Transgenic Disease Resistance in *Vitis vinifera*: Potential Use and Screening of Antimicrobial Peptides

Claire-Lise Rosenfield,[1] Suren Samuelian,[1,2] Jose R. Vidal,[1,3] and Bruce I. Reisch[1]*

Abstract: To increase resistance to important grapevine pathogens, Chardonnay was transformed with a construct containing two antimicrobial peptides (AMPs), Magainin-2 and PGL. Five lines expressing Magainin-2 transcripts showed increased resistance to two strains of *Agrobacterium vitis*, TM4 and CG450. These results led to a comprehensive study of the effects of nine AMPs on in vitro growth of four pathogens: two bacterial (*A. tumefaciens* and *A. vitis* strains CG450 and TM4) and two fungal (*Botrytis cinerea* and *Erysiphe necator*). Cecropin B, MSI-99, and Shiva 1 were most effective against the two *A. vitis* strains, but ESF-12 had no effect. The growth of TM4 was always more sensitive to AMPs than was CG450, a pattern commensurate with the observations of crown gall resistance among transformed vines expressing Magainin-2. *Botrytis cinerea* required more than 10 µM α-Purothionin, PGL, or ESF39 for effective growth inhibition. None of the AMPs studied could reproducibly control *E. necator* spore germination or lesion formation. MSI-99 and Cecropin B were most promising for future increased resistance to crown gall disease in transgenic grapevines.

Key words: antimicrobial peptide, *Vitis vinifera, Agrobacterium vitis, Botrytis cinerea*, genetic transformation, *Erysiphe necator*

Grapevines are susceptible to many plant pathogens and the resulting diseases cause major economic losses. Grapevine genetic transformation, now routinely achieved by both *Agrobacterium*- and biolistic-mediated DNA delivery systems (Burger et al. 2009, Colova-Tsolova et al. 2001, Kikkert et al. 2001, Reustle and Buchholz 2009), has been used to introduce genes coding for antimicrobial peptides (AMPs) and proteins into the genome of susceptible varieties. Chardonnay (*Vitis vinifera* L.) vines were stably co-transformed with *npt-II* and magainin transgenes using DNA constructs in circular plasmids (Vidal et al. 2003) or minimal gene cassettes (Vidal et al. 2006a). Regenerated transgenic plants actively transcribed those transgenes (Vidal et al. 2006b). Some transgenic lines had significant reductions in crown gall or were measurably less infested by powdery mildew (PM) (Vidal et al. 2006b). Seyval blanc plants were stably transformed with a gene combination encoding two antifungal proteins (chitinase and a ribosome-inactivating protein) and actively expressed those proteins, but they failed to show any improved resistance against downy and powdery mildew when tested in the field (Bornhoff et al. 2005). Those results show that grapevine transformation with genes coding for AMPs can confer partial resistance, but greater disease resistance is needed before transgenic lines can be considered for commercialization.

Analyzing the results of grapevine transformation is a lengthy process compared to tobacco (*Nicotiana tabacum* L.) and *Arabidopsis thaliana* (Xing et al. 2006) or other transgenic crops (Halpin 2005). Therefore, it is important to screen AMPs to select those most effective against targeted grapevine pathogens. Moreover, it is also worthwhile to determine in advance if transformation by more than one potential gene may confer greater resistance against grapevine diseases via an additive or synergistic effect. Screening of certain AMPs and other potentially useful compounds has already been reported (Ali and Reddy 2000, Ali et al. 2003, Li and Gray 2003). The number of known gene-encoded AMPs has increased greatly since Magainin-2 was discovered in the African clawed frog (Zasloff 1987). AMPs have been found in many insects, animals, and plants. By 2003 more than 500 AMPs were listed in the AntiMicrobial Sequence Database (Zasloff 2002); that number has now reached almost 900 peptides. Screening for AMPs most effective against grapevine pathogens does not guarantee that transformation with the corresponding genes will result in disease resistance. However, it does ensure that transgenic grapevines produce effective defense compounds and increases the likelihood that resistance will be improved.

[1]Department of Horticultural Sciences, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456; [2](present address) National Wine and Grape Industry Centre, Charles Sturt University, Locked Bag 588, Wagga Wagga NSW 2678, Australia; and [3](present address) Plant Physiology Department, Facultad de Biología, Universidad de Santiago de Compostela, Campus Sur, 15782, Santiago, Galicia, Spain.

*Corresponding author (email: bruce.reisch@cornell.edu; tel: 315 787-2239; fax: 315 787-2216)

Acknowledgments: Financial support for this project was provided by the USDA-CSREES Viticulture Consortium. The pSAN319 construct was kindly provided by John Sanford (FMS Foundation) and Franzine Smith (Sanford Scientific Inc.). J.R. Vidal was supported in part by Ramón y Cajal and AGR08-515/AGR grants from the Spanish Ministry of Science and Innovation.

The authors thank Lance Cadle-Davidson for sharing a technique to evaluate the formation of PM lesions on grapevine leaves in vitro and Steve Luce and Patricia Wallace for expert technical assistance.

Manuscript submitted Jul 2009, revised Feb 2010, accepted Apr 2010

Here we present previously unreported molecular analyses and evaluation of disease resistance in grapevines transformed with two AMP genes, Magainin-2 (*mag2*) and PGL. These results and earlier observations convinced us that a comprehensive study of AMPs and their effects on the growth of grapevine pathogens was necessary before undertaking further long-term efforts to transform vines for AMP production. To evaluate the antimicrobial activity of AMPs on grapevine pathogens, peptides that had been found effective against pathogens of other plant species were chosen (Table 1). Some peptides were naturally occurring compounds with documented bactericidal effects, like cecropins, magainins, and thionins (Zasloff 1987, Stec 2006). Other AMPs, such as MsrA3 and PGL (Maloy and Kari 1995), were designed to improve upon the effect of existing ones. The transfer and expression of the gene for MsrA3, a modified temporin, gave potatoes resistance against several bacterial and fungal pathogens (Osusky et al. 2004). ESF39, which has an amino acid (AA) core similar to that of ESF12 plus a carboxy end turn with a 6X HIS tail, conferred upon transgenic American elms significant resistance to Dutch elm disease (Powell et al. 1995, Merkle et al. 2007). Transgenic tobacco became resistant to pathogens when transformed with either MSI-99, an enhanced version of *mag2* (DeGray et al. 2001), or with Shiva-1, a modified Cecropin B (Jaynes et al. 1993). Shiva-1 was designed so that each cationic or anionic AA residue in Cecropin B is replaced by another AA of similar charge and the resulting peptide has only 46% sequence homology with Cecropin B.

The automated spectrophotometer-shaker-incubator Bioscreen C has been a powerful tool for more than 15 years to study simultaneous growth of up to 200 microbiological samples in small volumes. It has been used to follow the growth of both bacterial and fungal plant pathogens (Mourgues et al. 1998, Malnoy et al. 2003, Faize et al. 2006, Tauk-Tornisielo et al. 2007) and to determine the effectiveness of newly developed cyclic peptides against plant pathogenic bacteria (Monroc et al. 2006a, 2006b). This instrument was used in the present study to assess the effects of AMPs on growth of grapevine pathogens.

Materials and Methods

Antimicrobial peptides. All AMPs evaluated in this study are listed (Table 1). Cecropin B and Magainin-2 were purchased from Sigma Chemicals (St Louis, MO). ESF12 was obtained from William Powell (Powell et al. 2000), who also provided the ESF39 AA sequence. Pure α-Purothionin (α-PTH), approximately a 1:1 mixture of α1-PTH and α2-PTH (Jones and Mak 1975), was provided by Ronald Skadsen, of the USDA (Madison, WI). ESF39, MSI-99, MsrA3, PGL, and Shiva-1 were custom-synthesized (>95% purity) by Sigma Genosys (The Woodlands, TX). MSI-99 was also synthesized by Genscript (Piscataway, NJ). Lyophilized AMP powders were dissolved in sterile distilled water to obtain 10 µg/µL stocks stable at -20°C.

