MANAGEMENT OF PIERCE'S DISEASE OF GRAPE BY INTERFERING WITH CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA*

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ABSTRACT
Xylella fastidiosa (*Xf*) has homologs of the cell-cell signaling genes found in the important plant pathogen *Xanthomonas campestris* pathovar *campestris* (*Xcc*) and produces a similar alpha,beta unsaturated fatty acid signal molecule called DSF that coordinates gene expression. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease (PD). The *rpfF* gene is necessary and sufficient for DSF signal synthesis and *rpfF* mutants of *Xf* are hypervirulent and non-transmissible. Lack of transmissibility was linked to an inability of the *rpfF* mutant to form a biofilm in the insect foregut; while taken up by insects, the mutant strain is not retained. *Xf* strains that overproduce DSF produce disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF, by degrading DSF, or by in some way interfering with recognition of DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DSF-degrading strains greatly reduced the incidence and severity of disease in grape; DSF-producing strains consistently were the most effective in reducing disease. Disease was also reduced when some of these strains were simply sprayed onto grape before inoculation with *Xf,* indicating that they can alter behavior of the pathogen even when not co-inoculated. To verify that disease control is due to DSF interference, we have constructed mutants of these strains that disrupt the ability of these strains to produce or degrade DSF and show that the mutants are deficient in PD control. Both mutants unable to produce DSF as well as mutants deficient in degradation of DSF exhibited less ability to control PD when co-inoculated with *Xf*, suggesting that altering DSF abundance within the plant was a major factor contributing to disease control by these DSF-interfering strains. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in planta. Transgenic plants are being assayed for DSF production and susceptibility to *Xf* infection. The bacterial genes required for DSF degradation have been cloned and identified in antagonist Pseudomonas strain G, enabling their exploitation for disease control by over-expression in various bacterial endophytes of grape as well as by expression within plants themselves. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension. PD was reduced in plants after topical application of a DSF-producing strain of *Erwinia herbicola* (*E. herbicola)*.

INTRODUCTION
Endophytic bacteria such as *Xf* colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector. *Xf* shares sequence similarity with the plant pathogen *Xcc* (1). In *Xcc,* expression of pathogenicity genes is controlled by the *rpf* system of cell-cell communication, enabling a population of cells to launch a pathogenic attack in a coordinated manner (1). Two of the *rpf* proteins, *rpfB* and *rpfF*, work to produce a diffusible signal factor (DSF) (2) which has recently been described as an alpha,beta unsaturated fatty acid (3) (Figure 1).

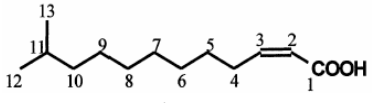

Figure 1

As the population grows, the local concentration of DSF increases. Other *rpf* proteins are thought to sense the increase in DSF concentration and transduce a signal, resulting in expression of pathogenicity factors. The *Xf* genome not only contains homologs of the *rpf* genes most essential for cell-cell signaling in *Xcc*, but also exhibits striking colinearity in the arrangement of these genes on the chromosome. We now have shown that *Xf* makes a molecule that is recognized by *Xcc* but

probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of *rpf* regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel. Similarly, we would expect the density-dependent genes to be expressed during the time when a population of *Xf* is ready to move into un-colonized areas.

Other organisms can apparently interfere with the density-dependent behaviors of *Xf*. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria. Our preliminary work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, initiated genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

OBJECTIVES

1. Identify bacteria that interfere with DSF-mediated cell-cell signaling in *Xf*, and conduct pathogenicity tests on grapevines colonized by DSF-interfering bacteria to determine potential for PD control
2. Isolation of mutant strains of DSF-degrading and DSF-producing bacteria that can no longer interfere in cell-cell signaling to verify that disease control is linked to cell-cell signal interference
3. Molecular identification of genes conferring DSF-degrading activity
4. Engineer the grapevine endophytes *Alcaligenes xylosoxidans denitrificans* and *Agrobacterium vitis* to express genes conferring DSF-degradation and DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control
5. Creation of grapevines expressing genes conferring DSF-degradation and DSF-synthesis activity to test for PD resistance
6. Evaluate topical application of DSF-degrading and DSF-producing bacteria with penetrating surfactants for PD control

RESULTS

We have isolated a variety of bacteria from grapevine vineyards affected by PD as well as tomato and cruciferous crop plants infected with the signal-producing pathogens *Xanthomonas campestris* pv. *vessicatoria* and *Xcc*, respectively and tested them for their ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strain described above. We found several strains that negatively affected signaling in *Xcc* while several strains were found to produce DSF. By adding purified DSF to either cell-free extracts of the strains with a negative influence on signaling or to whole cells we found that at least two mechanisms of interference with signaling could be observed. Some strains such as strains C,E,G, H, and J are able to degrade DSF while other inhibitor strains did not do so, and apparently have another means of interfering with DSF perception by *Xcc*. The several strains that produced DSF were all identified as *Xanthomonas* species. We sequenced the 16S rRNA gene from these strains to determine their species identity.

