CONTROL OF PIERCE'S DISEASE BY METHODS INVOLVING PATHOGEN CONFUSION

Principal Investigator:
Steven E. Lindow
Dept. of Plant & Microbial Biology
University of California
Berkeley, CA 94720

Cooperator:
Subhadeep Chatterjee
Dept. of Plant & Microbial Biology
University of California
Berkeley, CA 94720

Reporting Period: Most of the results reported here are from work conducted October 2007 to October 2008.

ABSTRACT

Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that changes its gene expression in cells as they reach high numbers in plants. 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). We have investigated both the role of DFS-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, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. *Xf* mutant strains that overproduce DSF cause 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. When co-inoculated into grape with *Xf* DSF-producing strains such as *Rhizobium etli,* harboring *rpfF* from *Xf* greatly reduced the incidence and severity of disease in grape; lesser effects were observed when these strains were inoculated into plants separately, suggesting that the biological control strains did not move efficiently within the plant and hence were not coincident with *Xf*. Topical application of DSF extracted from over-producing strains of *Erwinia herbicola* harboring *rpfF* cloned from *Xf* reduced the severity of Pierce's disease when applied shortly before inoculation with *Xf*. We have transformed tobacco, tomato and grape with the *rpfF* gene of *Xf* to enable DSF production in plants. While expression of *RpfF* in the cytoplasm has yielded modest levels of DSF that were sufficient to greatly reduce the movement of *Xf* in grape, and thus reduce Pierce's disease, targeting of *RpfF* to the chloroplast of plants has led to much higher levels of DSF production that should provide even high levels of disease control. Grafting studies are underway to determine if DSF produced by rootstocks can move to scions and confer disease control.

INTRODUCTION

We have found that the virulence of *Xylella fastidiosa* (*Xf)* is strongly regulated in a cell density-dependent fashion by accumulation of a signal molecule called DSF encoded by *rpfF* and involving signal transduction that requires other *rpf* genes. We now have shown that *Xf* makes a DSF molecule that is recognized by *Xanthomonas campestris* pv. *campestris (Xcc*) but slightly different than the DSF of *Xcc* (**Figure 1**).

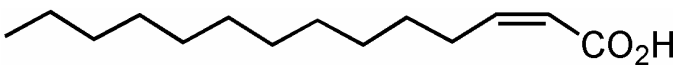

Figure 1

In striking contrast to that of *Xcc*, *rpfF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DSF-deficient mutants of *Xf* are consistent with the role of this density-dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged *Xf* are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf*. Importantly, *rpfF*- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow to excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of *Xf*, we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of *Rpf* regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the extent to which other endophytes can modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, obtained genetic transformation of grape and other hosts of *Xf* to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

OBJECTIVES

1. Evaluate plants with enhanced production of DSF for disease control.
2. Determine if DSF is transferable within plants – eg. whether DSF production in rootstocks can confer resistance to Pierce's disease in the scion.
3. Evaluate enhanced DSF-producing endophytic bacteria for control of Pierce's disease.
4. Investigate DSF-overproducing strains of *Xf* as biocontrol agents for Pierce's disease and whether *Xf* strains previously identified with biocontrol potential exhibit an elevated production of DSF.
5. Determine if resistance to Pierce's disease is associated with low rates of degradation of DSF by plants.
6. Determine those plant factors that confer induction of virulence genes in *Xf* and whether susceptibility to Pierce's disease is due to differences in induction of virulence factors in the pathogen by the plant.

RESULTS

Objective 1. Production of DSF in transgenic plants for disease control.

We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. In addition to grape, we have transformed genes conferring DSF production into tobacco since this species is colonized by *Xf* and disease symptoms can be produced (**Figure 2**). Because transformation of tobacco is much quicker than grape, we have used studies of *Xf* infection of tobacco as a surrogate for studies in grape to speed our assessment of different ways to produce DSF in grape. The various mutants of *Xf* that are hyper and hypo virulence on grape yield similar reactions on SR1 tobacco (**Figure 3**).

Figure 2. Symptoms caused by *Xf* on SR1 tobacco.

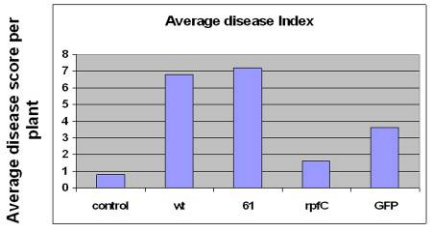

Figure 3. Severity of disease on SR1 tobacco inoculated with WT *Xf* and an *rpfF* mutant (61), an *rpfC* mutant, and a gfp-marked strain.