Plant material and evaluation of transgenic grapevine plants. Embryogenic cultures of Chardonnay (clone 95) were transformed via biolistic bombardment with a vector, pSAN319 (Figure 1A), harboring *mag2* and *PGL* genes according to procedures described (Vidal et al. 2006b). Molecular analyses (Southern blot and semiquantitative RT-PCR), evaluation of resistance to grapevine diseases (crown gall and PM), and data analyses were carried out as described previously (Vidal et al. 2006b). To determine the number of integration events of *mag2* and/or *PGL* genes into the genome of transgenic Chardonnay plants, genomic DNA from PCR-positive lines (Figure 1A) was digested with *Hind*III to obtain fragments between the *Hind*III restriction site in the pSAN319 construct and other such sites elsewhere in the plant genome. The DNA was fractionated by gel electrophoresis and resulting Southern blots were probed with the *Pubq3-mag2* fragment (Figure 1A). To more readily detect gene transcripts and evaluate their expression, RT-PCR of total RNA was used instead of the standard Northern blot technique because of the small size of the transcripts.

Each resistance evaluation was carried out in two consecutive years. To evaluate resistance against *Agrobacterium vitis*, plants with more than 20 nodes were inoculated following acclimation to greenhouse conditions (year 1) or after growth resumption following a period of dormancy

Table 1 Antimicrobial peptides (AMP) used in this study, grouped according to sequence similarity. Notable differences between peptides of similar sequences are highlighted. (Amino acids additions are shown in boldface and substitutions are indicated by shading. α-Purothionin is a mixture of α1- and α2-Purothionin.)

AMP	Amino acid sequence
Cecropin B	**[H]**K-W-K-V-F-K-K-I-E-K-M-G-R-N-I-R-N-G-I-V-K-A-G-P-A-I-A-V-L-G-E-A-K-A-L**[OH]**
Shiva-1	**[H]M-P**-R-W-R-L-F-R-R-I-D-R-V-G-K-Q-I-K-Q-G-I-L-R-A-G-P-A-I-A-L-V-G-D-A-R-A-V-**G[OH]**
ESF12	[H]**M**-A-S-R-A-A-G-L-A-A-R-L-A-R-L-A-L-R [OH]
ESF39	[H]A-S-R-A-A-G-L-A-R-R-L-A-R-L-A-R-R-**E-L-R-Y-A-Q-S-G-P-E-L-R-Y-A-Q-S-H-H-H-H-H-H**[OH]
Magainin-2	[H]G-I-G-K-F-L-H-S-A-K-K-F-G-K-A-F-V-G-E-I-**M**-N-S[OH]
MSI-99	[H]G-I-G-K-F-L-K-S-A-K-K-F-G-K-A-F-V-K-I-L-N-S[OH]
MsrA3	[H]M-A-S-R-H-M-F-L-P-L-I-G-R-V-L-S-G-I-L[OH]
PGL	[H]A-S-K-A-G-A-I-A-G-K-I-A-K-V-A-L-K-A-L[OH]
α1-Purothionin	[H]K-S-C-C-R-S-T-L-G-R-N-C-Y-N-L-C-R-A-R-G-A-Q-K-L-C-A-G-V-C-R-C-K-I-S-S-G-K-C-P-T-G-F-P-K[OH]
α2-Purothionin	[H]K-S-C-C-R-T-T-L-G-R-N-C-Y-N-L-C-R-S-R-G-A-Q-K-L-C-S-T-V-C-R-C-K-L-T-S-G-K-C-P-T-G-F-P-K[OH]

(year 2). Similar-sized plants from each transgenic line were inoculated with two strains, TM4 and CG450, and gall size was measured two months after inoculation (Vidal et al. 2006b). To evaluate resistance against PM, plants acclimated to the greenhouse for four months (year 1) or with actively growing shoots (year 2) were inoculated with *E. necator* conidia and, 14 days after inoculation, the percentage of PM infection on the leaf surface was recorded on nine leaves (four leaves inoculated initially and five younger leaves infected naturally via secondary inoculum). Disease resistance data were collected from three replicate plants from each transformed line, and from three plants of a nontransformed control line (Vidal et al. 2006b).

Bacterial strains and growth conditions. To test the direct effects of AMPs on the growth of *Agrobacterium tumefaciens* and *Agrobacterium vitis*, which both cause crown gall disease in grapevine, frozen stocks of *A. tumefaciens* LBA4404 and of *A. vitis* TM4 and CG450 were subcultured on Luria Bertani (LB) medium, then bacterial cultures were grown at 28°C in liquid LB medium to an OD_{600} of 0.1, corresponding to a cell density of ~10^8 cfu/mL for *A. tumefaciens* and of ~10^7 cfu/mL for *A. vitis*. A more accurate cell density was calculated in each experiment by streaking known amounts of serially diluted suspensions.

Growth studies of bacteria in the presence of AMPs. To test the effects of AMPs on bacterial growth, equal amounts of bacterial suspensions in 2x LB medium and of AMP solutions in sterile H_2O were mixed to reach the desired final concentrations of bacteria and AMP. 100-µL samples of each test (at least in triplicate) were grown at 28°C with continuous shaking at medium speed in an automated Bioscreen C Workstation (Growth Curves USA, Piscataway, NJ), measuring the OD_{600} of each well every 3 hr. Negative control readings were taken from at least 10 wells containing LB only and positive controls were obtained from at least 10 wells containing LB and bacteria without AMPs. Experiments were repeated until reliable results were obtained in a suitable range of AMP concentrations.

Fungal spore germination and hyphal growth. *Botrytis cinerea* spores (obtained from Wayne Wilcox, N.Y. State Agricultural Experiment Station, Geneva) were transferred to potato dextrose (PD) agar plates and left at 20°C with 12 hr dark and 12 hr light to germinate, develop hyphae, and then form spores. Spores were gently scraped off the plate and suspended in PD broth medium. The suspension was filtered through sterile cheesecloth to remove debris, and the spore concentration of the suspension was determined by counting spores in a diluted aliquot with a hemacytometer.

Spore germination and hyphal tube growth in the presence of AMPs. To test the effects of AMPs on spore germination and initial hyphal tube growth, AMP solutions and spore suspensions were mixed to desired concentrations in 600-µL siliconized Eppendorf tubes. Replicates of treated suspension (15 µL) were placed on clean glass slides, which were kept on wet paper towels in small germination boxes at 25°C in the dark. Samples were taken at different times and examined microscopically after gently lowering glass cover slips on the sample drops and sealing the cover slip edges with nail polish, which instantly stopped tube growth and prevented drying. Percent germination was determined by counting two samples of 100 spores each using a phase contrast microscope. Pictures were taken with an Olympus DP-12 Digital Microscope camera at 10x magnification (dark phase) and printed on paper at the same magnification. Tube lengths were measured with a digital Scalex MapWheel and the readings were converted to microns by measuring the picture of a hemacytometer square (200 µ), photographed and printed under the same conditions as the samples.

