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). Symbiotic control employs symbiotic bacteria to deliver anti-pathogen 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 and the xylem of plants, as does *Xf*. In this report, we describe the relationship between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control. Additionally, 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 principal 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.

Symbiotic control approaches have been developed to disrupt Triatomid transmission of *Trypanosoma cruzi* [3], to prevent colitis in mammals [4, 11], and to interfere with transmission of HIV [8]. 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 [10, 12]. *Axd*, genetically marked with DsRed or EGFP protein, colonized the cibarium of GWSS for up to 35 days, the longest period tested [6]. *Axd* readily colonized the xylem vessels of several plants with citrus being the most hospitable to the bacterium. [5]

Two categories of 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 relationships between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control.
2. Test the ability of anti-pathogens to disrupt *Xf* disease cycle.

RESULTS

Axd Movement and Colonization within Host Plants

In two trials, chrysanthemums (n=20) were needle inoculated with DsRed *Axd,* which contains a kanamycin-resistance gene. One week later phloem and xylem fluid samples were collected independent of one another using a Scholander pressure bomb [7]. The collection resulted in about 20-50 µl of phloem fluid and 100-150 µl of xylem fluid per stem. DNA was extracted from the remaining half of each phloem and xylem fluid sample from each plant using the Extract-N-Amp™ Plant kit (Sigma Aldrich, Steinheim, Germany). Presence of *Axd* was then determined using QRT PCR. The other half of each phloem and xylem fluid sample was inoculated into LB broth containing kanamycin and incubated for 48 h at 37ºC. After the incubation period, bacteria were screened for red fluorescence using a MZ12 fluorescent microscope (Leica Microsystems Inc., Heerbrugg, Switzerland). Positive samples were confirmed by QRT PCR.

A higher proportion of xylem fluid samples tested positive for the presence of *Axd* than phloem samples in both trials: in trial 1 xylem 8/20, phloem 2/20 (χ^2=4.8, 1df, p=0.0284); in trial 2 xylem 15/20, phloem 8/20 (χ^2=5.013, 1df, p=0.025). In all

cases, positive phloem samples were detected only when the corresponding xylem samples was positive, whereas, most xylem samples were positive when phloem samples were negative. This indicated that positive detection of *Axd* in the xylem was due to actual presence of the bacterium; detection in phloem may have been due to contamination. Of the samples that tested positive, xylem samples contained 10X more cells on average than phloem although these values were not significant at the p=0.05 level (Trial 1: F=0.911, 1df, p=0.368.Trail 2: F=3.123, 1df, *p*=0.092). All plant samples which tested positive by RT PCR were confirmed by culturing followed by visualization under fluorescent microscopy.

Movement of Axd into GWSS Populations

After being exposed to an artificial feeding system containing DsRed *Axd* for 48h [6], 2 GWSS were marked with paint and placed on an individually caged chrysanthemum with 10 naive GWSS for 2 weeks. At the end of this period, all GWSS were collected from the cage and analyzed for the presence of DsRed *Axd* by QRT PCR. In two trial, each with 10 replicates (10 individually caged plants), 81% of the test insects survived through the studies. In both trials, more than 57% of the surviving, previously "naïve", GWSS tested positive for the presence of *Axd* (Trial 1, 51.2%; Trial 2, 64.3%). Therefore, through passive delivery of the symbiont in a finite period of time, more then ½ of the insects acquired the bacterium

Effect of Axd or Xf on GWSS Biology

Colonies of GWSS which were orally inoculated with DsRed *Axd*, wild-type *Axd*, S1 *Axd* (bacterium expressing an antibody), *Xf*, or no introduced bacteria (control) were maintained under laboratory conditions. Feeding ability, natural mortality, and dry weight post-mortem were compared between groups to determine if the presence of bacterium influenced any of these biological factors. In preliminary studies, mean g consumed after 5 was not significantly different for any of the 5 groups (n=20, p<0.001). Mortality rates of GWSS maintained in the laboratory were plotted over an 18 day period (Figure 1). Decline of the colony was consistent over time and no significant differences in mortality rates were detected (p<0.001). Randomly, individual GWSS were collected from each colony, dried in a desiccating oven for 48 h, and weighed. The average weight of a dried GWSS was 0.01g and no significant differences in dry weight were revealed (*p*=0.7). In two trials, 50 wild GWSS that were collected on the UCR campus were microinjected with different amounts either, DsRed *Axd* or H_2O. Bacteria were detected in the hemocoel; however, based on Chi-square analysis there were no significant differences in the mortality rates between the two groups.