Further tests of SR1tobacco as a surrogate host to evaluate transgenic expression of *rpfF* as a means to increase DSF abundance in plants were performed. SR1 tobacco which had been transformed with the untargeted *rpfF* genes from either *Xf* or *Xcc* were inoculated with *Xf* ; the incidence of disease was dramatically reduced in *rpfF*-expressing SR1 compared to untransformed tobacco (**Table 1**). Some of the more mature leaves on the base of the plant had exhibited leaf scorching even on uninoculated plants (**Table 1**), suggesting that the extent to disease control conferred by expression of *rpfF* was much greater than 50%.

Table 1. Proportion of leaves of wild-type and DSF-producing SR1 tobacco with marginal leaf scorch after inoculation with *Xf*

Treatment	Fraction of leaves	
Wild-type SR1		0.52 a
Xf rpfF-expressing SR1	0.38 b	
Xcc rpfF-expressing SR1		0.27 b
No *Xf* control	0.22 c	

Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis with a non-targetted *rpfF* construct. These plants produced only very low levels of DSF but are MUCH less susceptible to Pierce's disease (**Figure 4**). While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. We thus expect to find that *Xf* is limited in its movement in plants having even higher levels of DSF due to the expression of *rpfF*, in a manner similar to what we have observed in DFS-overproducing strains of *Xf*.

We have recently transformed tobacco and *Arabidopsis* with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of *RpfF* in the current transformed plants. Assay of DSF in transgenic SRI tobacco plants-where the *RpfF* is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpfF* are much higher as compared to the plants in which the *RpfF* is expressed in the cytosol. Transcription analysis of the chloroplast targated *rpfF* transformed plants indicates high level expression of the *rpfF* gene (**Figure 5**). We have generated seeds from the transgenic

SRI tobacco plants and we are conducting pathogenicity assays with *Xf* comparing these enhanced producing plants with normal and untargeted *RpfF* plants.

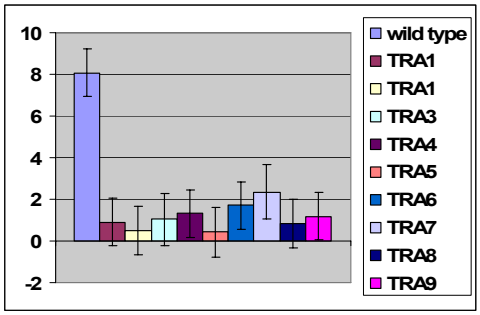

Figure 4. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

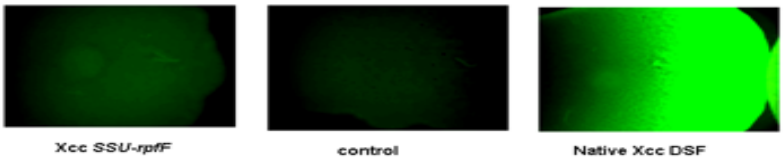

Figure 5. DSF extracted from transgenic tobacco SR1 plants harboring a chloroplast targeted *RpfF* (left) or from WT tobacco (center) or purified DSF from *Xcc* (right). DSF is spotted on a paper disc on the right side of each image and the *Xcc* DSF bioindicator is to the left. gfp fluorescence is evidence of DSF.

Further tests of the efficacy of chloroplast targeting of *rpfF* implants were preformed by evaluating DSF production in transgenic Moneymaker tomato. Substantial levels of DSF could be detected in the chloroplast-targeted tomato and sufficient amounts of DSF were present to alter the behavior of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) that was inoculated onto leaves. While an average of 323 lesions formed when *Xcv* was inoculated onto normal tomato, 570 lesions formed per leaf on the DSF-producing plants, a finding expected if DSF was present since virulence of *Xcv* is enhanced by DSF. We have also initiated transformation of grapes with a chloroplast targeted *rpfF* construct. We expect to receive the transformed plants by December, 2008, and then will grow them to larger sizes, make green cuttings to produce enough plants for pathogenicity testing by mid-2009. Although *RpfB* is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by *RpfF*. We expected that co-expression of *RpfB* and *RpfF* in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants can complement the virulence of the non pathogenic *rpfF* mutant of *Xcc* (**Table 2**). Importantly, transgenic plants expressing both *rpfB* and *rpfF* were more susceptible to the *rpfF* mutant of *Xcc*, indicating enhanced DSF levels. Given this evidence of enhanced DSF production in transgenic *Arabidopsis*, and recent results with similarly-transformed tomato, we are initiating transformation of grape with similar constructs.