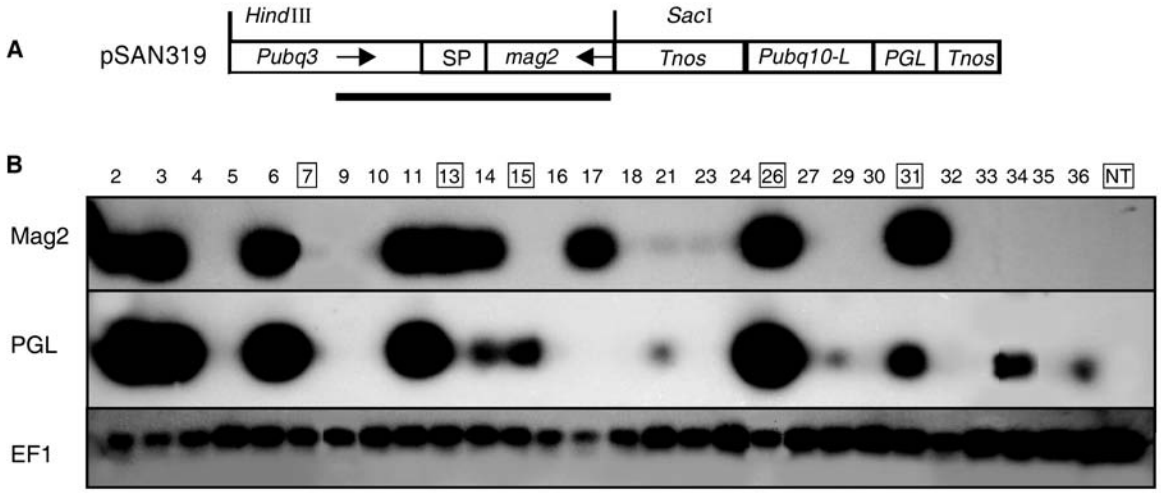

Figure 1 pSAN319 construct used in grapevine transformation (**A**). Cassette elements (not drawn to scale): *Pubq3* and *Pubq10-L* (*Arabidopsis thaliana* ubiquitin promoters); *Tnos* (*Agrobacterium tumefaciens* T-DNA nopaline synthase terminator); *SP* (signal peptide from pea vicilin protein); *mag2* (Magainin-2 gene); *PGL* (PGL gene); *Hind*III and *Sac*I (unique restriction sites). Arrows indicate primer annealing sites. Bar corresponds to the PCR probe (~400 bp). Gene transcription *in planta* (**B**). Hybridization signals from digoxigenin-labeled probes in semiquantitative RT-PCR analyses of transgenic PCR-positive lines and a nontransformed (NT) line, comparing transcription of *mag2*, *PGL*, and *EF1*-α genes in pSAN319-derived lines using leaves of plants grown in vitro. Quantitative results from framed lines are given in Table 2.

To test the effects of AMPs on hyphal tube growth of *B. cinerea*, equal amounts of spore suspensions in 2x PD broth and of AMP solutions in sterile H_2O were mixed to the desired final concentrations of spore and AMP. Triplicate 100-µL samples of each mixture were incubated at 25°C in a Bioscreen C Workstation, set to shake at low speed for 15 seconds just before measuring the OD_{600} of each well, every 3 hr.

In vitro testing of AMP effects on PM. Grapevines were grown from seed of Chardonnay (presumed to result primarily from self-pollination) in a growth chamber kept at 20°C (12 hr light/12 hr dark). Some plants were transferred to another enclosed chamber and inoculated with *E. necator* conidia to provide PM spores two weeks later as a suspension in 0.0005% Tween 20. Spore concentration was determined using a diluted aliquot with a hemacytometer. From the remaining healthy plants, one to three fully expanded leaves closest to the growing tip were removed, sterilized for 2 min in 10% bleach, rinsed with sterile deionized water, blotted dry, and placed on the surface of sterile 1% agar plates. To test the effects of AMPs on PM spore germination and lesion formation on grapevine leaves, equal amounts of freshly prepared spore suspension and of AMP solutions were mixed to desired final concentrations of spores (10^4 spores/mL) and AMP, and 1.5 µL droplets were applied in duplicate to six preselected areas on each leaf. Plates containing PM-inoculated leaves were kept in plastic sleeves in the growth chamber and PM lesions were measured after 21 days.

Results

Integration and transcription of Magainin-2 and PGL. Grapevines transformed with construct pSAN319 (Vidal et al. 2003) were examined to confirm the integration of the *mag2* and PGL genes (Figure 1A), determine the number of integration events, and evaluate gene expression. Most pSAN319-transformed lines showed positive Southern hybridization signals, but nontransformed Chardonnay negative controls did not (data not shown). Band patterns were different among all lines, indicating independent transformation and multiple integration events ranging from 1 to 3 for the *Pubq3-mag2* sequence (Table 2). The expression of gene transcripts was evaluated by RT-PCR, and cDNA bands of the expected size (120 bp for *mag2* and 63 bp for *PGL*) were obtained for most Southern blot positive lines, while no signals were detected for the nontransgenic control. Out of 28 PCR-positive lines, 16 were positive for transcription of the *mag2* gene and 15 lines for the *PGL* gene, but only 10 lines were positive for both *mag2* and *PGL* gene transcription (Figure 1B, Table 2).

Evaluation of transgenic lines for resistance to crown gall and PM. Two months after inoculation with *A. vitis* strains TM4 and CG450, all six transgenic lines showed crown gall symptoms but most had smaller galls than did the nontransgenic control. In general, gall size in year 2 after inoculation was larger than in year 1, probably due to better plant growth. Among the six transgenic lines, five that were inoculated with strain TM4 and two that were inoculated with strain CG450 showed statistically significant reductions in gall size when compared to the nontransgenic control (Table 2). The only line inoculated with TM4 that did not show reduced galling (line 319-15) was also negative for transcription of the *mag2* gene.

Transgenic lines were also evaluated for resistance to PM infection during two consecutive years. There was no significant difference between transgenic lines and nontransgenic controls in the first year (data not shown), but in the second year, line 319-7 had significantly reduced symptom development compared to the nontransgenic control (Table 2).

Table 2 Transcription of transgenes and disease resistance of AMP-transgenic Chardonnay lines and nontransformed controls, assayed in greenhouse trials for resistance to crown gall and powdery mildew diseases.

Line[a]	Integration *Mag2* probe[b]	RT-PCR AMP/EF1-α ratio[c]		Disease resistance (year 2)		
				Crown gall[d]		Powdery mildew[e]
		PGL	Mag2	TM4	CG450	
319-7	1	0.32	0.10	-2.0 ± 0.6 *	-1.2 ± 0.6	34.8 ±0.6 *
319-13	2	0.41	1.79	-2.9 ± 0.6 **	-2.2 ± 0.6 **	41.5 ± 0.1
319-15	1	2.10	0.00	1.0 ± 0.6	-0.3 ± 0.7	nt
319-21	2	0.74	0.23	-1.4 ± 0.6 *	-0.4 ± 0.6	44.6 ± 0.6
319-26	3	2.93	2.62	-3.6 ± 0.7 **	-3.2 ± 0.7 **	nt
319-31	2	3.59	5.55	-3.4 ± 0.7 **	-0.8 ± 0.6	44.5 ± 0.3
NT-2	0	0.00	0.00	0	0	49.8 ± 2.5

[a]PCR-positive transgenic lines containing two antimicrobial peptide (AMP) genes, Magainin-2 and PGL, and a nontransformed (NT-2) line (framed numbers in Figure 1B).

[b]Number of AMP gene integrations in genomic DNA after digestion with *Hind*III and probe of Southern blots with a magainin2 (*mag2*) containing fragment.

[c]Transcription ratio of AMP gene as compared with that of the alpha subunit of translation elongation factor 1 (*EF1-α*) gene, estimated on equivalent total cDNA amount at 20 cycles of RT-PCR (Figure 1B).

