An engineered innate immune defense protects grapevines from Pierce disease

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Edited by Eugene W. Nester, University of Washington, Seattle, WA, and approved January 20, 2012 (received for review September 29, 2011)

We postulated that a synergistic combination of two innate immune functions, pathogen surface recognition and lysis, in a protein chimera would lead to a robust class of engineered antimicrobial therapeutics for protection against pathogens. In support of our hypothesis, we have engineered such a chimera to protect against the Gram-negative *Xylella fastidiosa* (Xf), which causes diseases in multiple plants of economic importance. Here we report the design and delivery of this chimera to target the Xf subspecies *fastidiosa* (Xff), which causes Pierce disease in grapevines and poses a great threat to the wine-growing regions of California. One domain of this chimera is an elastase that recognizes and cleaves MopB, a conserved outer membrane protein of Xff. The second domain is a lytic peptide, cecropin B, which targets conserved lipid moieties and creates pores in the Xff outer membrane. A flexible linker joins the recognition and lysis domains, thereby ensuring correct folding of the individual domains and synergistic combination of their functions. The chimera transgene is fused with an amino-terminal signal sequence to facilitate delivery of the chimera to the plant xylem, the site of Xff colonization. We demonstrate that the protein chimera expressed in the xylem is able to directly target Xff, suppress its growth, and significantly decrease the leaf scorching and xylem clogging commonly associated with Pierce disease in grapevines. We believe that similar strategies involving protein chimeras can be developed to protect against many diseases caused by human and plant pathogens.

disease protection | pathogen clearance | symptom suppression

Innate immune response is the first line of host defense against invading pathogens. This response occurs readily after pathogen recognition by the host cell through intracellular signaling and subsequent expression of effector molecules, such as lytic antimicrobial peptides, cytokines, and reactive oxygen species, that are involved directly or indirectly in pathogen clearance (1). However, many pathogens successfully circumvent the innate immune defense and manage to grow inside the host, establish infection, and cause disease (2, 3). It is our hypothesis that although a pathogen might be able to block the individual functions (i.e., pathogen recognition by host cells, intracellular host signaling, and function of the effector molecules), it would have difficulty overcoming a combination of two innate immune functions in the same molecule (4). Specifically, we believe that the synergistic combination of pathogen recognition protein and lytic peptide in a chimera would be very effective in blocking infection and thus represent a unique class of protein therapeutics. The efficacy of these chimeras would depend on the degree of synergism achieved between the chimera components and how effectively these chimeras were delivered to sites of pathogen colonization.

For a demonstration of chimera efficacy, we targeted *Xylella fastidiosa* (Xf), a Gram-negative bacterial pathogen with a wide range of plant hosts of economic importance (5–8). Xf is vectored primarily by sharpshooter insects and colonizes in the plant xylem (6, 7). The Xf subspecies *fastidiosa* (Xff), as exemplified by the California strain Temecula1, causes Pierce disease (PD) in grape. In this paper, we report that a transgene expressing a protein chimera containing recognition and lysis domains, expressed in multiple grape lines, significantly reduced the Xff level in the xylem and ameliorated the symptoms of PD. Transgenic model and crop plants expressing antimicrobial peptides (e.g., insect cecropins) have been shown to display resistance against various bacterial and fungal pathogens (9). Given that the level of protection by a single antimicrobial peptide is only moderate at best, efforts have been made to fuse two antimicrobial peptides (e.g., cecropin and melittin) to improve their efficacy in disease protection (10). Moreover, because antimicrobial peptides are inherently unstable due to their susceptibility to plant proteases, amino acids have been altered to improve their stability in plant tissues (11). However, to the best of our knowledge, an effective protein chimera combining recognition and lysis domains and targeting two different but conserved moieties on the pathogen surface has not yet been reported. We describe the anti-Xff properties of our chimera and propose an explanation for its much greater effectiveness compared with an antimicrobial peptide alone.