Table 2. Disease severity from topical application of bacteria varying in DSF production to *Arabidopsis*. Bacteria were inoculated on different *Arabidopsis* genotypes transformed with *rpfF* or with both-*rpfB* and *rpfF*

Arabidopsis genotype	*Xcc* strains	
	Wild type	*rpfF*-
Col (WT)	++++	-
rpfF transformed	++++	+
rpfF & *rpfB* transformed	++++	++

Direct application of DSF to non-transgenic grape can also confer disease control. While we have very recently determined the chemical structure and have synthesized DSF of *Xf,* for these studies we used crude ethyl acetate extracts of a DSF-producing *E. herbicola* strain as a source of DSF. The DSF was either topically applied as well as needle inoculated into the stems of grape either once a day before inoculation with *Xf* or weekly. While a single needle application of DSF reduces disease index, a weekly application of DSF into the stem of the plant was much more effective (**Figure 6**). These results are exciting in that they suggest that disease control from external applications of DSF might be a practical means of disease control. We have recently been successful in determining the structure of *Xf* DSF and have synthesized gram quantities of DSF. Plants have recently been treated with topical and injected synthetic DSF and then inoculated with *Xf*; disease assessment will commence in mid-November.

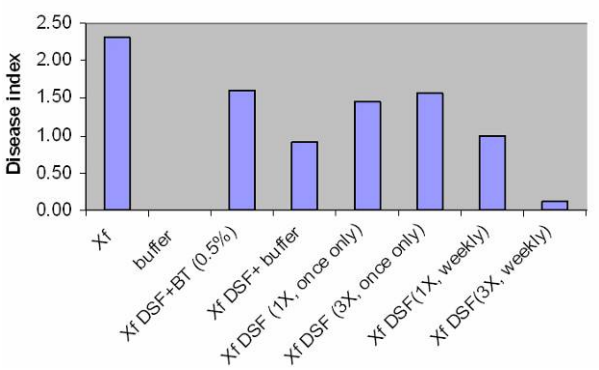

Figure 6. Disease incidence-severity relation (disease index) for grape inoculated with *Xf* and to which DSF was topically applied or introduced into the stem.

Objective 2. Graft transmissibility of DSF.
To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the *rpfF* of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (**Figure 7**). Over 100 of such grafted plants have now been made, and they have been inoculated with *Xf* to test whether normal SR1 scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization; disease will be rated by mid-November. Non-chloroplast targeted *RpfF*-expressing transgenic Freedom grape plants have been propagated in sufficiently large numbers to produce enough plants to serve as rootstocks to test with *Xf* inoculations in larger scale studies. Over 100 such plants have now been propagated and green-grafting of Cabernet Sauvignon has been successfully employed to produce grafted plants with a normal Freedom and a DSF-producing Freedom rootstock (**Figure 7**). Initial attempts at green grafting of grape produced a low frequency of successful grafts, but a new procedure has provided a satisfactory level of graft success; the grafted plants have been inoculated with *Xf* to test for graft transmissibility of DFS as evidenced by reduced movement of *Xf* and disease severity.

Figure 7. Grafted SR1 tobacco plants (left) and Cabernet Sauvignon grape grafted onto DSF producing Freedom rootstocks (right) onto which *Xf* has been inoculated. The plants are as yet asymptomatic.

Objectives 3 and 4. Disease control with endophytic bacteria.
We have been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (**Table 2**). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* G12 with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained production of DSF in this strain. This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process. The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured four weeks after inoculation by puncture inoculation into one site in the stem, measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation (**Figure 8**). While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape; we are excited about this result since no other of several bacterial strains that we have investigated for the ability to move within grape has ever exhibited any ability to move beyond the point of inoculation. Further studies are underway to determine the population sizes to which *R. etli* will grow given more time after inoculation. *R. etli* also has the ability to move within grape leaves and multiply to high population sizes. When applied as a point source to leaves using a penetrating surfactant, cells of *R. etli* could be found up to three cm away within one week, and population sizes of this strain increased 100-fold within three weeks after inoculation (**Figure 9**). Studies are continuing to determine the maximum population size that this strain can achieve in grape leaves.