[d]Estimate (log of odds) ± SE of the gene-expression effect on gall size in transgenic lines as compared with nontransformed control (*$p < 0.05$; **$p < 0.001$) inoculated with *A. vitis* strains TM4 or CG450 (3 plants/line).

[e]Percentage leaf area infected with powdery mildew ± SE 14 days after inoculation with *U. necator*. Significant reduction of disease symptoms (*$p < 0.05$) (nt: not tested).

Effect of nine AMPs on growth of *A. vitis* and *A. tumefaciens*. The effect of Cecropin B on growth of *A. tumefaciens* strain LBA4404 and on two strains of *A. vitis*, CG450 and TM4, was evaluated as follows. Six concentrations of Cecropin B, 0.0 to 1.0 µM, were tested with the three bacterial strains starting at 10^6, 10^5, and 10^4 cfu/mL (Figure 2). The shapes of the positive control growth curves (0.0 µM Cecropin B) were typical for each bacterium. Bacterial growth of the 10x and 100x dilutions could be recorded after an approximately 12- and 24-hour delay, respectively. Cecropin B affected all three strains of *Agrobacterium* similarly at each bacterial concentration. It had no effect at or below 0.01 µM but delayed growth between 0.05 and 0.1 µM. Those delays were much longer in the two *A. vitis* strains than in *A. tumefaciens*, and they were also clearly longer in *A. vitis* TM4 than in *A. vitis* CG450, especially at 0.05 µM. At 0.5 µM Cecropin B, no growth was observed, not even after streaking aliquots of the treated suspensions on LB agar plates, demonstrating that 0.5 µM Cecropin B effectively killed all three types of *Agrobacterium* at all three initial bacterial concentrations.

Similar experiments were done with all nine AMPs (15 µM was the highest concentration tested). To provide direct comparisons among AMPs, their effects on the growth of *Agrobacterium* are summarized for *A. vitis* CG450 and TM4 (Table 3) and for *A. tumefaciens* (Table 4). The most effective AMP against *A. vitis* CG450 was Cecropin B, but MSI-99 was almost as effective: both kill the bacteria at 0.5 µM. Shiva-1 was less effective (killing at 1.0 µM), as were α-PTH (killing at 5.0 µM), Magainin-2 (stopping growth at 2.0 µM), and ESF39 (killing at 5.0 µM). PGL stopped TM4 growth at 10 µM, but did not stop CG450 even at 15 µM. Similarly, MsrA3 stopped TM4 growth at 15 µM, but did not stop CG450. ESF12 had almost no effect at the highest concentration tested. AMPs inhibited the growth of *A. vitis* TM4 in the same order of effectiveness as they did *A. vitis* CG450, but the TM4 strain was more sensitive to AMP growth inhibition than the CG450 strain. MSI-99 killed TM4 at 0.25 µM, Shiva-1 at 0.5 µM, and α-PTH at 1.0 µM. Magainin-2 stopped TM4 growth at 1.0 µM and ESF39 at 1.0 µM. Cecropin B slowed the growth of both TM4 and CG450 at 0.05 and 0.1 µM (Table 3), but TM4 growth delays were longer than those of CG450 (Figure 2).

Effect of Cecropin B and MSI-99 combinations on growth of *A. vitis* TM4. Cecropin B and MSI-99 were the most promising of the nine AMPs tested for control of crown gall bacteria. Those results raised the possibility of transforming Chardonnay with genes coding for both peptides to enhance resistance to crown gall disease. However, before attempting a double transformation, it was important to determine if the combination of those two AMPs would inhibit bacterial growth in an additive or synergistic manner or if the two AMPs would have an antagonistic interaction. To understand their interaction, a mixture of both AMPs had to be used, and the concentration chosen for each AMP should cause a 50% growth inhibition. Choosing

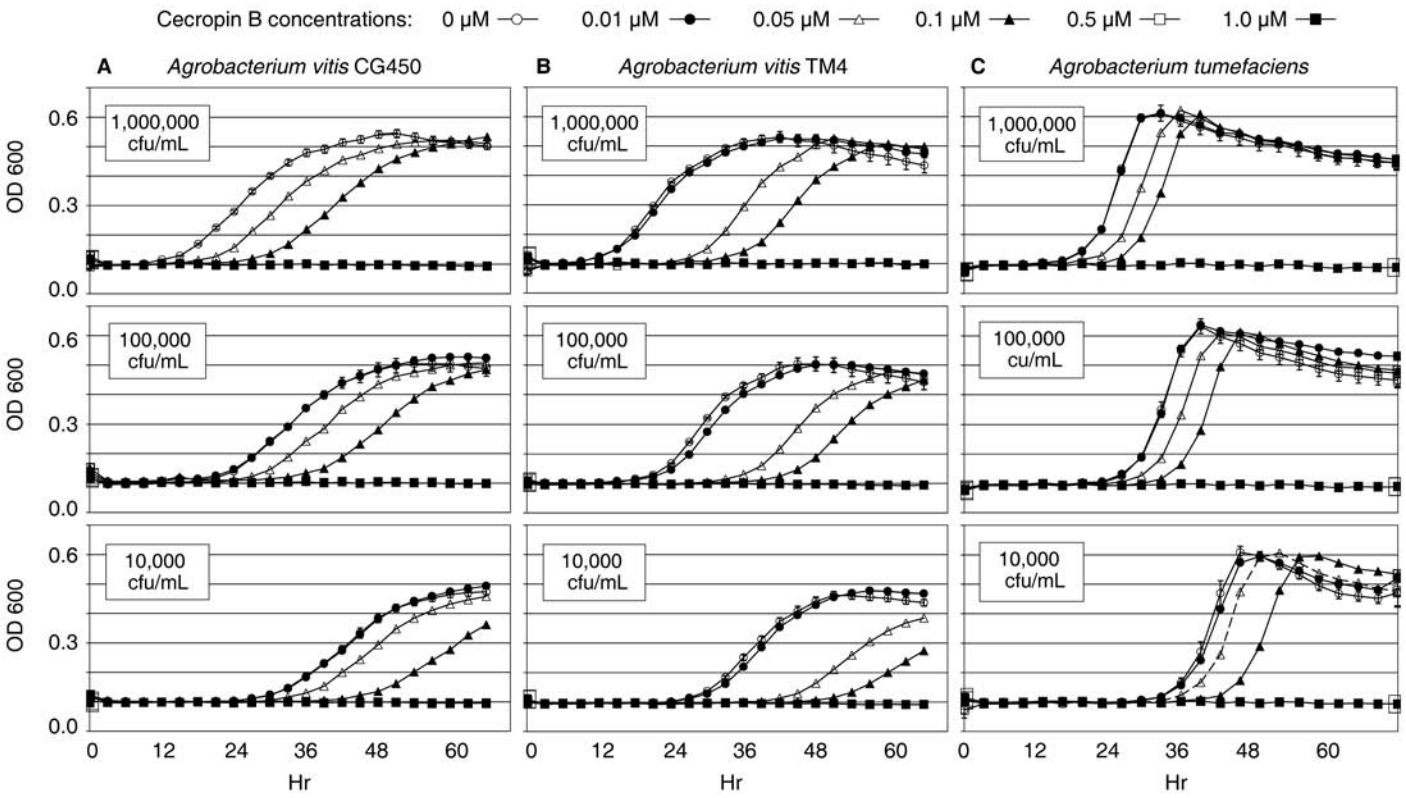

Figure 2 Effect of increasing concentrations of Cecropin B on the growth of *A. vitis* CG450 (**A**) and TM4 (**B**), and *A. tumefaciens* (**C**). 100-µL samples (10^6, 10^5, 10^4 cfu/mL) were incubated in LB medium containing Cecropin B at the indicated concentrations from 0 to 1.0 µM. Bacterial growth was recorded spectrophotometrically in a Bioscreen C Workstation. The standard deviation of the growth curve is indicated only for the control (0 µM).

that concentration was difficult because the percent growth inhibition of both AMPs increases rapidly from 10 to 90% and the precise point of 50% growth inhibition varies between experiments (horizontal double arrows in Figure 3A). A simultaneous experiment was performed with Cecropin B alone, MSI-99 alone, and four series of mixtures of the two AMPs. Two of those series gave results within the desired range (Figure 3B) and the observed interaction was clearly additive: whereas 0.3 µM MSI-99 alone and 0.2 µM Cecropin B alone caused ~30% growth inhibition of TM4, mixtures of half those concentrations also resulted in a 30% growth inhibition. The interaction ratio between the two AMPs was calculated using the Abbott formula (Ali and Reddy 2000), and in all instances the calculated interaction ratios were between 0.5 and 1.5, indicating that combinations of Cecropin B and MSI-99 interact additively.

