TESTING TRANSGENIC GRAPEVINES FOR RESISTANCE TO PIERCE'S DISEASE

Project Leaders:
Bruce I. Reisch and Julie Kikkert
Department of Horticultural Sciences
NYSAES, Cornell University
Geneva, NY 14456

Cooperators:
Andrew Walker
Department of Viticulture and Enology
University of California
Davis, CA 95616

Claire-Lise Rosenfield
Department of Horticultural Sciences
NYSAES, Cornell University
Geneva, NY 14456

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ABSTRACT
Magainins are small antimicrobial peptides (AMPs) that inhibit growth of numerous bacteria and fungi. Some AMP-transgenic lines of 'Chardonnay' have improved resistance to tumorigenic strains of crown gall (*Agrobacterium vitis*). Other researchers have claimed that similar AMPs induce grapevine resistance to Pierce's disease (PD). Sixteen 'Chardonnay' lines transformed with the magainin-type AMP genes, mag-2 and MSI99, and with a PGL class gene, were produced and tested for PD resistance using a greenhouse needle inoculation technique. Most lines were susceptible, but several showed reductions in symptom development and reductions in plant tissue bacterial counts. These vines are being propagated for a field trial to test for resistance under conditions of natural inoculation. Tests are also underway to quantify the level of peptide production in each transgenic line. In addition, in vitro assays are being conducted to evaluate the relative effect of these and other peptides on growth of *Xylella fastidiosa* (*Xf*). MSI99 and ESF39 inhibit *Xylella* growth more effectively than do the other peptides tested, according to results obtained to date.

INTRODUCTION
Numerous genes involved in plant disease defense have been isolated (Punja 2001; Mourgues et al. 1998). When disease resistance genes are introduced and expressed in transgenic plants, fungal and bacterial diseases have been greatly reduced (Mourges et al. 1998; Punja, 2001; Van der Biezen 2001). We have developed a set of transformed grapevines in which AMP genes are transcribed into RNA. About 80 'Chardonnay' lines transformed with the magainin-type genes, mag-2 and MSI99, and with a PGL class gene, were produced (Vidal et al. 2003). Magainins are small peptides with strong inhibitory activity against numerous bacteria and fungi (Zasloff et al. 1988; Smith et al. 1998; DeGray et al. 2001; Smith et al. 2001). The MSI99 peptide expressed in tobacco and banana was shown to be highly effective against several pathogens (Chakrabarti et al. 2003). Some AMP-transgenic lines of 'Chardonnay' demonstrated improved resistance to tumorigenic strains of crown gall (*Agrobacterium vitis*) (Vidal et al. 2005), suggesting that these lines may harbor resistance to other bacterial diseases, as well.

Some AMP producing genes such as Shiva-1 are effective against PD (Scorza and Gray, 2001) but the subject warrants further study. It is the purpose of the present project to study the potential resistance of our AMP-producing vines to PD; learn more about the effects of various AMPs on *Xf* growth; and develop new sets of transgenic vines with the potential to resist PD.

OBJECTIVES
1. Analyze AMP (anti-microbial peptide) expression in transgenic 'Chardonnay' vines.
2. Understand the relationship between AMP levels and disease resistance; design improved transformation vectors based on results.
3. Evaluate resistance to PD among these transgenic vines.

RESULTS
Objective 1: Analyze AMP (anti-microbial peptide) expression in transgenic 'Chardonnay' vines
Transgene expression in leaves was quantified by ELISA. For the mag-2 and MSI99 peptides, an antibody was developed that recognized an antigenic sequence common to both. In a series of preliminary ELISA tests (during 2003; methods per Li et al. 2001), low levels of peptide production were detected in 8 of 22 lines, in agreement with previous RT-PCR results. However we were unable to detect the peptide consistently, suggesting the methodology required some improvement. In spring 2004, a series of ELISA tests for peptide detection were carried out using very young leaves from greenhouse plants. Chardonnay lines transformed with either the gene for mag-2 or for MSI99 production (ten of each), plus two non transformed lines, were assayed in three separate experiments. Despite rapid sample preparation, oxidation was an erratic problem among samples, and there were inconsistencies in the data collected. Among the ten lines expressing mag-2, lines

167-3 and 167-9 were significantly different from the non transgenic controls. There were no significant differences in the ELISA assay among lines transformed with MSI99, however the highest ELISA readings were with lines 168-8 and 168-15.