Results

Outer-Membrane Protein MopB Is a Logical Xf Recognition Target for Our Strategy. Our first step in designing a chimera was selecting an appropriate recognition target on the Xf surface. We selected MopB as our recognition target based on four main observations. First, MopB is the most abundant protein in Xff membrane preparations detected on mass spectrometry analysis (Fig. S1). Second, the sequence of MopB was identical in all Xf subspecies sequenced thus far (12–14). Third, MopB is accessible on the Xff cell surface, as evidenced by strong staining of the Xf cell surface by Alexa Fluor 488-labeled rabbit anti-MopB polyclonal antibody (Fig. 1*A*). Fourth, sequence and structure analysis revealed that MopB contains recognition and cleavage sites for human neutrophil elastase (HNE), a host defense protein. Our cleavage studies confirmed that MopB is indeed a target for HNE (Fig. 1*B*), although the membrane-bound MopB appears to be partially protected relative to the soluble MopB. In addition, as a member of the multifunctional porin family, MopB is an attractive Xff target because it is likely critical for bacterial growth and viability (15, 16) and, like other porins, is an expected target for host defense (17, 18). The observation that porin deletion

Author contributions: A.M.D., G.B., E.C., and G.G. designed research; H.G., A.M.I., S.L.U., C.B.A., S.M., Y.B., P.A.F., G.B., R.N., P.E.P., A.C., and M.N. performed research; C.B.A. performed plant transformations; L.R.G. supervised the mass spectrometric work of R.N.; L.R.G. analyzed data; and A.M.D., G.B., and G.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1116027109/-/DCSupplemental.

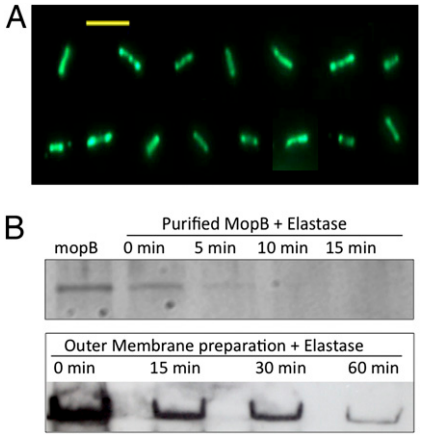

Fig. 1. HNE–MopB interactions. (*A*) Staining of *X. fastidiosa* sbsp. *fastidiosa* Temecula1 strain (Xff, causal agent of PD disease of grapevines) by fluorescently labeled IgG from rabbit polyclonal antibody raised against Xf-MopB purified from bacterial culture. The images were assembled from seven fields to be representative of cells with their long axis parallel to the substrate. (Scale bar: 5 μm.) (*B*) Visualization of the digestion of Xff MopB by HNE on denaturing gels using purified MopB (detection by Coomassie stain) (*Upper*) and an Xf outer membrane preparation (detection by immunoblotting with anti-MopB polyclonal antibody) (*Lower*). Approximately 5 ng of purified MopB was incubated with 0.5 μg of HNE for the indicated times before analysis by electrophoresis. Aliquots of Xff cell suspension were incubated with 0, 0.1, 0.3, and 1 μg of elastase (Calbiochem) at 37 °C for 1 h before immunoblotting.

mutants of bacteria are more susceptible to bactericidal effects (19) is particularly noteworthy for our strategy.

HNE Enhances the Bactericidal Effect of Cecropin B. As the lysis component of our chimera, we selected the peptide cecropin B, which shows a preference in its lytic action for Gram-negative bacteria (20). Because HNE, through cleavage, can make Xff more vulnerable to bactericidal agents, as was observed for porin deletion (19), it was of interest to test whether HNE does indeed enhance the bactericidal effect of cecropin B on Xff. Fig. 2*A* shows the effect of HNE, cecropin B, and HNE plus cecropin B on survival of Xff over a 1-h period as assessed by subsequent plating on solid periwinkle wilt (PW) medium (Fig. S2). We found that 50 nM HNE had a negligible effect on Xff viability relative to the untreated control, but that 5 μM cecropin B reduced the number of viable Xff cells. More interestingly, treatment with HNE plus cecropin B produced a synergistic effect, as evidenced by the dramatic reduction in the number of viable Xff cells (from 10^7 to 10^3 CFU/mL, compared with reductions to 10^6 for HNE alone and 10^5 for cecropin B alone). The synergy may be due to MopB cleavage by HNE facilitating Xff lysis and/or Xff lysis exposing MopB cleavage sites in the Xf outer membrane and periplasm.

