# TESTING TRANSGENIC GRAPEVINES FOR RESISTANCE TO PIERCE'S DISEASE

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## ABSTRACT
About 80 'Chardonnay' lines transformed with the magainin-type antimicrobial peptide (AMP) genes, mag-2 and MSI99, and with a PGL class gene, were produced and about 40 were chosen for detailed analysis. Magainins are small peptides that inhibit growth of numerous bacteria and fungi. Some of these AMP-transgenic lines 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). The goal of our project was to characterize gene insertion, expression, and disease resistance in 'Chardonnay' grapevines harboring mag-2, MSI99, and a PGL class gene. These lines were shown to harbor between 1 and 5 copies of the foreign gene. RT-PCR testing showed that the genes are transcribed into RNA but efforts to directly detect peptide production in leaf tissues have been hampered by technical difficulties. We were able to show that some lines do produce the peptide, and that several have improved resistance to crown gall disease. The Walker Lab (UC Davis) has tested the same set of vines for resistance to PD. Symptom development was delayed in a few lines, which also showed improved lignification and re-growth following symptom development. Using an ELISA test to quantify the presence of the causal bacterium, 15 lines were classified as 'susceptible' while one line was classified as 'intermediate' in terms of resistance. Plants have been grafted to determine whether resistance (and/or transgene products) can be transmitted from a transformed rootstock to a non transformed scion cultivar. These tests are now underway.

## INTRODUCTION
Numerous genes involved in plant disease defense have been isolated (Punja 2001; Mourges 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 (DeGray et al. 2001; Zasloff et al. 1988; Smith et al. 1998; 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). In preliminary studies in 2002, some AMP-transgenic lines of 'Chardonnay' demonstrated improved resistance to tumorigenic strains of crown gall (*Agrobacterium vitis*), 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. Scorza and Gray described a trial of two lines of 'Thompson Seedless' expressing the Shiva-1 peptide; both eventually succumbed to PD, but one had milder symptoms, which did not include the typical signs of marginal leaf burn when compared to the non-transformed control plant. However, data are not available in the literature to determine if mag-2, PGL, and MSI99 peptides are effective against *Xylella fastidiosa*. It is the purpose of the present project to study the potential resistance of our AMP-producing vines to PD.

## OBJECTIVES
1. Quantify the expression of AMPs (antimicrobial peptides) in transgenic 'Chardonnay' vines.
2. Evaluate resistance to Pierce's Disease among these transgenic vines.
3. Determine the extent to which an AMP transgenic rootstock can confer PD resistance to the scion.

## RESULTS
***Objective 1 - Quantify the Expression of AMPs (Antimicrobial Peptides) in Transgenic 'Chardonnay' Vines:***
Southern blots were used to determine the number of integration events in each positive line, as well as to determine which lines have full-length copies of the promoter + gene combination. Digested genomic DNA was separated by electrophoresis and visualized by chemiluminescence using digoxigenin-labeled probes. We tested 35 PCR positive lines and hybridization signals were detected in 34 lines. Between 1 and 5 AMP gene integration sites per line were detected. Hybridization banding

patterns differed among the lines, indicating independent transformation events. The entire non-fragmented promoter/signal peptide/AMP gene sequence was detected in twelve lines.

Transgene expression in leaves was quantified by ELISA. For the mag2 (23 amino acids) and MSI99 (22 amino acids) peptides, an antibody was developed (by Sigma-Genosys) 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 mag2 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 mag2, 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. All four of these lines showed both mRNA transcription (via RT-PCR assays) and resistance to crown gall. Future attention was turned toward assaying peptide activity in bioassays to detect direct effects of plant extracts on bacterial growth.

***Crown Gall*** *(cooperative work with Tom Burr; included here since it shows relative resistance to a bacterial disease among the same set of AMP transformed vines)*
Crown gall resistance was assayed in two separate experiments. Thirty-one transgenic lines (4 plants/line) were inoculated with two different *Agrobacterium vitis* strains (TM4 and CG450). (Table 1 shows results for 16 lines.) Resistance was evaluated 60 d post inoculation based on gall size of 20 inoculation sites per line following a disease index (DI): 0 = no symptoms, 1 = small gall; 2 = medium gall; 3 = large gall; and 4 = very large gall. Among the lines tested, 6 harboring the *mag2* gene, 5 with the *MSI99* gene, 5 with the *PGL* gene and 5 with the combination of the *mag-2 + PGL* fusion gene showed statistically significant gall size reductions ($P<0.05$) compared to non-transformed controls (Table 1).

In the present study, a correlation was found between transcription level and resistance to the crown gall disease. We used a constitutive ubiquitin promoter from *Arabidopsis* to drive AMP gene expression. Although ubiquitin promoters are functional in *V. vinifera*, stronger promoters could be more useful for effective accumulation in plant tissue of small antimicrobial peptides. Finally, the level of resistance under potentially lower levels of inoculum in field conditions remains to be determined; greenhouse tests were done with high concentrations of inoculum.