With the inconsistency of results from the ELISA assays, two other methods for detecting peptide expression were investigated. Using the Bioscreen C Microbiological Workstation, conditions to bioassay for peptide activity in plant tissue extracts were investigated. Tissue extracts from non transgenic vines plus known amounts of mag-2 peptide were incubated with bacterial cultures and growth following the incubation period was measured. Peptide degradation was reduced with a protease inhibitor cocktail. Small amounts of plant extract were found to stimulate bacterial growth and large amounts completely inhibited growth. So this line of research is proceeding to establish the proper conditions to bioassay for peptide activity in transgenic plant tissues.

Direct quantification of peptide production is also being investigated using a BioLC Chromatograph in the chemistry laboratory of Dr. Terry Spittler, Horticultural Sciences, Cornell University. This system can be used to determine species and quantity of peptides by highly sensitive ion chromatographic techniques and electrochemical detection. Plant tissue extracts have been collected and stored at –20 C. Control experiments are underway to determine elution times for detection of the peptides of interest. Results are not yet available.

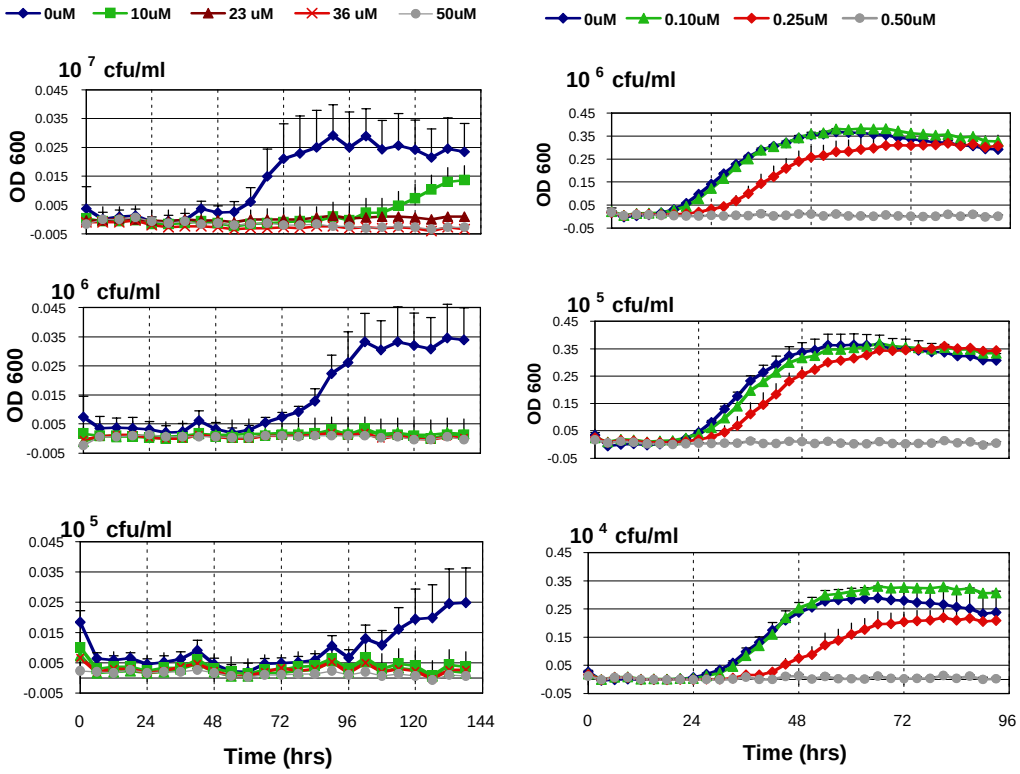

Figure 1. Growth of *Xf* (left side) and *Agrobacterium vitis* (right side) in the presence of varying concentrations of MSI 99. Initial bacterial concentrations ranged from 10^7 to 10^5 cfu/ml.