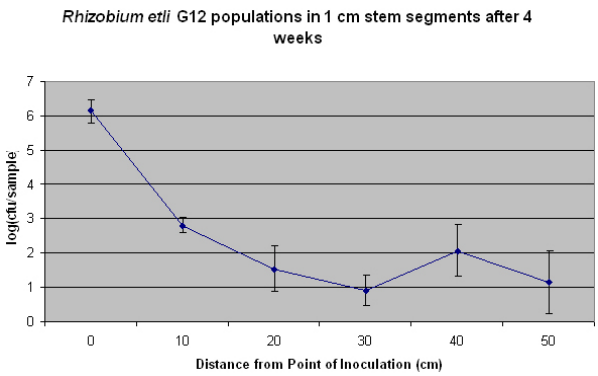

Figure 8. Population size of *R. etli* in stems four weeks after inoculation at one point.

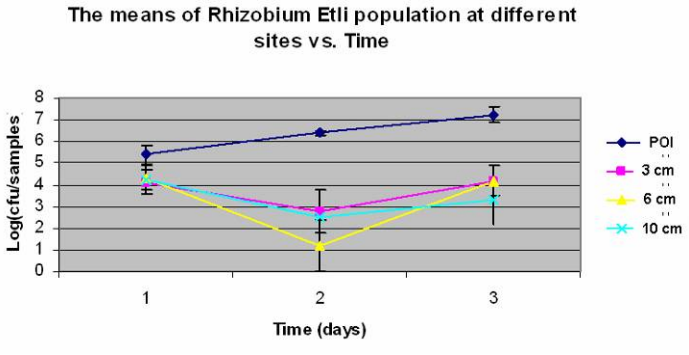

Figure 9. Population size of *R. etli* in leaves one, two, and three weeks after inoculation at a single point.

Various DSF-producing bacteria were tested for their ability to control Pierce's disease when applied to grape in different ways. DSF-producing *R. etli* were both needle inoculated one or more times at sites near where *Xf* was subsequently inoculated, as well as co-inoculated with *Xf* into grape stems and sprayed onto leaves with 0.5% of the penetrating surfactant Breakthru 1 week before *Xf* was inoculated into stems. The co-inoculation of *R. etli* with *Xf* greatly decreased the incidence of colonization of grape petioles compared to control plants inoculated with *Xf* alone (**Figure 10**) while topical application or injection elsewhere in the stem provided little control. We presume that the relatively slow movement of *R. etli* in the stems of plants (**Figure 8**) explains why co-inoculation was most effective. *R. etli* was somewhat susceptible to damage from Breakthru and its population sizes were reduced during application with this detergent. We will continue to test different ways in which *R. etli* can be introduced into plants to determine its ability to control PD. We expect that introduction of *R. etli* into stems far in advance of *Xf* will provide much better disease control. *RpfC*- mutants of *Xf* greatly over-produce DSF so we tested them for their ability to control PD when applied in various ways as discussed above for *R. etli*. The incidence of colonization of grape petioles with *Xf* was greatly reduced when plants were needle inoculated into grape either two weeks before plants were inoculated with *Xf* or when coinoculated with the pathogen (**Figure 11**). While the *RpfC* mutant does not move as well within grape as the wild-type *Xf*, its presence locally in plants can suppress the movement of wild-type *Xf* and thus lead to control of PD. These studies are promising and are being repeated.

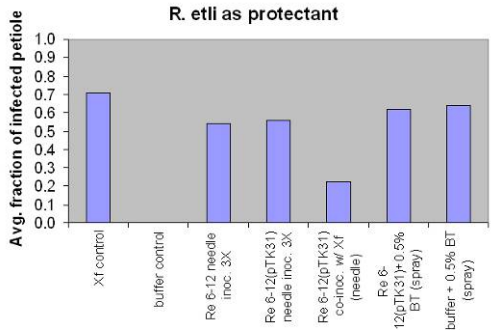

Figure 10. Incidence of colonization of petiols of grape by *Xf* when plants were treated with DSF-producing *R. etli* in various ways.

