete suppression of disease symptoms was observed at a concentration of 1.5 μM peptide compared to rose petals inoculated with pathogen only (pairwise Wilcoxon test, corrected $P < 0.0001$) (SI Appendix, Fig. S8 C and D).

**Spray-Applied NCR044 Confers Gray Mold Resistance in N. benthamiana and Tomato Plants and Is Not Internalized by Plant Cells.** We tested the potential of NCR044 for use as an antifungal peptide for controlling gray mold disease in young N. benthamiana and tomato plants. At a concentration of 24 μM, NCR044 was sprayed onto the leaves of 4-wk-old N. benthamiana and 15-d-old tomato plants and allowed to dry. The Kruskal–Wallis test showed significant differences in disease severity between different treatment groups of N. benthamiana ($K_{W_{\text{statistic}}} = 22.75$, df = 2, $P < 0.0001$) and tomato plants ($K_{W_{\text{statistic}}} = 29.06$, df = 2, $P < 0.0001$). N. benthamiana and tomato plants sprayed with the peptide had significantly reduced disease symptoms at 48 h (pairwise Wilcoxon test, corrected $P < 0.01$, Fig. 8 A and B) and 60 h (pairwise Wilcoxon test, corrected $P < 0.01$, Fig. 8 C and D) postinoculation, respectively, compared to control plants sprayed with pathogen only. The photosynthetic efficiency (variable fluorescence over saturation level of fluorescence [Fv/Fm]) of the control plants (no peptide) exposed to pathogen was significantly lower compared to plants sprayed with NCR044 prior to pathogen exposure. In an effort to determine if defenses internalization/localization observed in fungi also occurred with plant cells, we applied a droplet of 24 μM DyLight550-NCR044 to the leaf surface of N. benthamiana and imaged it by confocal microscopy. Our results indicated that DyLight550-NCR044 remained on the plant surface, concentrating at anticlinal walls, with no evidence of internalization within the leaf epidermal cells (SI Appendix, Fig. S9 A–I).

**Discussion.** M. truncatula expresses ~700 NCR peptides during the establishment of successful symbiosis with S. melloti (2–4). These peptides are thought to be involved primarily in causing differentiation of the rhizobacteria into bacteroids. However, a subset of these peptides with high cationicity exhibit antimicrobial activity in vitro and in planta (30). Several NCR peptides have now been shown to exhibit bactericidal activity against various...
Among the hundreds of NCR peptides expressed are two NCR044 homolog peptides that share 83% sequence identity and high cationicity. In this study, we report the structure, antifungal activity, and MOA of one of these peptides, NCR044. In addition, we demonstrate its potential for control of pre- and postharvest fungal diseases.

Pre- and postharvest fungal diseases cause substantial losses in the yields of many important crops. These diseases are currently mitigated primarily through application of chemical fungicides multiple times. Unfortunately, such chemical fungicides pose considerable health and environmental risks and become ineffective over time due to the evolution of resistance. Alternative means for control of fungal infections are peptide-based antifungal agents applied topically to plants (31). The oxidized form of the NCR044 peptide exhibits potent broad-spectrum antifungal activity in vitro against four economically important fungal pathogens tested in this study. The antifungal activity of the oxidized form of the NCR044 peptide exhibited potency similar to that of other antifungal peptides (25, 26, 31). However, other factors such as a growth medium used in testing the antimicrobial activities can strongly affect the efficacy of antifungal peptides (32). Our initial experiments with a reduced form of the NCR044 peptide showed over a two-fold decrease in the in vitro antifungal activity against B. cinerea, indicating the importance of disulfide bonds for increased potency of the oxidized form of NCR044. For deployment of NCR044 in crop protection, one could envisage further manipulating the cationicity/hydrophobicity of the oxidized or reduced NCR044 through site-directed modifications.

Fig. 8. Spray application of NCR044 confers gray mold resistance in N. benthamiana and tomato plants. (A and B) Four-wk-old N. benthamiana and (C and D) 2-wk-old Solanum lycopersicum L. cv. Mountain Spring plants sprayed with either 2 mL water or NCR044 (24 μM) prior to exposure with a spray containing 1 mL of a 5 × 10⁶ B. cinerea fungal spore suspension. Maximum quantum yield of PSI photochemistry (Fv/Fm) and the chlorophyll (Chl) index were measured after 48 h (N. benthamiana) and 60 h (tomato), after fungal exposure. The false color gradient scale next to the figure indicates the efficiency of photosynthesis. Red (Fv/Fm) or black (Chl) colors represent a low efficiency of photosynthesis, characteristic of stress caused by the interaction between the pathogen and plant. Green (Fv/Fm) or gray (Chl) colors indicate higher efficiency of photosynthesis. NCR044 significantly protected tobacco and tomato plants from gray mold disease caused by B. cinerea. The calculated photosynthetic quantum yield (Fv/Fm) of (B) tobacco and (D) tomato plants. Each colored boxplot represents the 25th and 75th percentiles (box), and the whisker represents 1.5 times the interquartile range (IQR) from the 25th and 75th percentiles. The horizontal line in the box plot represents the median with the mean ± SEM denoted by a dot. Outliers are indicated by large dots outside 1.5*IQR above the first and third quartile. The individual measurements of Fv/Fm are indicated on the Left of each boxplot and represent the variances of the samples. The number (n = 12) of plants tested is indicated below each group in the boxplot and is from at least two independent experiments for tobacco. Similar results were observed in a third independent experiment, except the data were collected 60 h after fungal exposure. The number (n = 15) of plants tested is indicated below each group in the boxplot and is from at least three independent experiments for tomato. Asterisks represent significant differences between different groups (**P < 0.01, ****P < 0.0001, pairwise Wilcoxon test with Holm correction, Kruskal–Wallis for multiple groups).
mutagenesis and increases its antifungal potency on the surface of plant tissues without causing phytotoxicity.