Design of (HNE-Cecropin B) Chimeras to Facilitate Pathogen Recognition and Lysis and Delivery into the Plant Xylem. The results of our Xff cell viability studies prompted us to design a chimera of covalently linked HNE and cecropin B with the intent of enhancing the synergistic effect of the two modules. The design of such a chimera was subject to two criteria: (*i*) Each domain should retain its native folding and function, and (*ii*) the two domains should be joined in such way that both are able to act simultaneously on their target sites on Xff. Clearly, an appropriate choice of a linker is critical to meeting these criteria. We performed molecular dynamics and energy minimization (21) to obtain stable structures of the chimera, in which a flexible linker with the amino acid sequence GSTA joins the HNE and cecropin B domains. Fig. 2*B* shows a ribbon diagram of such a chimera. The flexibility of GSTA and neighboring amino acids were used to model a stable structure of the chimera in which HNE and cecropin B assumed their correct folding. A chimeric gene was constructed by fusing DNA sequences encoding the amino-end secretory signal peptide of HNE to the HNE-GSTA-cecropin B chimera. This construct is designated HNEsp-HNE-GSTA-cecropin B (Fig. S3). The secretory pathway is expected to facilitate the native folding of the secreted HNE and cecropin B domains in the chimera (22, 23). We tested the HNE activity by spectrophotometric enzyme assay (24), and tested the folding of cecropin B by binding to polyclonal antibody (Fig. S4). As expected, the secreted HNEsp-HNE-GSTA-cecropin B construct was more effective than HNE plus cecropin B in killing Xff (Fig. S5). In addition to the observed synergy, HNEsp-HNE-GSTA-cecropin B was delivered in the grape xylem, suggesting that the HNE secretion signal, HNEsp, is sufficient for the xylem delivery in a heterologous host (see below).

We created a second construct, designated PGIPsp-HNE-GSTA-cecropin B, using a plant-specific signal sequence for delivery of the chimera into the plant xylem. In this construct, we replaced the HNEsp with the signal peptide sequence from the pear polygalacturonase-inhibiting protein (PGIP) gene. PGIP, an innate defense protein, is secreted to the apoplast of plants and blocks cleavage of the pectin component of the middle lamella between plant cells by bacterial and fungal polygalactouranases (25). In a previous study (26), we demonstrated that the expression of PGIP in grapevines resulted in the secretion of PGIP first to the apoplast and then to the xylem. The chimeric genes (optimized for plant codon use) were cloned into a plant vector to create the two binary plasmids pDA05.0525 with PGIP signal peptide PGIPsp and pDU04.6105 with HNEsp (Fig. S6).

Our Chimera Protects Against Development of PD-Like Symptoms in Tobacco Plants. Disarmed *Agrobacterium tumefaciens* strain EHA 105 was electroporated with binary plasmid pDA05.0525, containing the PGIP signal peptide PGIPsp, and then used to transform *Nicotiana tabacum* cv. SR1, which has been established as a model host for the bioassay of Xf strains (27, 28). A total volume of 250 μL containing 2.5×10^7 Xff cells was injected at three sites, one on either side of the base of the midrib and the third in the middle of the midrib. As shown in Fig. 2*C*, at 2 mo after inoculation, extreme leaf scorching was observed on the untransformed plants, but only minimal scorching was seen on the transformed plants. Thus, expression of PGIPsp-HNE-GSTA-cecropin B in the xylem provided significant protection against the development of Xff-induced symptoms in tobacco.

Expression and Activity of the Chimera in Transgenic Grape Lines. Our results from the model tobacco system encouraged us to generate transgenic grapevines expressing the chimera with HNE signal peptide sequences (Fig. S6). As described previously, preembryogenic callus of *Vitis vinifera* variety Thompson seedless (TS) was used for plant transformation (29). Thirty-eight independent transgenic grape lines were generated from pDU04.6105 and grown in a greenhouse. Fig. 3*A* shows the expression of the HNEsp-HNE-GSTA-cecropin B gene as determined by real-time PCR in two transformed TS lines (89 and 146) in leaf and stem samples. A higher level of chimera expression is observed in leaf tissue compared with stem tissue. Plant transgenes under control of the CaMV 35S promoter reportedly are expressed at higher levels in the leaves than in other tissues (30). However, as shown in Fig. 3*B*, even the relatively low expression level in the stem was sufficient to provide in vitro anti-Xff activity in xylem sap. The presence and activity of the chimera in the xylem sap of the transgenic grape lines were examined by monitoring the growth of Xff for 5 h in the corresponding xylem saps. Fig. 3*B* shows the mortality of Xff in the