To test the ability of bacteria that alter *Xf* signaling to alter the process of disease in plants, we co-inoculated grapevines with *Xf* and strains that either inhibit or activate cell-cell signaling in greenhouse studies. The incidence of PD was greatly reduced by all of the signaling interfering strains that we tested (Figures 2 and 3). As we had expected, DSF-producing strains generally reduced disease severity more than did strains that interfered with signaling in *Xf*. These results were highly repeatable, having been observed in five separate experiments. We find these results to be very exciting in that they suggest that alteration of signal molecules within plants can have a profound effect on the disease process.

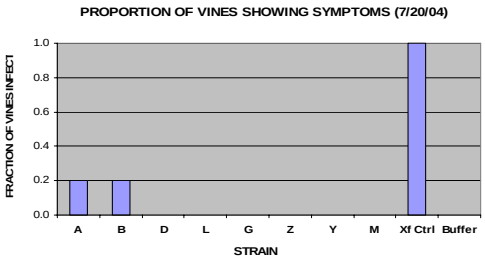

Figure 2. Incidence of PD in grape co-inoculated with *Xf* Temecula and various DSF-producing and degrading bacterial strains.

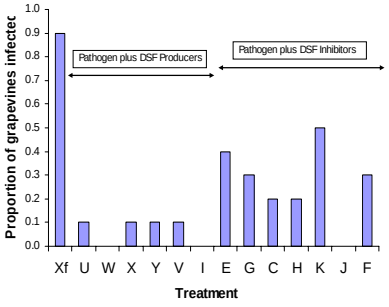

Figure 3. Incidence of PD in grape co-inoculated with *Xf* Temecula and various DSF-producing and degrading bacterial strains.

We also have been able to provide disease control by topical inoculation of DSF-producing bacteria such as DSF-producing strains X and 8004 to the foliage of plants where they colonize and presumably produce DSF as well as by pre-treatment of plants by injection of these antagonists before inoculation with *Xf* (Figure 4).

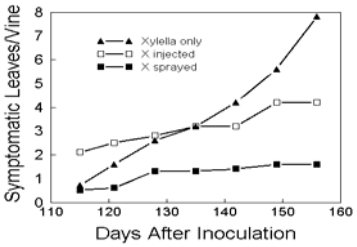

Figure 4. Severity of PD in grape co-inoculated with DSF-producing strain X or sprayed with this strain before inoculation with Temecula compared to plants inoculated only with Temecula.

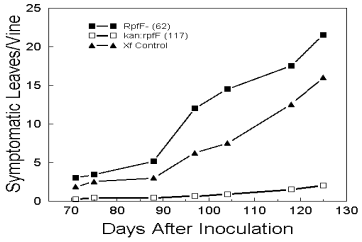

Figure 5. Severity of PD in grape co-inoculated with a DSF over-producing strain of *Xf* or with an *rpfF* mutant compared to plants inoculated with Temecula.

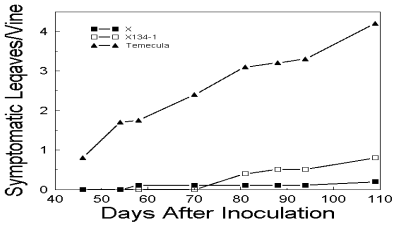

Figure 6. Severity of PD in grape co-inoculated with DSF-producing strain X or a mutant of X that does not produce DSF compared to plants inoculated only with Temecula.

To determine the extent to which altered DSF abundance in plants would alter the progress of PD we also made mutants of *Xf* that were either blocked in DSF production or over-expressed DSF. A strain of *Xf* Temecula in which the *rpfF* gene, which is required for production of the signal in *Xcc*, is knocked out was constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. The *rpfF* mutant of *Xf* does not make DSF as determined using previously constructed "signal-sensing" strains of *Xcc* to determine DSF production by *Xf* and other bacterial strains. We also over-expressed DSF by introducing the *rpfF* gene driven by a constitutive *kan* promoter into the genome of *Xf*. This strain produced much higher levels of DSF than the parental strain. The strains altered in DSF production were tested for their ability to infect and move within host plants and to cause PD symptoms. The *rpfF* gene appears to play a role in modulating disease progress because the timing and severity of symptom development are greatly exacerbated in grapevines infected with *rpf*-deficient mutants when compared to the wild type (Figure 5). In contrast, the *Xf* strain that overproduced DSF caused disease symptoms in grape, but only at the site of inoculation. The mutant cells did not move within the plant as did wild-type strains (Figure 5). These results all support our model that DSF regulates genes required for movement of *Xf* from colonized vessels. We hypothesize that *rpfF*-deficient mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression.