Objective 2: Understand the relationship between AMP levels and disease resistance; design improved transformation vectors based on results

The following AMPs were grown in the presence of varying concentrations of *Xf* (Stag's Leap strain) in vitro: ESF12, ESF39, mag-2, MSI99 and PGL. At least one additional AMP, MsrA3, is still to be tested. Replicated testing was conducted in a Bioscreen C Microbiological Workstation. Initial *Xf* concentrations were adjusted to 10^5, 10^6 and 10^7 cfu/ml in PW (Periwinkle Wilt) liquid medium.

The Bioscreen C microplate reader was found to be suitable for automated measurements of growth of *Xf*. At OD600, the range of readings was very low as compared to the range obtained with *Agrobacterium vitis* (see Figure 1), yet the readings were consistent and indicative of the effects of increasing concentrations of antibacterial compounds. Among the five peptides tested to date, MSI99 was the most effective inhibitor of growth (Table 1). Since it was only tested at concentrations ranging from 10 to 50 µM, further testing will be necessary at lower concentrations. The amino acid sequence of MSI99 is very similar to that of mag-2. However, it is a much more potent inhibitor of *Xf,* and this is consistent with

reports that it was developed to be a more potent analog of mag-2. PGL and ESF12 had very little effect on the growth of *Xf*, even at the very high concentration of 50 μM. ESF39, reported to be a more potent analog of ESF12, was much more inhibitory to *Xf* growth than was ESF12.

Table 1. Effects of five antimicrobial peptides on growth of *Xylella fastidiosa*.

AMP (μM)	*Xylella* conc. (cfu/ml)	grows well at or below:	grows slowly or erratically at:	does not grow at or above:
Magainin-2 1; 5; 10; 15	10^7	1 μM	5 to 15 μM	n.a.
	10^6	n.a.	1 to 15 μM	n.a.
	10^5	1 to 5 μM	10 μM	15 μM
MSI-99 10; 23; 36; 50	10^7	n.a.	10 μM	23 μM
	10^6	n.a.	n.a.	10 μM
	10^5	n.a.	n.a.	10 μM
PGL 10; 23; 36; 50	10^7	50 μM	n.a.	n.a.
	10^6	36 μM	50 μM	n.a.
	10^5	23 μM	36 μM	50 μM
ESF-12 10; 23; 36; 50	10^7	50 μM	n.a.	n.a.
	10^6	50 μM	n.a.	n.a.
	10^5	n.a.	50 μM	n.a.
ESF-39 10; 23; 36; 50	10^7	n.a.	10 μM	23 μM
	10^6	n.a.	10 μM	23 μM
	10^5	n.a.	10 μM	23 μM

n.a. = not applicable

Further work during the course of the present project will focus on the development of improved transformation vectors for resistance to PD. The present work to assess the four groups of transgenic vines plus the ongoing project to evaluate the effects of a range of AMPs on growth of *Xf* in vitro will be used as a knowledge base to contribute to the design of new plasmids or gene cassettes. Consideration will be given toward optimizing the promoters and signal peptides in each construct.

Objective 3 - Evaluate resistance to PD among these transgenic vines

Previous efforts to test peptide-producing transgenic lines of Chardonnay for resistance to *Xf* showed that, using the greenhouse needle-inoculation technique, most lines were susceptible to PD, and just a few showed reduced symptom development and reductions in the number of *Xf* bacteria (Reisch et al. 2004). It is not yet known how these vines will respond in the field under conditions of natural inoculation. Vines are now being propagated for planting of a trial of AMP-producing vines in Texas, where they will be observed for field resistance to PD. This trial will include at least two lines of each of four types of transformants. The four types are those transformed with genes for the production of peptides mag-2, MSI99, PGL, and mag-2 + PGL. The trial will be replicated and will include control susceptible and resistant vines. Planting is scheduled for spring 2006.

CONCLUSIONS

Transgenic vines harboring genes that produce substances inhibitory to growth of *Xf* are being propagated to test for field resistance to PD. Work is underway to quantify the production of these inhibitory substances in grapevine tissues. These and other similar substances are being tested in vitro for their relative effects on the growth of *Xf*. Based on the data being produced, new gene constructs will be designed with the goal of providing improved levels of resistance to PD.

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