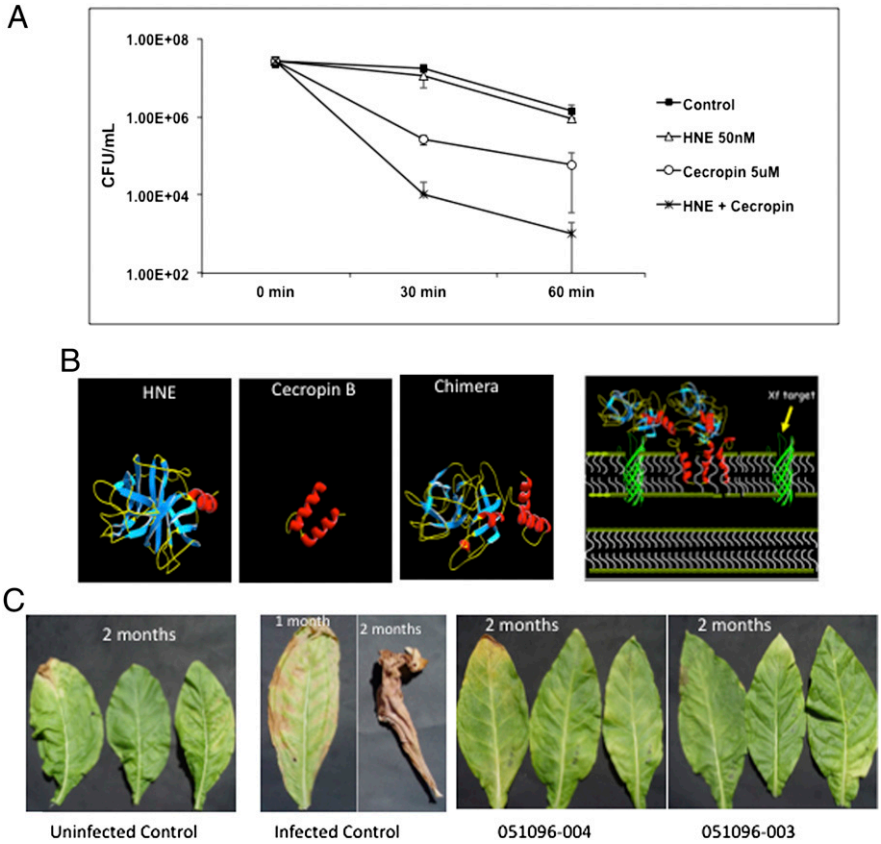

Fig. 2. Structures and biological activities of HNE, cecropin B, and HNE-GSTA-cecropin B. (*A*) The Xff-killing activity of HNE and cecropin B against 10^7 CFU/mL of Xff at indicated times after addition of buffer (control), 50 nM HNE, 5 μM cecropin B, and 50 nM HNE plus 5 μM cecropin B. Viable Xf was estimated by diluting aliquots and plating them on PW solid plates. (*B*) (*Left*) Ribbon diagrams of HNE (PDB ID: 1HNE) and cecropin B (PDB ID: 2IGR) generated from their coordinates and the energy-minimized model of the HNE-GSTA-cecropin B chimera. The flexibility of GSTA and a few residues of HNE and cecropin B were used to obtain a stable model using the AMBER force field (21) with the objective of having both HNE and cecropin B retain their native fold (and function). (*Right*) A schematic model of how oligomerization of the hydrophobic part of cecropin B leads to creation of pores in the outer membrane and also can allow cleavage of MopB. (*C*) Protection against development of PD symptoms demonstrated by two transgenic tobacco lines (051096-004 and 051096-003) expressing the HNE-GSTA-cecropin B chimera in the xylem and generated by *Agrobacterium*-mediated transformation using the PGIPst-HNE-GSTA-cecropin B–containing pDA05.0525 binary vector. Photographs were taken 1 or 2 mo postinoculation with Xff Temecula1 strain, as indicated.

xylem of the transgenic TS (89 and 146) lines relative to that in the xylem of the untransformed TS or in buffer. It appears that the endogenous signal sequence of HNE is sufficient to secrete the HNE-GSTA-cecropin B chimera to the xylem, where it confers the expected anti-Xff effect.