To establish a rigorous connection between DSF production and disease control, we have constructed mutant strains of those DSF-producing bacteria that perform best in the disease control assays that no longer could produce DSF. These mutants were then compared to their parent strains in disease control assays. We also made mutants of DSF-degrading strains that no longer could degrade DSF. We expected that if DSF interference can provide disease control, then strains no longer able to interfere with DSF signaling will also no longer be able to control disease. All mutants unable to produce DSF were diminished in ability to reduce PD when co-inoculated with *Xf* compared to their DSF-producing wild-type strain (Figures 7-8).

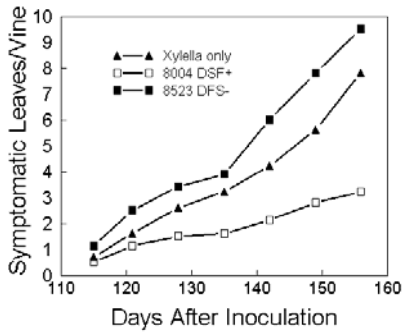

Figure 7. Severity of PD on grape co-inoculated with an *Xcc* DSF-producing strain or a mutant *Xcc* strain unable to produce DSF and Temecula compared plants inoculated only with Temecula.

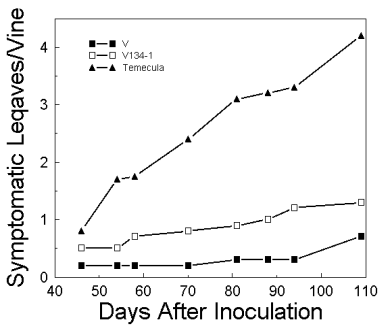

Figure 8. Severity of PD on grape co-inoculated with DSF-producing strain V or a mutant unable to produce DSF and Temecula compared to plants inoculated only with Temecula.

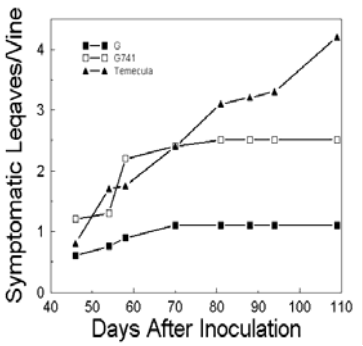

Figure 9. Severity of PD on grape co-inoculated with DSF-degrading strain G or a mutant unable to degrade DSF and Temecula compared to plants inoculated only with Temecula.

Likewise, mutant strain G741, a mutant of DSF-degrading parental strain G that no longer could degrade DSF also was greatly reduced in ability to control PD when co-inoculated with *Xf* compared to its parental strain (Figure 9). These results suggest strongly that it is the production of, or degradation of DSF in plants by these antagonistic bacteria that makes a large contribution to their ability to reduce PD. The results thus strongly suggest that any method that either increases or decreases DSF abundance in *Xf*-infected plants will have a large effect on the incidence and/or severity of PD.

We have recently made a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and will compare it to that of the wild type. Preliminary results show that this hypervirulent mutant moves more rapidly through grape and also more rapidly fills xylem vessels, suggesting that virulence factors are de-repressed in an *rpfF*- mutant (Figures 10 and 11).

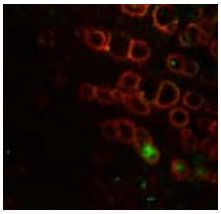

Figure 10. Presence of gfp-marked cells of wild-type *Xf* strain Temecula visualized as green fluorescence in cross sections of grape petiole viewed with confocal microscopy.

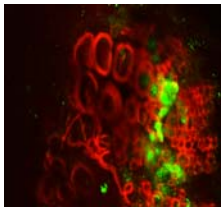

Figure 11. Presence of cells of an *rpfF* mutant of *Xf* Temecula visualized as green fluorescence in cross sections of grape viewed with confocal microscopy.

To increase the usefulness of any interfering agents identified in this screen, we are molecularly identifying the genes conferring the DSF-interference phenotypes. We have inactivated the genes for interference in Pseudomonas strain G individually by random Tn5 mutagenesis and cloned the disrupted loci. Mutations of the *carAB* genes, encoding carbamoyl-phosphate synthetase activity, in antagonist G abolishes DSF degradation. Multiple mutants of these two genes (and only these two genes) have been found to disrupt DSF production; we are currently investigating how this enzyme confers DSF degradation by over expressing it. The *carAB* genes have been cloned, shown to restore DSF interference in strain G mutants, and are being assessed for their ability to confer DSF interference in other bacterial strains when over expressed.