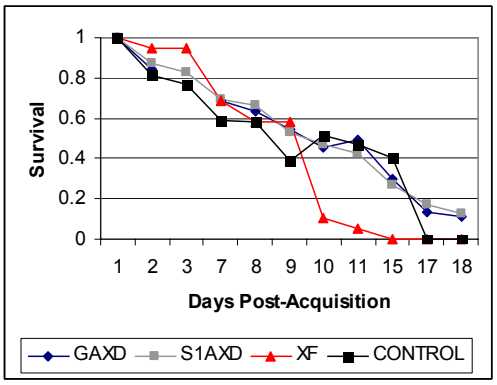

Figure 1. Mortality rate in four GWSS colonies maintained in the lab oratory.

Laboratory-Based Artificial Disease Cycle

A simple and efficient transmission cycle was developed for the study of *Xf* transmission by GWSS which allowed collection of sufficient transmission data in 1 wk. Specific numbers of cells were detected both in plant tissue and within the insect vector by QRT PCR. *Xf* cells were scraped from a PD3 plate and suspended in sterile ½ strength PBS (OD^{600}=2.0). Five cm sections of cut chrysanthemum stems were used for bacterial inoculations [6]. Five GWSS per 5 cm of stem were caged in snap cap vials for 48 h. After the acquisition access period (AAP), GWSS were placed on *Xf*-free chrysanthemums for 48 h, so that any detection of bacteria would be associated with transmission and not stylet contamination. Pairs of GWSS were transferred to sterile vials containing a fresh chrysanthemum stem cutting. The insects were exposed to a stem for an inoculation access period (IAP) of 48 or 96 h. DNA was extracted from the inoculation targets with the XNAR Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO) and PCR was run following a standard QRT-PCR protocol. Across 9 replicates using a 48h IAP, the mean transmission rate of *Xf* by GWSS was 0.508±0.122, while the mean rate when given a 96h IAP was 0.341±0.138. Using Chi-square analysis, these ratios were significantly different (χ^2=16.281, df=1, p≤0.001). The lower rate associated with the longer IAP is probably due to the non-hospitable environment of the test plant stems.

Interruption of PD Cycle

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 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, respectively), 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=14) at 500µg/mL. These experiments are currently being replicated. While the rate of *Xf* transmission was higher than previously reported [1, 2, 9], we feel this is a fair assessment of the insects' ability to transmit.

CONCLUSIONS

Several major biological associations were found which support the feasibility of symbiotic control to reduce transmission of *Xf* by GWSS:

1. Natural populations of GWSS are commonly found thriving on several citrus varieties.
2. *Axd* colonized and grew best in the citrus varieties tested.
3. *Axd* colonized the xylem vessels of test plants, the same tissue from which GWSS feed.
4. *Axd* passively moved through populations of GWSS.
5. *Axd* did **not** negatively affect GWSS.

Interestingly, *Axd* appears to mirror the host range of GWSS. 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 in California, two other native sharpshooters also transmit the vehicle bacterium, *Axd*, and several plants can serve as hosts.

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.

We concluded that *Axd* will be an effective delivery agent of a symbiont control strategy for combating *Xf*. GWSS readily acquired *Axd* from a plant source and this bacterium translocated and colonized a variety of plants tested. We have yet to determine the effect of the reagents on *Xf* in infected grapevines.

Previously, plant symptoms confirmed by ELISA or PCR detection were used to determine if transmission had occurred. Unfortunately, these systems require the bacterium to colonize and infect the host plant to determine transmission. If an infected plant is asymptomatic, important but less obvious transmission events may be missed. Our system removes the plant "unknowns" from the equation. However, we recognize the importance of actual plant infection as a measure of transmission importance, but suggest using the artificial disease cycle as an initial rapid measure of vector competence.

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