When applied on the surface of lettuce leaves and rose petals, the oxidized form of the NCR044 peptide significantly reduces gray mold disease lesions caused by *B. cinerea* infection. Moreover, spray application of this peptide controls gray mold disease symptoms in young tomato and *N. benthamiana* plants. These data warrant additional studies to investigate the potential of NCR044 as a spray-on peptide antifungal agent for the control of gray mold disease in vegetables, flowers and other economically important crops. In contrast to chemical fungicides, peptide-based “green chemistry” antifungal agents are sustainable and ideal for protection of the environment and consumer health (31).

In the inverted repeat-lacking clade legumes such as *M. truncatula*, two families of cysteine-rich peptides, namely defensins and defensin-like NCRs, are expressed. These two families have likely evolved in parallel in this legume to play different biological roles. While defensins have evolved for defense against harmful pathogens early in the evolution of land plants, NCRs have evolved relatively recently and act as effectors inducing differentiation of rhizobia into nitrogen-fixing bacteria (5). Therefore, antifungal activity is a secondary attribute of NCR044. However, the possibility cannot be ruled out that it acts in concert with other NCRs to defend differentiating nodules from harmful fungal and bacterial pathogens in *M. truncatula* (33). Antifungal plant defensins with four disulfide bonds are diverse in their amino acid sequences but, their 3D structures with a highly conserved cysteine-stabilized α/β motif are strikingly similar (34, 35). To our knowledge, no 3D structure of an NCR peptide has been determined to date. The structure of NCR044 reported here is dominated by largely disordered regions. It remains to be determined if this disordered structure adopts an ordered secondary structure in membrane-mimetic solutions and contributes to the biochemical function and/or stability of this peptide. It is interesting to note that the predominant disulfide pattern of the oxidized NCR044 (C1 to C4, C2 to C3) is the same as the C1 to C4, C2 to C3 pattern of the predominant derivative of NCR247 oxidized in vitro (33). Several hundred NCR peptides expressed in the nodules of *M. truncatula* also exhibit significant variation in their amino acid sequences but the possibility cannot be ruled out that similar patterns are shared in their structures and antifungal MOA (36).

A common characteristic of antifungal peptides is their ability to permeabilize the plasma membrane of fungal pathogens. In plant defensins, membrane permeabilization is also an important early step in their MOA (34, 35). Recently, the peptides NCR192, NCR247, and NCR335 have been reported to disrupt the plasma membrane of *C. albicans* (8). In the mechanistic studies employing NCR044 here, we determined that NCR044 quickly binds to the cell walls of *B. cinerea* spores and germlings and subsequently breaches the plasma membrane of these cells near the attachment sites. Within 30 min after peptide exposure, SYTOX Green uptake was observed at a dose-dependent rate. Thus, membrane permeabilization is also an early step in fungal cell death induced by NCR044. This was further supported by our observation that as cytoplasmic loading of DyLight550-labeled NCR044 peptide occurred, it coincided with concomitant loss of turgor. However, spores temporarily exposed to NCR044 for a period of 1 h or overnight could subsequently germinate. It was noted that peptide localization in uncongested spores appeared to be largely restricted to the spore wall with few exceptions, (presumed to be compromised dead spores). Taken together, these data showed that the peptide inhibited germination and additionally, the quiescent spores appeared to lack the physiological activity to allow sufficient membrane permeabilization and induce cell death as observed in germlings.

Since several plant defensins bind to plasma membrane resident bioactive phosphoinositides or related phospholipids to oligomerize and induce membrane permeabilization (26, 35), we tested whether NCR044 also bound to specific phospholipids. Like the plant defensins NaD1 (26) and MtDe55 (24), NCR044 bound to several phospholipids in the phospholipid–protein overlay assay. Of note, it bound more effectively to PI mono/diphosphates, in particular, PI(3,5)P2. Further studies are needed to decipher the mechanism by which membrane permeabilization is induced by NCR044 and to understand the role phospholipid binding plays in peptide-induced membrane permeabilization of fungal pathogens.