Disease control by DSF-interfering strains will be optimized if they are good colonists of grapevine. To maximize disease control we are expressing the various genes conferring DSF interference in effective non-pathogenic endophytic colonists of grapevine such as *Alcaligenes xylosoxidans denitrificans* (*Axd*) and *Agrobacterium vitis* (*Av*). We expect that this strategy will deliver the disease control agent directly to the site of the pathogen and result in highly effective control. Since the *rpfF* gene of *Xf* is sufficient to confer expression of DSF in other bacteria we are introducing it into these two species. Preliminary studies showed that while *Av* strains established large populations in grape near the inoculation site, they did not move extensively in the plant (Figure 12).

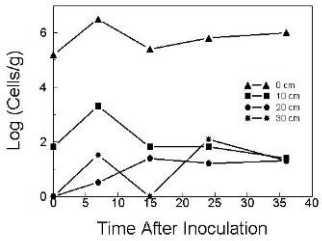

Figure 12. Population size of *A. vitis* strain 210R sampled at different distances from point of inoculation at different times after inoculation into stems of grape.

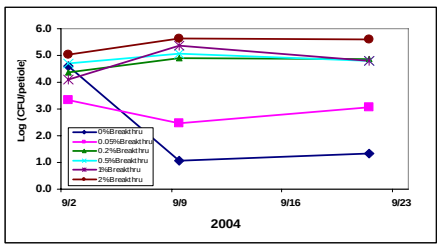

Figure 13. Population size of *E. herbicola* strain 299R in petioles at different times after spray inoculation with different concentrations of Breakthru.

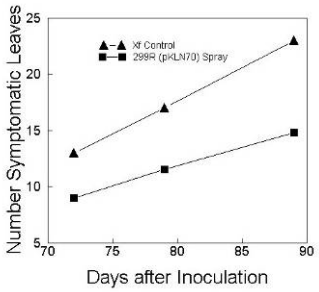

Figure 14. Severity of PD in grape sprayed with DSF-producing *E. herbicola* 299R harboring the *rpfF* of *Xf* Temecula compared to plants inoculated only with Temecula.

We have initiated expression of the *rpfF* gene in grape at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California, Davis. Initially, we submitted a tested but un-optimized *rpfF* construct to the facility; the first transformed plants are now mature and are being tested for DSF production. Initial assays reveal that DSF is rapidly degraded by damaged plant tissue. Therefore assays are being developed to avoid this complication in assessing DSF abundance. Mature plants have now been rooted to produce large numbers of clonal plants that will be inoculated with *Xf* as they grow large enough (late November).

We have found that it is possible to establish large populations of bacteria within grape leaves, stems and petioles by simple topical applications of bacterial suspensions to plants in solutions of organosilicon surfactants having very low surface tensions. A variety of bacteria were found to colonize grape at very high population sizes (> 10^6 cells/petiole) for extended

periods of time following topical application (Figure 13). While these bacteria apparently do not spread throughout the plant after inoculation as does *Xf*, by introducing it into the intercellular spaces and perhaps even the xylem of the plant by use of the surfactants that stimulate spontaneous infiltration of the plant, we can inoculate the bacteria into all sites within the plant. Initial studies have shown that topical applications of an *Erwinia herbicola* strain harboring the *Xf rfpF* gene can provide some control of PD (Figure 14).

CONCLUSIONS

Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of *Xf* and that disruption of cell signaling is a promising means of controlling PD. Strikingly, *Xf* strains that cannot signal are also not transmissible by nor colonize an efficient insect vector. This result reveals an important and previously unappreciated connection between cell-cell signaling and transmission as well as the requirement for biofilm formation for transmission. These new findings will be helpful for those interested in targeting transmission as a means of disease control. We also found that mutants unable to signal are hypervirulent. Conversely, strains of *Xf* that overproduce DSF have low virulence and do not move within grape. This suggests that, it will be more efficient to elucidate and target *Xf*'s colonization strategies rather than traits predicted to contribute to virulence based on studies of other plant pathogens. We have identified bacterial strains that can interfere with *Xf* signaling. These strains proved very effective as protective agents for grapevines when co-inoculated with *Xf*. Both positive and negative interference with DSF signaling reduced disease in grape suggesting that signaling is normally finely balanced in the disease process; such a finely balanced process might be readily disrupted. Since in bacteria *rpfF* is sufficient to encode a synthase capable of DSF production, expression of DSF directly in plants is an attractive approach for disease control. Preliminary results are very encouraging that DSF can be made in plants. Alternatively, the use of various bacteria to express DSF in plants may prove equally effective in altering *Xf* behavior and hence disease control. Our observation that large numbers of bacteria could be introduced into grape plants by simple topical applications of cell suspensions in a penetrating surfactant has enabled us to pursue a new strategy of disease control that will enable us to efficiently test those strains that are found to be effective in PD control in Objective 1 by a method that should prove practical for commercial use. Thus our investigation of the fundamental issues associated with interactions of *Xf* with grape has led to several very practical possible control measures of PD that can be evaluated over the short term.

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