Transgenic Grape Lines Expressing the Chimera Are Protected Against Xff-Induced PD. The protection of the transgenic TS grapevine against PD was demonstrated by visual monitoring of leaf scorching (Fig. 4*A*) and NMR imaging of xylem blocking (Fig. 4*B*) after stem inoculation with Xff. Fifteen individuals each from 14 transgenic TS plant lines and one untransformed (control) plant were examined for 7–14 wk after Xff inoculation in a greenhouse. Representative leaves were selected from the eighth node above the point of inoculation at 10–14 wk postinoculation. Fig. 4*A* shows representative leaves from the untransformed TS and transgenic TS lines (89 and 146). We used a scaling system that assigned a score of 0 to a leaf with no scorching and 5 to a leaf with 100% scorching, a leaf that usually had dropped. The average "leaf scorching" scores for the transformed lines were ≤ 2 at the 10th week postinoculation and ~2.5 at the 14th week postinoculation, compared with ≥ 4.2 for the untransformed plants at 10 wk or later postinoculation. Differences between the scores for transformed and untransformed plants were statistically significant with P values < 0.001.

Blocking of the xylem vessels was monitored by proton density NMR imaging of stem cross-sections (31) obtained from each plant at 15–20 cm above the point of inoculation. The bright patches in the proton image correspond to the presence of water, whereas the dark patches imply the absence of water. Fig. 4*B* shows the proton density maps of the stem cross-sections from the untransformed TS and transgenic TS lines at 10 wk after Xf inoculation. The stem cross-sections from the infected untransformed TS show central dark patches, suggesting lack of water flow due to xylem clogging, whereas the corresponding stem cross-sections in the transgenic lines show higher water density, implying relatively open xylem.

A selected set of "PD-protected" grape lines bearing the HNEsp-HNE-GSTA-cecropin B construct is currently being tested in the field. Grape lines bearing the PGIPsp-HNE-GSTA-cecropin B construct have been generated and are ready to be tested in the greenhouse and in the field.

Discussion

Here we describe a therapeutic approach for clearing a pathogen from the site of colonization in the host. This approach is based on two key steps: (*i*) designing a protein chimera containing recognition and lysis domains targeting conserved moieties on the pathogen surface, and (*ii*) specifically expressing this chimera at the site of pathogen colonization in the host. Such a protein chimera has been successfully designed to protect against PD caused

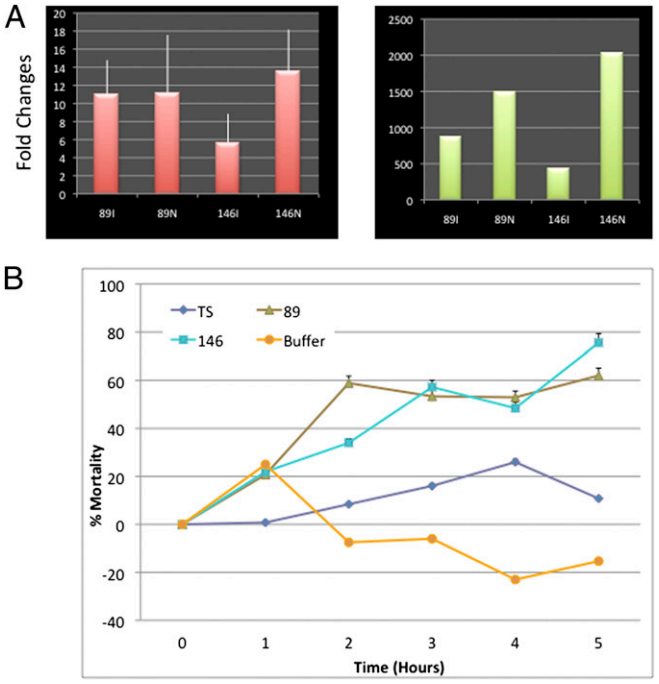

Fig. 3. Expression of HNEsp-HNE-GSTA-cecropin B and the biological effect of its gene product. (*A*) Level of expression of the chimera HNEsp-HNE-GSTA-cecropin B gene in the stem (red bars) and leaf tissues (green bars) of the transgenic TS-89 and TS-146 lines in inoculated (89I and 146I) and non-inoculated (89N and 146N) states, as determined by quantitative real-time PCR. The fold changes are relative to the expression levels in the untransformed TS. (*B*) The effect of the xylem sap collected from the transgenic TS lines 89 and 146 on the in vitro accumulation of the Xff strain KLN59.3 cells (28), which express GFP. For each sample extruded under pressure from stem sections, 450 μL of xylem sap was mixed with 50 μL of PBS (pH 7.4) containing 10^6 KLN59.3 cells. Three different dilutions of Xf/xylem sap mixture were plated on PW nutrient agar plates at 1-h intervals for 5 h. Xylem sap from the untransformed TS and buffer was used as controls.