***Objective 2 - Evaluate Resistance to Pierce's Diseasea Among These Transgenic Vines:***
AMP-transgenic greenhouse-grown vines were tested for resistance to Pierce's disease. There were four groups chosen for testing, as shown in Table 1. Four lines were chosen to represent each of the four groups, and four vines of each line were tested. Vines were inoculated using the pin-prick needle inoculation technique of Hopkins (1980, 1984). Controls included two tissue-culture-produced non-transformed lines from the same set of experiments, plus a line of Chardonnay that was propagated from conventional cuttings. Transformed vines for this experiment were selected from among those with moderate to high rates of AMP gene transcription.

Results are not yet fully analyzed, but there is some variation for PD resistance among the 16 transgenic lines tested. All lines showed leaf symptoms of PD, but some lines had better cane lignification and new growth despite infection. Though symptom development was delayed in a number of lines, by about 3 months after collecting data on PD symptoms and samples for the ELISA assays, all vines had severe symptoms of PD or were dead. ELISA testing placed all lines but one in the 'susceptible' category, while one line harboring two AMP genes (319-13) was placed in the 'intermediate' category (Table 1). All control lines were classified as 'susceptible'. The two tissue cultured control lines did relatively well compared to conventionally propagated Chardonnay.

***Objective 3 - Determine the Extent to Which an AMP Transgenic Rootstock can Confer PD Resistance to the Scion:***
Green grafting was used to connect five replicates of each transgenic line with non transgenic scions. Shoot growth on the scion 'Chardonnay' will be needle inoculated with the Stag's Leap *Xylella fastidiosa* strain. This work is still ongoing. About 70% of the grafted transgenic lines have been successfully produced and will soon be inoculated with *Xf*.

**CONCLUSIONS**
Some indications of elevated resistance to PD are provided by the delays observed in symptom development and, for one line, the ELISA assays for bacterial concentration. However, after 3 months, all vines had severe symptoms of PD. Under natural field conditions with reduced inoculum concentrations, it is not yet known how these vines will perform. Use of AMP-transgenic vines to generate PD tolerant lines of important cultivars still seems to hold some promise, and warrants further testing. Confirmation of these initial results is still pending. The use of various means to target expression to the xylem may hold promise in future trials.

**Table 1.** Resistance to two bacterial diseases in AMP-transgenic 'Chardonnay' lines.

| | *Disease resistance* | | | | |
|---|---|---|---|---|---|
| | *Crown Gall* [a] | | *Pierce's Disease* | | |
| Line | TM4 | CG450 | Mean cfu/ml ($\times 10^5$) ± std. error | PD Class [b] | Avg. visual symptoms ± std. error |
| ***with Mag2*** | | | | | |
| **167-2** | ** | * | 15.8±7.1 | S | 3.9±0.9 |
| **3** | ** | ** | 38.8±10.4 | S | 4.8±0.2 |
| **9** | ** | * | 6.7±1.7 | S | 2.3±0.5 |
| **17** | * | | 4.6±1.0 | S | 2.0±0.4 |
| ***with MSI99*** | | | | | |
| **168-8** | * | | 16.3±6.6 | S | 3.3±1.1 |
| **15** | ** | | 8.1±1.8 | S | 1.3±0.8 |
| **32** | ** | * | 5.3±1.2 | S | 2.4±0.8 |
| **37** | * | * | 15.5±7.2 | S | 2.6±1.1 |
| ***with PGL*** | | | | | |
| **315-5** | ** | | 20.2±6.1 | S | 4.7±0.7 |
| **17** | * | * | 12.0±5.9 | S | 3.4±1.0 |
| **19** | * | * | 15.2±2.6 | S | 4.4±0.4 |
| **20** | ** | ** | 11.4±1.7 | S | 2.5±0.9 |
| ***with mag2 + PGL*** | | | | | |
| **319-7** | * | | 11.6±13.3 | S | 2.7±1.6 |
| **13** | ** | ** | 3.5±0.4 | I | 3.0±0.8 |
| **26** | ** | ** | 13.1±1.8 | S | 2.6±0.8 |
| **31** | ** | | 4.7±1.8 | S | 1.9±0.7 |
| ***controls:*** | | | | | |
| **NT8.1** | | | 8.2±2.4 | S | 3.1±0.6 |
| **NT8.2** | | | 7.5±2.6 | S | 2.5±0.7 |
| **Chardonnay** | | | 27.2±6.7 | S | 3.7±0.4 |

[a] Significantly resistant (* $P<0.05$; ** $P<0.01$) when inoculated with TM4 or CG450 strains of *A. vitis*.

[b] PD Class

Resistant (R): ELISA mean cfu/ml $<1 \times 10^5$

Intermediate (I): ELISA mean cfu/ml $>1 \times 10^5$ and sum of ELISA mean cfu/ml + Std Err $<5 \times 10^5$

susceptible (S): Sum of ELISA mean cfu/ml + Std Err $>5 \times 10^5$

Visual Symptoms Score

Genotypes with an average score of 2.0 or less can be considered resistant if ELISA values do not contradict.

Scores higher than 2.0 are indicative of susceptible genotypes

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