PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: BIOLOGY OF ENDOPHYTIC BACTERIA IN GRAPE PLANTS AND BIOASSAY OF REAGENTS TO DISRUPT PIERCE'S DISEASE

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ABSTRACT
Xylella fastidiosa (*Xf*), which causes Pierce's disease (PD) in grapevines, is transmitted by the glassy-winged sharpshooter (GWSS). Paratransgenesis employs symbiotic bacteria to deliver anti-*Xf* compounds to disrupt transmission of the pathogen to new host plants. *Alcaligenes xylosoxidans denitrificans* (*Axd*) was identified as a potential agent for paratransgenesis because it inhabits the foregut of GWSS, as does *Xf*, and the xylem of plants. In this report, we describe the interaction of *Axd* with plants, GWSS predators, and alternate *Xf*-vectors and report preliminary data on inhibition of transmission by anti-*Xf* factors. *Axd* colonized and traveled within 6 host plants, reaching the highest titers in lemon. *Axd* colonized the foregut region of two alternate *Xf*-vectors, the blue green and smoke tree sharpshooters, and was not identified in predatory arthropods that fed on *Axd*-positive GWSS. Disruption of *Xf*-transmission by GWSS was demonstrated using two reagents, a single chained antibody fragment and an antibiotic peptide.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the principle vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapevines. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission would control the disease.

Paratransgenic approaches to pathogen control are currently being developed to deliver anti-pathogen strategies to disrupt Triatomid transmission of *Trypanosoma cruzi* (1), to prevent colitis in mammals (2, 7), and to interfere with transmission of HIV(4). Candidate microbes that live in close proximity to the pathogen in the vector insects and in host plant tissues would by ideal vehicles to control *Xf*.

Alcaligenes xylosoxidans denitrificans (Axd), originally isolated from the cibarium of GWSS, has been described as a non-pathogenic plant endophyte and a non-pathogenic soil-borne microbe (5, 8). *Axd*, genetically marked with DsRed or EGFP protein, colonized the cibarium of GWSS for up to 35 days, the longest period tested (3).

Two categories anti-pathogen reagents, single-chained antibodies (scFV) and antibiotic peptides, were tested for activity against *Xf*. Screening of scFV uncovered an antibody fragment that was specific to *Xf* and may be specific to the PD-causing strain of *Xf*. Four toxic peptides were identified that inhibited the growth of *Xf*, but did not inhibit the growth of *Axd*.

OBJECTIVES
1. Identify environmental endpoints of *Axd* in plants, GWSS predators, and alternate insect vectors.
2. Test the ability of anti-pathogens to disrupt *Xf* disease cycle.

RESULTS
Axd movement within and colonization of six host plants.
Plants treated with *Axd* showed no abnormalities (stunting, chlorosis, necrosis, etc.) two weeks after inoculation, despite relatively high titers in several hosts as compared to control plants. The melting temperature of the 199 bp *Axd* EGFP insert sequence is 92.1°C. Comparing PCR product melting temperature to that of the primer dimer (80.6°C), conformations were made by increasing the temperature from 72 to 99°C at a rate of 1°C/45s and measuring fluorescence every 45s, then plotting results on the melting curve. A standard curve and a regression line (R=0.983, R2=0.967, efficiency=0.73, M=0.239, B=10.362) was constructed based the amplification of known standards with plant background. Standards were tested in the presence of each host plant and no significant differences were found, therefore differences in quantification were not attributed to deleterious plant effects on PCR.

Axd was found most consistently (44 of 45) and in the highest concentrations in lemon plants in both replications (Table 1). In both replications, lemon plants were found to have greater than 3 million *Axd* cells per 2 cm of tissue, almost one order of

magnitude higher than in other hosts tested. While all inoculated sweet orange plants (25 of 25) were positive with relatively high titers (943,305 cells/2 cm) of *Axd* in the first replication, only 13 of 20 were positive in replication 2 with a lower mean titer (19,458 cells/2 cm). Grapevine, periwinkle, and crepe myrtle had lower numbers of positive plants and fewer cells per 2 cm cut. Chrysanthemum was only replicated once and 19 of 20 plants were positive with a relatively high titer of *Axd* (151,108 cells/2 cm).