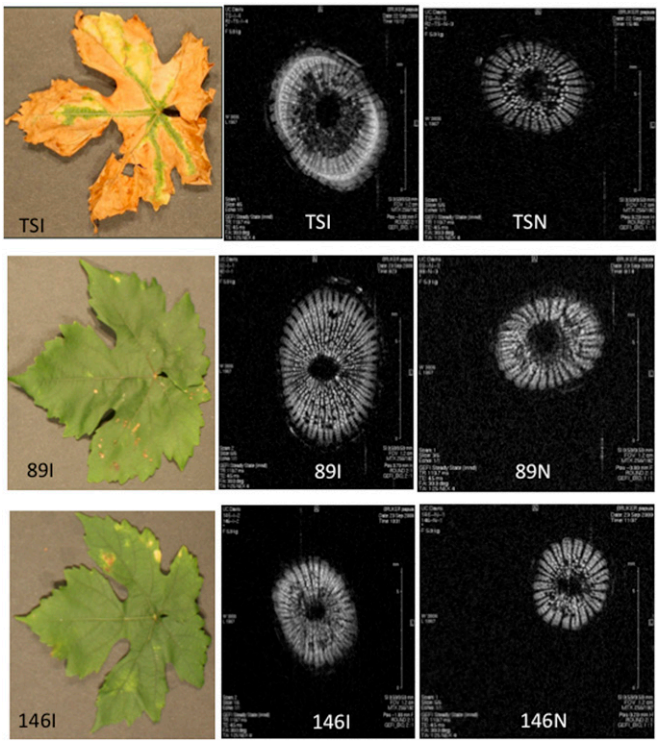

Fig. 4. Protective effects of HNE-GSTA-cecropin B in transgenic grapes. (*Left*) Monitoring of leaf scorching in the untransformed TS line and in the transgenic TS-89 and TS-146 lines expressing the HNE-GSTA-cecropin B chimera. Each leaf scorch monitoring experiment was performed using 30 plants from each transgenic line, 15 for Xff KLN59.3 inoculation and 15 for untreated controls. Transgenic TS and control TS plants were stem-inoculated with a total of 20 μL of the GFP-expressing Xff KLN59.3. The symptoms of leaf scorching were monitored after 10–14 wk of Xff KLN59.3 inoculation. A 10-μL drop of inoculum containing 10^7 Xf CFU/mL was placed near the base of the main stem 10 cm above the soil, and the stem was pricked through the liquid 5–10 times with a number 0 entomological pin. Fifteen plants were examined for the untransformed TS and for each of the transgenic TS lines. Representative leaves were selected from the eighth node above the point of inoculation at 10–14 wk postinoculation. (*Right*) ^{1}H-MRI images of the stems from the untransformed TS and the transgenic lines (89 and 146) inoculated with Xff strain KLN59.3 and of the stems from the same lines without inoculation. The images were obtained using an Avance 400 spectrometer equipped with Bruker DRX console microimaging accessories. The magnet is an Oxford vertical wide-bore (89 mm) magnet at 9.4 T (proton frequency, 400.1 MHz). At 10 wk postinoculation, the stems were cut under water from sections 10–20 cm above the point of inoculation and then completely wrapped with paraffin sheets to prevent further cavitation. Wrapped sections (10-12 cm) were placed inside the volume coil, and six transverse image slices were acquired simultaneously using a gradient-echo pulse sequence with a tip angle of 30 degrees, a repetition time of 110.7 ms, and an echo time of 4.5 ms. The images with the highest resolution were selected for further analysis. The images were acquired using 256 × 192 pixels with a field of view of 1.2 cm and slice thickness of 1.2 mm.