Effect of nine AMPs on growth of *B. cinerea*. Since optical density measurements can be used to record growth of *B. cinerea* hyphae (Ali et al. 2003), the Bioscreen C was used to determine the effects of nine AMPs on hyphal development (Table 4). The growth of *B. cinerea* hyphae was much less sensitive to AMPs than were the three strains of *Agrobacterium* tested. Fifteen µM α-PTH stopped hyphal growth even at the highest spore concentration studied (Figure 4). PGL and ESF39 were also effective, but all other AMPs were effective only at or above 23 µM.

To determine whether PGL, ESF39, and α-PTH inhibited hyphal development by reducing spore germination or by slowing hyphal growth, 15-µL replicates of AMP-treated spore suspensions were placed on clean glass slides, incubated at 25°C in the dark, and samples were examined at different times. Percent germination was determined until, after 10 hr incubation, accurate counts could no longer be determined as ungerminated spores would stick to hyphal tubes. Tube lengths were measured from pictures taken with a digital microscope camera until, after 16 hr incubation, hyphal tubes become too long to measure accurately (Figure 5). PGL inhibited spore germination but had little effect on hyphal growth; ESF39 did not prevent spore germination but severely decreased hyphal growth, causing distortions in initial tube development; and α-PTH steadily decreased hyphal growth without affecting spore germination below 10 µM and stopped both germination and hyphal growth at 15 µM.

Effect of nine AMPs on development of powdery mildew. The germination of PM conidia in the presence of nine AMPs (0 to 50 µM) was studied repeatedly, but no reproducible trend could be obtained (results not shown). Similarly, when PM conidia mixed with increasing concentrations of AMPs (0 to 20 µM) were used to inoculate in vitro sets of five grapevine leaves, each leaf treated in six predetermined locations with the same test suspension, no

Table 3 Effect of nine antimicrobial peptides on the growth of *Agrobacterium vitis* strains CG450 and TM4. AMPs are listed in alphabetical order.

	Growth *A. vitis* CG450				Growth *A. vitis* TM4			
AMP	Concn (cfu/mL)	Good at or below (µM)	Poor at (µM)	None at or above (µM)	Concn (cfu/mL)	Good at or below (µM)	Poor at (µM)	None at or above (µM)
Cecropin B	10^6	0.01	0.05-0.1	0.5	10^6	0.01	0.05-0.1	0.5
	10^5	0.01	0.05-0.1	0.5	10^5	0.01	0.05-0.1	0.5
	10^4	0.01	0.05-0.1	0.5	10^4	0.01	0.05-0.1	0.5
ESF-12	10^6	15	na[a]	na	10^6	15	na	na
	10^5	15	na	na	10^5	15	na	na
	10^4	15	na	na	10^4	15	na	na
ESF-39	10^6	0.5	1.0	5.0	10^6	na	0.5	1.0
	10^5	0.5	1.0	5.0	10^5	na	0.5	1.0
	10^4	0.5	1.0	5.0	10^4	na	0.5	1.0
Magainin-2	10^6	1.0	na	2.0	10^6	0.5	na	1.0
	10^5	0.5	1.0	2.0	10^5	0.5	na	1.0
	10^4	0.5	1.0	2.0	10^4	0.2	0.5	1.0
MSI-99	10^6	0.10	0.25	0.5	10^6	0.10	0.25	0.25
	10^5	0.10	0.25	0.5	10^5	0.10	0.25	0.25
	10^4	0.10	0.25	0.5	10^4	0.10	0.25	0.25
MsrA3	10^6	1.0	5-15	na	10^6	1.0	5-10	15.0
	10^5	1.0	5-10	15	10^5	1.0	5-10	15.0
	10^4	1.0	5-10	15	10^4	1.0	5.0	10.0
PGL	10^6	5.0	15	na	10^6	1.0	5.0	10.0
	10^5	5.0	10-15	na	10^5	1.0	5.0	10.0
	10^4	1.0	5-15	na	10^4	0.5	1.0	5.0
α-PTH	10^6	na	0.5-1.0	5.0	10^6	na	0.5	1.0
	10^5	na	0.5-1.0	5.0	10^5	na	0.5	1.0
	10^4	na	0.5-1.0	5.0	10^4	na	0.5	1.0
Shiva-1	10^6	0.5	na	1.0	10^6	0.10	na	0.5
	10^5	na	0.5	1.0	10^5	0.10	na	0.5
	10^4	na	0.5	1.0	10^4	0.10	na	0.5

[a]na: not applicable.

reproducible trend was observed because leaves obtained from seedlings grown from seed were shown to vary for susceptibility to PM infection (results not shown). Finally, the three AMPs that had given the most promising results in the previous PM experiments, α-PTH, PGL, and Cecropin B, were retested on a set of 50 leaves, and each leaf was inoculated in duplicate with PM conidia mixed with only three AMP concentrations (0 µM control, 2.5 µM, and 5.0 µM). Three weeks after inoculation, the three AMPs tested had no effect on average lesion size (Table 5).

Discussion

The ectopic expression of AMPs in transgenic plants has resulted in enhanced resistance to some plant pathogens (Chakrabarti et al. 2003), but not all (DeGray et al. 2001). Transgenic Chardonnay plants expressing either Magainin-2 or MSI-99 showed enhanced resistance to two *A. vitis* strains, TM4 and CG450, and a slight reduction of PM symptoms (Vidal et al. 2006b). The finding that mixtures of AMPs, such as Magainin-2 and PGL, have synergistic effects when added together (Westerhoff et al. 1995)

Table 4 Effect of nine antimicrobial peptides on the growth of *Agrobacterium tumefaciens* and *Botrytis cinerea*. AMPs are listed in alphabetical order.