Inhibition of mitochondrial respiration leads to the generation of ROS that play an important role in fungal cell death (34, 35). Using the dye H2DCFDA, ROS generation was readily detected in NCR044-challenged germlings, but under the same conditions, not detected in spores of *B. cinerea*. However, very low levels of ROS were observed in spores after long exposure and histogram rescaling. This observation is consistent with the overall reduced peptide uptake in quiescent spores relative to germlings. This was further supported by our observation that *B. cinerea* spores treated with DyLight550-labeled NCR044 following overnight incubation were able to resume their growth after removing the unbound peptide from the media. However, further studies are needed to unambiguously establish NCR044 as an induced ROS generation as a cause of cell death. In particular, it will be important to obtain evidence for the induction of the markers of ROS-mediated oxidative damage, such as protein carbonylation and DNA laddering, that lead to cell death.

Internalization of NCR044 into the spores and germlings of *B. cinerea* has been studied in relation to its antifungal activity. While NCR044 internalization by spores was slow and limited, it was internalized rapidly by germlings of this pathogen. Our colocalization experiments with FM4-64, an amphiphilic styryl dye that serves as a marker for endocytosis and vesicle trafficking, provided important clues about NCR044 initial entry into fungal cells. The earliest signs of internalization of DyLight550-labeled NCR044 were often indicated by a single patch near the germination tip (Fig. 6 G and J, T = 0) and smaller foci just beneath the cell wall (Fig. 6 G–I) which colabeled with FM6-64. This pattern was similar to that reported for *M. truncatula* defensin MtDef55 (37, 38). Once internalized, the peptide appeared to diffuse through the cytoplasm and NCR044 gradients formed (Fig. 6J) that coincided with origins of initial cell surface foci but no longer matched the classical membrane/vesicle-labeled pattern of FM4-64 (Fig. 6 D–I). Once cytoplasmic, the peptide localized to nucleoli, the site of ribosomal biogenesis. Interestingly, NCR247 has also been shown previously to interact with ribosomal proteins in the symbiont *S. melliloti* (12). While the exact mechanism for nucleolar binding is unknown, electrostatic maps of ribosomal subunits show large areas of negative potential (39) and this property significantly influences interactions with positively charged proteins (40), and conceivably, NCR044 with a net charge of +9. It will be important to determine if other antifungal NCR peptides from IRLC legumes also target nucleoli in fungal cells. Interaction with ribosomes and inhibition of translation are likely to be a shared mechanism used by some antifungal NCR peptides (12). While it is intriguing to speculate that this could be a possible mechanism used by NCR044, further supporting experiments are required to understand the significance of this observation. However, the possibility remains that these peptides have multiple intracellular targets to induce fungal cell death, a notion recently proposed for a highly effective fungicide strategy using monoalkyl chain lipophilic cations (41).
Based on the results reported here, we propose a multistep model for the antifungal action of NCR044 against *B. cinerea* (Fig. 9 A–C). The first step involves binding of the peptide to the cell wall and inhibition of germination (when applied to spores) followed by binding and internalization at putative sites of endocytosis and then disruption of the plasma membrane via interaction with membrane-resident phospholipids (in germlings). The peptide eventually gains entry into the cytoplasm, inducing the formation of ROS and localization to nucleoli, where it is hypothesized to interact with ribosomal RNA and/or ribosomal proteins and inhibit protein translation. Our data show that NCR044 interacts with several major and diverse cellular components, perhaps permitted by its highly dynamic structure as determined by NMR, thus allowing broad effectiveness against fungal pathogens. These insights necessitate further studies to better understand the relative contribution of each step to the antifungal action of NCR044. Hundreds of NCR peptides expressed in nodules of certain legumes offer highly diverse antifungal peptide sequence space and this work on NCR044 peptide structure and its MOA will help in the design of more effective antifungal peptides using protein engineering for future use in agriculture.

**Materials and Methods**

Details of each experimental method, including statistical analysis, fungal cultures, and spore suspensions, recombinant expression and purification of NCR044, 15N and 13C isotopic labeling of NCR044 for NMR structural analysis, NMR spectroscopy of NCR044, NMR solution structure calculations for the oxidized form of NCR044, antifungal activity assay, 5G membrane permeabilization assay, quantification of intracellular ROS, NCR044 uptake into fungal cells, FRAP experimental setup and analysis, peptide distribution on the plant surface, NCR peptide–phospholipid interactions, semi-in planta antifungal activity of NCR044 against *B. cinerea*, and evaluation of NCR044 as a sprayable peptide for protection against gray mold disease are given in SI Appendix.

**Data Deposition.** The coordinates of the NMR structure have been deposited in the Protein Data Bank, www.rcsb.org (PDB code: 6U6G) and the NMR chemical shifts have been deposited in the BioMagResBank, www.bmrbr.wisc.edu (acccession no. 30660).

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