by Xff. We have demonstrated anti-Xff activity of the chimera in the grape xylem (Fig. 3*B*) and protection against PD in grapevine (Fig. 4 *A* and *B*). Our approach provides a viable alternative to conventional breeding of PD-resistant grapes. Note that in general, multiple genes clusters control PD resistance in natural grape cultivars, and these genes assort independently, resulting in very few resistant progeny in breeding populations. However, the recent discovery of PD resistance in the *Vitis arizonica* grape occurring in a single locus (designated *Pdr1*) suggests much promise for conventional breeding because of single-gene inheritance (32). In contrast to a conventional gene for resistance, the chimeric transgene described here has a defined mode of action, does not result in the introduction of adverse traits through linkage drag, and can be manipulated to alter its strength of action and other characteristics. Finally, because MopB is identical in all Xf strains, the same chimera also should effectively protect against Xf-induced diseases of economically important tree fruits and nuts, such as citrus, almond, coffee, peach, and plum.

Traditionally, the lytic antimicrobial peptides have been characterized as the next-generation antibiotics for their ability to target and kill bacteria. However, these peptides have limitations, being active only at high (μM) concentration (33) and often rendered inactive by bacterial resistance (34, 35). The appropriate linking of the recognition protein with a lytic peptide increases the overall antimicrobial activity of the chimera by several-fold over a lytic peptide alone (such as cecropin) or a combination of two lytic peptides (such as cecropin and melittin) with similar modes of action. The use of a protein chimera of recognition and lysis domains targeting two different but conserved moieties on a pathogen's surface should significantly overcome the problem of pathogenic resistance, because simultaneous mutations of the two sites are likely to occur at a very low frequency. Although proteins with multiple functional domains are quite common, antimicrobial protein chimeras synthetically designed by linking of pathogen recognition and lysis domains are not. This strategy of creating antipathogen chimeric proteins is applicable to a broad spectrum of pathogen-induced diseases, as we advocated several few years ago (4). We have recently completed preliminary studies showing that a protein chimera with recognition and clearance domains can completely inhibit the in vitro growth of *Staphylococcus aureus* at nM concentrations.

Materials and Methods

MopB was extracted and purified from the Xff grown on PD3 agar. IgG polyclonal antibody was purified from New Zealand White rabbit anti-sera collected after 100 d of MopB injection. The distribution of MopB on the Xff accessible on the surface was assessed by fluorescent imaging of Xff cells stained with Alexa Fluor-labeled anti-MopB IgG. The digestion of MopB was studied for the purified protein fraction, intact Xff, and membrane fraction by monitoring the product on a denaturing gel detected by either Coomassie blue staining or anti-MopB polyclonal IgG.

The HNE-GSTA-cecropin B chimera gene was cloned into pBacPAK8 baculovirus vector and expressed in High Five insect cells. The chimera was purified from the supernatant using elastin affinity beads that bound to HNE in the chimera. The antimicrobial activity of HNE, cecropin B, and the chimera was determined by measuring the CFU/mL of Xff plated on PW medium.

Two binary plasmids carrying HNEsp-HNE-GSTA-cecropin B and PGIPsp-HNE-GSTA-cecropin B genes were constructed. Each binary plasmid was introduced into a disarmed *Agrobacterium* strain to create a functional plant transformation system. Tobacco (*N. tabacum* cv. SR1) was transformed using the binary plasmid carrying PGIPsp-HNE-GSTA-cecropin B. TS grapevine embryogenic callus cultures were transformed using both binary plasmids.

mRNA levels of the chimera in the leaf, petiole, and stem tissues of the transformed and untransformed grape lines were measured by quantitative real-time PCR. Xylem sap was collected from the transformed and untransformed grapevines, and the activity of the chimera in xylem sap was tested by measuring the bactericidal effect on the Xff KLN59.3 strain grown in PW medium. The *in planta* efficacy of the HNEsp-HNE-GSTA-cecropin B chimera was determined by comparing (*i*) leaf scorching in transformed and untransformed grape lines and (*ii*) blockage of water by NMR in the xylem of transformed and untransformed grape lines.

More details are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This project was supported by funding from the Pierce's Disease Control Board of the California Department of Food and Agriculture (various awards to A.M.D., G.B., and P.A.F.), the US Department of Energy, and the US Department of Agriculture (USDA-ARS Agreement 58-5302-4-544).

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