	Growth *A. tumefaciens*				Growth *B. cinerea*			
AMP	Concn (cfu/mL)	Good at or below (µM)	Poor at (µM)	None at or above (µM)	Concn (spore/mL)	Good at or below (µM)	Poor at (µM)	None at or above (µM)
Cecropin B	10^6	0.01	.05-0.1	0.5	10^5	10	15	na
	10^5	0.01	.05-0.1	0.5	10^4	10	15	na
	10^4	0.01	.05-0.1	0.5	10^3	5	10-15	na
ESF-12	10^6	15	na[a]	na	10^5	23	36-50	na
	10^5	15	na	na	10^4	23	36	50
	10^4	15	na	na	10^3	10	23-36	50
ESF-39	10^6	1.0	5.0	10.0	10^5	na	10-36	50
	10^5	1.0	na	5.0	10^4	na	10	23
	10^4	1.0	na	5.0	10^3	na	10	23
Magainin-2	10^6	1.0	5.0	10.0	10^5	36	50	na
	10^5	1.0	5.0	10.0	10^4	na	10-23	36
	10^4	1.0	na	5.0	10^3	na	10	23
MSI-99	10^6	0.25	0.5	na	10^5	10	23-50	na
	10^5	0.25	0.5	na	10^4	10	23-36	50
	10^4	0.25	0.5	na	10^3	10	23-36	50
MsrA3	10^6	5.0	10-15	na	10^5	10	23-50	na
	10^5	5.0	10-15	na	10^4	na	10-36	50
	10^4	5.0	10-15	na	10^3	10	23-36	50
PGL	10^6	15	na	na	10^5	10	23-36	50
	10^5	15	na	na	10^4	na	10	23
	10^4	15	na	na	10^3	na	na	10
α-PTH	10^6	1.0	5-15	na	10^5	1-5	10	15
	10^5	1.0	5-15	na	10^4	1-5	10	15
	10^4	1.0	5-15	na	10^3	1	5-10	15
Shiva-1	10^6	0.1	0.5-1.0	2.0	10^5	50	na	na
	10^5	0.1	0.5-1.0	2.0	10^4	10	23-50	na
	10^4	0.1	0.5-1.0	2.0	10^3	5	10-50	na

[a]na: not applicable.

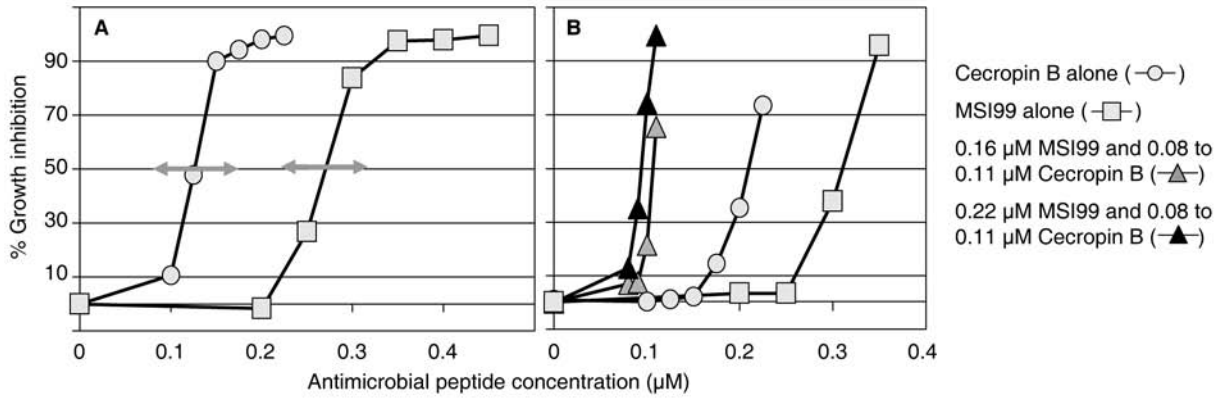

Figure 3 Growth inhibition of *Agrobacterium vitis* TM4 by Cecropin B and by MSI-99 alone (**A**, **B**) and in mixtures at the indicated concentrations (**B**).

prompted the transformation of Chardonnay with the combination of Magainin-2 and PGL to obtain broader antimicrobial protection. We report here the ectopic coexpression of two genes, *mag2* and *PGL*, both linked in a single construct used in transformation. Southern blot analyses and RT-PCR data from 28 transgenic lines showed that *mag2* and PGL genes were integrated in the plant genome with a positive correlation between the number of integration events and the level of transcription for *mag2* (r = 0.57) and *PGL* (r = 0.58) genes. Resistance against crown gall disease was linked to the expression of *mag2*, and greater symptom reduction was observed when the inoculating strain was TM4 (mean of the gene expression effect on gall size (log of odds) = -2.06) rather than CG450 (mean = -1.34), in agreement with previous work on transgenic Chardonnay lines expressing only the *mag2* gene (Vidal et al. 2006b). The correlation between the level of transcription of *mag2* and the symptom reduction of crown gall was higher for the TM4 strain (r = 0.73) than for the CG450 strain (r = 0.35).

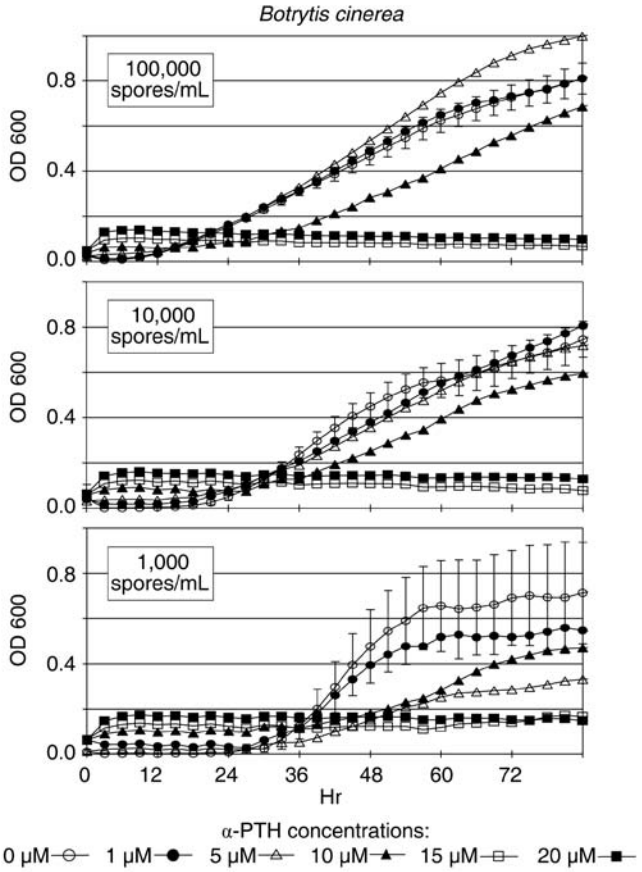

Figure 4 Effect of increasing concentrations of α-Purothionin (α-PTH) on the growth of *B. cinerea*. 100-μL samples (10^5, 10^4, 10^3 spores/mL) were incubated in PD medium containing α-PTH at the indicated concentrations. Hyphal growth was followed spectrophotometrically in a Bioscreen C Workstation. The standard deviation of the growth curve is indicated only for the control (0 μM).

Table 5 Effect of three AMPs on the average diameter of powdery mildew lesions (± standard error) on leaves inoculated in vitro, 21 days after inoculation (mm diam).