Table 1. Axd populations per 2 cm stem tissue (5 cm from inoculation point) in different host plants determined by QRT-PCR (2 wks post inoculation).

Host Plant	Replication 1		Replication 2	
	Number of cells per positive plant	Positive hosts	Number of cells per positive plant	Positive hosts
Lemon	3,591,427	25/25	3,034,792	19/20
Sweet Orange	943,305	25/25	19,458	13/20
Crepe Myrtle	884,770	8/25	29,235	2/20
Periwinkle	304,820	10/25	284,164	16/20
Grape	18,225	24/25	71,982	6/20
Chrysanthemum	NA	NA	151,108	19/20

*All negative controls were negative.

Acquisition of Axd by GWSS predators

Using a plant-based artificial feeding system (AFS) (3), GWSS adults were fed *Axd* marked with a dsRed protein. After feeding from the AFS, GWSS were maintained on chrysanthemum, and fed to predators over a 2 week period. Samples GWSS from this colony all tested positive for the presence of dsRed-*Axd*. Predators tested negative for the presence of *Axd*; 3 arachnid species (n=5), 1 Mantidae species (n=5), and 1 Reduviidae species (n=5) by fluorescent microscopy.

Acquisition of Axd by alternate Xf-vectors

In replication one, blue green sharpshooters were offered dsRed *Axd* through an artificial membrane (6) and smoke tree sharpshooters were offered dsRed *Axd* through the plant-based AFS (3). The foreguts of all dsRed *Axd*-fed insects were viewed under fluorescent microscopy and were positive at 1d, 7d, and 14d post-exposure, while all control (non-dsRed Axd-fed) insects were negative. In replication two, both blue green and smoke tree sharpshooters were offered dsRed *Axd* through the plant-based AFS and tested positive by fluorescent microscopy on all dates.

Axd in California vineyards

In July of 2003, we received a permit from the EPA to test for the environmental endpoint of *Axd* when inoculated into grapevines by needle inoculation, foliar application, and soil drench. We are currently analyzing samples from these experiments, which were conducted in Riverside, Bakersfield, Temecula, and Napa. In these caged field trials, our goal is to determine if *Axd* colonized different tissues of these grapevines, especially fruit.

Interruption of PD cycle

In a preliminary experiment, transmission of *Xf* from infected grapevine to healthy grapevine by GWSS was blocked by feeding GWSS on the plant-based AFS containing an *Xf*-specific antibody fragment (scFV S1) expressed in the coat of a M13 bacteriophage, between a 5 d acquisition access period (AAP) and the 5 d inoculation access period (IAP). At two concentrations of phage/antibody (10^{14} and 10^{15}) transmission of *Xf* was 0% (n=10 and n=13, respectfully), compared to 50% transmission in the control group (n=8). Transmission of *Xf* was reduced when GWSS were fed Indolicidin (American Peptide Company, Inc., Sunnyvale, CA) between the AAP and IAP from 50% in the control group to 35% (n=14) at 100µg/ml and 7% (n=X14) at 500µg/ml. These experiments are being replicated.

CONCLUSIONS

Genetically-marked *Axd* colonizes several host plants. This suggests that genetic modification does not interfere with the biology of *Axd*, which should enter into the insect-plant cycle and be transmitted along with the pathogenic bacteria target. While GWSS is the vector of greatest interest at this time, two other native sharpshooters also transmit the vehicle bacterium, *Axd* and several plants can serve as hosts.

Movement of *Axd* beyond grapevines is an issue that has to be addressed, not just for permitting purposes, but also to gain acceptance from the producers and consumers. Predatory arthropods feed GWSS containing high titers of *Axd* did not acquire the marked bacteria.

In the laboratory, inhibition of *Xf*-transmission by GWSS was demonstrated using two different categories of reagents, a surface antibody fragment and an antibiotic peptide (Indolicidin). The antibody fragment was specific to *Xf*. In our trials the antibody fragment was being expressed in the coat of a phage, so the effects on transmission might be greater when the antibody fragment is expressed on the surface of *Axd*. Indolicidin inhibited *Xf* growth *in vitro*, but did not affect growth of *Axd*. Transformation of *Axd* to produce each/or both of these reagents is currently under way.

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