AMP	0 μM	2.5 μM	5.0 μM
α-Purothionin	3.3 ± 1.4	3.4 ± 1.4	3.3 ± 1.2
PGL	3.0 ± 1.1	2.6 ± 1.5	3.0 ± 1.3
Cecropin B	2.2 ± 1.1	2.6 ± 1.3	2.3 ± 1.7

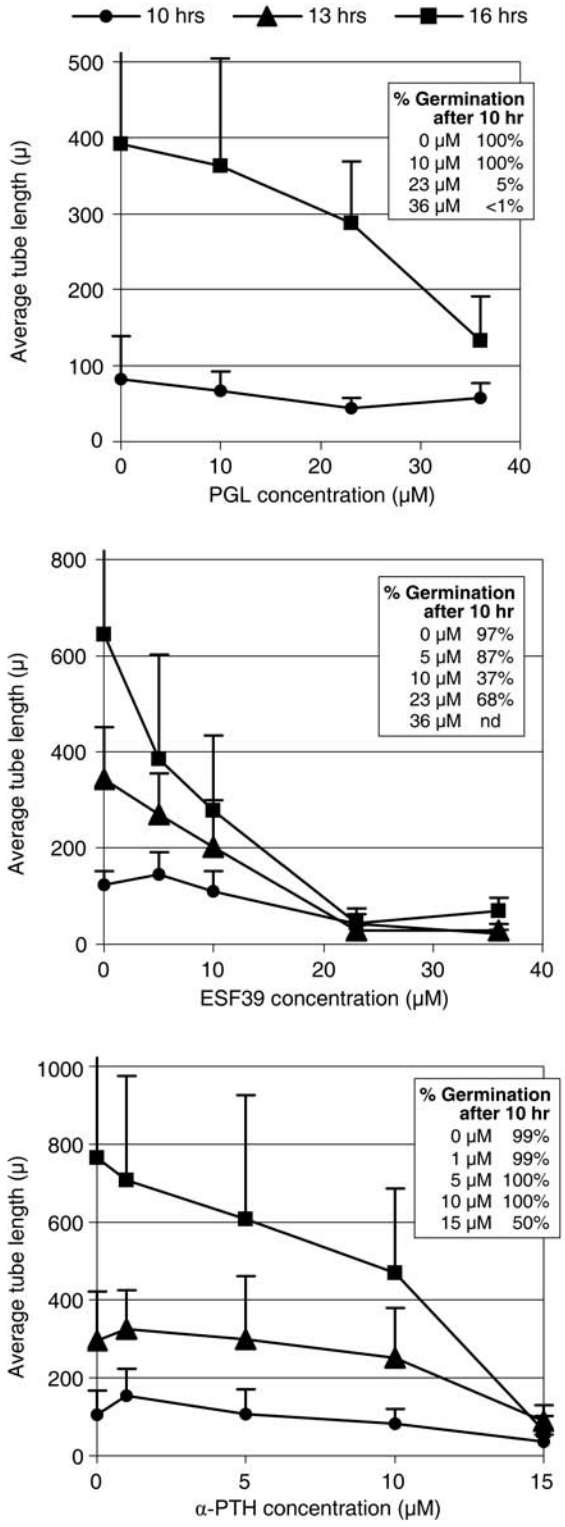

Figure 5 Effect of PGL, ESF39, and α-Purothionin on germination and hyphal tube growth from *B. cinerea* spores (10^5 spores/mL) after 10, 13, and 16 hr incubation.

Transgenic line 319-15 was positive for *PGL* transcription, but negative for *mag2* transcription. This line was also negative for crown gall resistance (Table 2), consistent with our observation that PGL is 10-fold less effective than Magainin-2 at controlling growth of *A. vitis* (Table 3).

The detection of AMP peptides in transgenic plants has been reported using immuno-absorbent assays (Li et al. 2001), but we have been unable to determine either by chemical analyses or by biological assays how much AMP is produced by transgenic grapevine plants (data not shown). It is possible that the patterns of pathogen resistance displayed by the transformed grapevines indicate AMP presence in the plant. This prompted the current study on the effect of AMPs on the growth of grapevine pathogens. One goal was to determine if Magainin-2, MSI-99, or other AMPs cause greater growth inhibition of *A. vitis* strain TM4 than strain CG450. In a more general sense, determining which AMP (or AMP combination) is best suited for controlling the growth of grapevine pathogens would assist in planning future grapevine transformations.

When the effects of nine AMPs on the growth of strains TM4 and CG450 are compared (Table 3), the growth of TM4 is always inhibited by slightly lower AMP concentrations than that of CG450, but the order of AMP effectiveness against *A. vitis* TM4 and CG450 is the same. Therefore, transgenic plants expressing the most effective AMP genes should have enhanced resistance to crown gall disease, but the level of resistance may depend on the bacterial strain present. That is commensurate with what was observed in two separate sets of transgenic grapevines expressing MSI-99 (Vidal et al. 2006b) or Magainin-2 (this study) and is also evidence of AMP expression.

The action of AMPs is concentration-dependent and varies with the membrane composition of the targeted pathogen (Gallucci et al. 2003). MSI-99, created to enhance the lytic ability of Magainin-2 by changing just a few amino acids in the sequence of the AMP (DeGray et al. 2001), was much more effective than Magainin-2 against the growth of *A. vitis*. Cecropin B was more effective against *A. vitis* growth than the related peptide, Shiva-1. Therefore, Shiva-1 is not a promising alternative for use in developing crown gall disease resistance, contrary to other results with tobacco and *P. solanacearum* (Jaynes et al. 1993). α-PTH, which has a rigid structure including four sets of intertwined cystines, was much more effective against the two *A. vitis* strains than against *A. tumefaciens*. ESF39, designed to resemble the secondary structure of magainins while having insignificant activity on plant and animal cells (Merkle et al. 2007), was moderately effective against *A. vitis,* especially strain TM4. With only two positively charged residues on the same side of the α-helix, MsrA3 inhibited the growth of *Agrobacterium* only at concentrations close to the maximum tested, as did PGL, another AMP with a short amino acid chain length. ESF12 was not effective even at the highest concentration tested, 15 μM, possibly because its 18-AA chain is not long enough to form an α-helix that can span the width of a biological membrane (Powell et al. 1995). Another *Agrobacterium* species, *A. tumefaciens*, was less sensitive to the inhibitory effects of AMPs than *A. vitis*, but the order of AMP effectiveness against it was almost the same.

Antimicrobial peptides also inhibited the hyphal development of *B. cinerea*, but much higher concentrations were needed than with *Agrobacterium* (Tables 3, 4). The order of AMP effectiveness against *B. cinerea* (α-PTH > PGL = ESF39 > Magainin-2 > ESF12 = MsrA3 > MSI-99 = Cecropin B = Shiva-1) was almost the reverse of what was found effective against *Agrobacterium*. α-PTH, PGL, and ESF39 were the most effective against *B. cinerea*: PGL via inhibition of spore germination and ESF39 by severely decreasing hyphal growth. However, the required concentrations, 15 μM or higher, seem beyond what can be produced in transformed grapevines (Li et al. 2001). When tested with wide ranges of AMP concentrations, none of the AMPs studied had reproducible effects on *E. necator* spore germination or on the in vitro formation of PM lesions on grapevine leaves, but when more than 50 leaves were tested in duplicate with only three concentrations of α-PTH, Cecropin B, or PGL, those AMPs had no effect on the in vitro formation of PM lesions. The highest concentration tested, 5 μM, was the highest concentration that could be expected from a transgenic plant (Li et al. 2001). Others have noted that the growth of fungal pathogens is much more difficult to inhibit than that of bacterial pathogens (Ali and Reddy 2000). While lytic peptides can be used to enhance disease resistance in crop plants, engineering transgenic plants to produce concentrations of lytic peptides required to inhibit fungal diseases will be very difficult to achieve (Alan and Earle 2002).

Conclusion

The use of the Bioscreen C Workstation made it possible to compare the effects of nine AMPs on five different pathogens of *V. vinifera* under carefully controlled conditions, facilitated the search for peptides with either antibacterial and/or antifungal activity, and improved our understanding of the differential resistance of transgenic vines to two strains of *A. vitis*. MSI-99 and Cecropin B were most inhibitory to *A. vitis* growth and, when added together into the growth medium of *A. vitis* TM4, the two AMPs had a desirable additive inhibitory effect. These findings suggest that future transformations with constructs containing these two AMPs may be useful to improve disease resistance in *Vitis*.

Literature Cited

Alan, A.R., and E.D. Earle. 2002. Sensitivity of bacterial and fungal plant pathogens to the lytic peptides, MSI-99, Magainin II, and Cecropin B. Mol. Plant-Microbe Interact. 15:701-708.

Ali, G.S., and A.S.N. Reddy. 2000. Inhibition of fungal and bacterial plant pathogens by synthetic peptides: In vitro growth inhibition, interaction between peptides and inhibition of disease progression. Mol. Plant-Microbe Interact. 13:847-859.

Ali, G.S., G.E. Harman, and B.I. Reisch. 2003. The interaction of endochitinase, a synthetic peptide and resveratrol in controlling fungi in vitro. Eur. J. Plant Pathol. 109:639-644.

Bornhoff, B.A., M. Harst, E. Zyprian, and R. Töpfer. 2005. Transgenic plants of *Vitis vinifera* cv. Seyval blanc. Plant Cell Rep. 24:433-438.

Burger, P., A. Bouquet, and M.J. Striem. 2009. Grape breeding. *In* Breeding Plantation Tree Crops: Tropical Species. S.M. Jain and P.M. Priyadarshan (eds.), pp. 161-189. Springer, New York.

Chakrabarti, A., T.R. Ganapathi, P.K. Mukherjee, and V.A. Bapat. 2003. MSI-99, a magainin analogue, imparts enhanced disease resistance in transgenic tobacco and banana. Planta 216:587-596.

Colova-Tsolova, V., A. Perl, S. Krastanova, S. Samuelian, and A. Atanassov. 2009. Progress in genetic engineering of grapevine for disease and stress tolerance. *In* Grapevine Molecular Physiology and Biotechnology. K.A. Roubelakis-Angelakis (ed.), pp. 509-533. Springer, Dordrecht.

DeGray, G., K. Rajasekaran, F. Smith, J. Sanford, and H. Daniell. 2001. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol. 127:852-862.

Faize, M., M.N. Brisset, C. Perino, B. Vian, M.A. Barny, J.P. Paulin, and M. Tharaud. 2006. Protection of apple against fire blight induced by an *hrp*L mutant of *Erwinia amylovora*. Biol. Plant. 50:667-674.

Gallucci, E., D. Meleleo, S. Micelli, and V. Picciarelli. 2003. Magainin-2 channel formation in planar lipid membranes: the role of lipid polar groups and ergosterol. Eur. Biophys. J. 32:22-32.

Halpin, H. 2005. Review–Gene stacking in transgenic plants–the challenge for 21st century plant biotechnology. Plant Biotechnol. J. 3:141-155.

Jaynes, J.M., P. Nagpala, L. Destefano-Beltran, J.H. Huang, J.H. Kim, T. Denny, and S. Cetiner. 1993. Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. Plant Sci. 89:43-53.

Jones, B.L., and A.S. Mak. 1975. Amino acid sequences of the two α-purothionins of hexaploid wheat. Cereal Chem. 54:511-523.

Kikkert, J.R., M.R. Thomas, and B.I. Reisch. 2001. Grapevine genetic engineering. *In* Molecular Biology and Biotechnology of the Grapevine. K.A. Roubelakis-Angelakis (ed.), pp. 387-404. Kluwer, Dordrecht.

Li, Z.T., and D.J. Gray. 2003. Effect of five antimicrobial peptides on the growth of *Agrobacterium tumefaciens*, *Escherichia coli* and *Xylella fastidiosa*. Vitis 42:95-97.

Li, Z., S. Jayasankar, and D. Gray. 2001. An improved enzyme-linked immunoabsorbent assay protocol for the detection of small lytic peptides in transgenic grapevines (*Vitis vinifera*). Plant Mol. Biol. Rep. 19:341-351.

Maloy, W.L., and U.P. Kari. 1995. Structure-activity studies on magainins and other host defense peptides. Biopolymers 37:105-122.

Malnoy, M., J.S. Venisse, M.N. Brisset, and E. Chevreau. 2003. Expression of bovine lactoferrin cDNA confers resistance to *Erwinia amylovora* in transgenic pear. Mol. Breed. 12:231-244.

Merkle, S.A., G.M. Andrade, C.J. Nairn, W. Powell, and C.A. Maynard. 2007. Restoration of threatened species: A noble cause for transgenic trees. Tree Genet. Genomes 3:111-118.

Monroc, S., E. Badosa, L. Feliu, M. Planas, E. Montesinos, and E. Bardaji. 2006a. De novo designed cyclic cationic peptides as inhibitors of plant pathogenic bacteria. Peptides 27:2567-2574.

Monroc, S., E. Badosa, E. Besalu, M. Planas, E. Bardaji, E. Montesinos, and L. Feliu. 2006b. Improvement of cyclic decapeptides against plant pathogenic bacteria using a combinatorial chemistry approach. Peptides 27:2575-2584.

Mourgues, F., M.N. Brisset, and E. Chevreau. 1998. Activity of different antibacterial peptides on *Erwinia amylovora* growth, and evaluation of the phytotoxicity and stability of cecropins. Plant Sci. 139:83-91.

Osusky, M., L. Osuska, R.E. Hancock, W.W. Kay, and S. Misra. 2004. Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot. Transgenic Res. 13:181-190.

Powell, W.A., C.M. Catranis, and C.A. Maynard. 1995. Synthetic antimicrobial peptide design. Mol. Plant-Microbe Interact. 8:792-794.

Powell, W.A., C.M. Catranis, and C.A. Maynard. 2000. Design of self-processing antimicrobial peptides for plant protection. Lett. Appl. Microbiol. 31:163-168.

Reustle, G.M., and G. Buchholz. 2009. Trends in grapevine genetic engineering. *In* Grapevine Molecular Physiology and Biotechnology. K.A. Roubelakis-Angelakis (ed.), pp. 495-508. Springer, Dordrecht.

Stec, B. 2006. Review: Plant thionins–the structural perspective. Cell Mol. Life Sci. 63:1370-1385.

Tauk-Tornisielo, S.M., J.M. Vieira, and J.S. Govone. 2007. Use of Bioscreen C for growth of *Mucor hiemalis* in different carbon and nitrogen sources. Braz. J. Microbiol. 38:113-117.

Vidal, J.R., J.R. Kikkert, P.G. Wallace, and B.I. Reisch. 2003. High-efficiency biolistic co-transformation and regeneration of 'Chardonnay' (*Vitis vinifera* L.) containing *npt-II* and antimicrobial peptide genes. Plant Cell Rep. 22:252-260.

Vidal, J.R., J.R. Kikkert, B.D. Donzelli, P.G. Wallace, and B.I. Reisch. 2006a. Biolistic transformation of grapevine using minimal gene cassette technology. Plant Cell Rep. 25:807-814.

Vidal, J.R., J.R. Kikkert, M.A. Malnoy, P.G. Wallace, J. Barnard, and B.I. Reisch. 2006b. Evaluation of transgenic 'Chardonnay' (*Vitis vinifera*) containing magainin genes for resistance to crown gall and powdery mildew. Transgenic Res. 15:69-82.

Westerhoff, H.V., M. Zasloff, J.L. Rosner, R.W. Hendler, A.D.E. Waal, A.V. Gomes, A.P.M. Jongsma, A. Riethorst, and D. Juretic. 1995. Functional synergism of the magainins PGLa and magainin-2 in *Escherichia coli,* tumor cells and liposomes. Eur. J. Biochem. 228:257-264.

Xing, H., C.B. Lawrence, O. Chambers, H.M. Davies, N.P. Everett, and Q.Q. Li. 2006. Increased pathogen resistance and yield in transgenic plants expressing combinations of the modified antimicrobial peptides based on indolicidin and magainin. Planta 23:1024-1032.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA 84:5449-5453.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389-395.