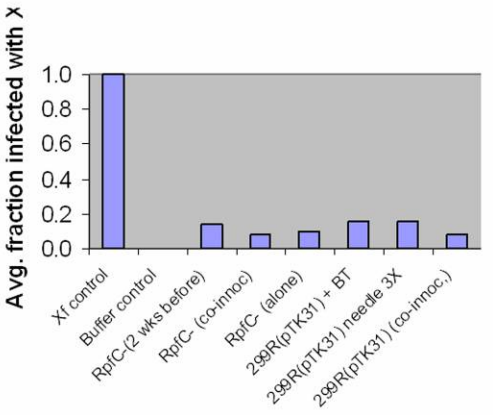

Figure 11. Incidence of colonization of petioles of grape by *Xf* when plants were treated with *RpfC* mutants of *Xf* in various ways.

Objective 5. Degradation of DSF by plants. Development of an Xcc biosensor efficient in detecting Xylella DSF.
For many of the objectives of this project, in addition to the study of DSF degradation in plants an improved bioindicator for DSF would be very valuable. We are presently using an *Xcc*-based biosensor in which the endoglucanase gene is linked to a green fluorescent protein (GFP) reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. We have devised a strategy to develop a surrogate *Xcc* biosensor system which will express all the

components of DSF signal transduction of *Xf*. This should give rise to a system which is close to DSF signal transduction system in *Xcc*. We have made two different *Xanthomonas* strains in which the endogenous signal synthesis as well as signal recognition system (consisting of the hybrid two component *RpfC* and *RpfG* response regulators) has been knocked out. In one of these strains the DSF signal synthase *RpfF* and the DSF signal sensor *RpfC* has been knocked out (**Figure 12**). We have also made an *Xcc* strain in which the DSF synthase gene *RpfF* has been knocked out in a background of a RpfCHG deletion.

These mutants will enable us to express the *Xf RpfC-RpfG* two component system and should serve as a more sensitive surrogate host biosensor. Completion of the biosensor is expected within three more months. It then will be applied to the study of *Xf* DSF stability in plant extracts as originally proposed.

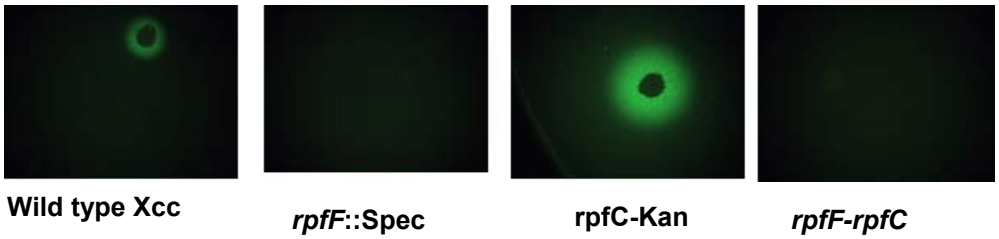

Figure 12. Different *Xcc* mutants constructed to serve as surrogate host for expressing the *Xf RpfC-RpfG* two component DSF signal transduction system. The presently used *Xcc* biosensor 8523/pKLN55 is sprayed over the colonies. Presence of DSF is detected by the GFP fluorescence of the biosensor.

We also are investigating the use of *Xf* itself to detect DSF**.** Among the several genes that we know to be regulated by DSF, those genes most strongly regulated include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. We thus have examined the phenotypes of an *rpfF*- mutant of *Xf* exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. Initial results are encouraging. Suppression of twitching motility of the *rpfF*- mutant was observed when DSF was added at concentrations greater than about 10 uM (**Figure 13**). Likewise, cells of the *rpfF*- mutant which were not adherent, and thus which did not form cell-cell aggregations became much more adherent to each other when DSF was added at concentrations greater than about 10 uM (**Figure 14**). Thus it appears that we can assess the concentration of DSF in samples using either a cell twitching assay or a cell adhesion assay using *Xf* cells, although both assays are time consuming and somewhat qualitative.

Initial results have shown relatively little induction of EPS production in an *rpfF*- mutant of *Xf* by the addition of DSF; little EPS was observed whether DSF was added to culture medium or not. We are investigating, in cooperation with Rodrigo Almeida, other medium contents which might be needed for EPS production and have very preliminary evidence that EPS production can be stimulated by DSF under the correct culture conditions. EPS abundance will then be measured both chemically and immunologically as an estimator of DSF abundance.

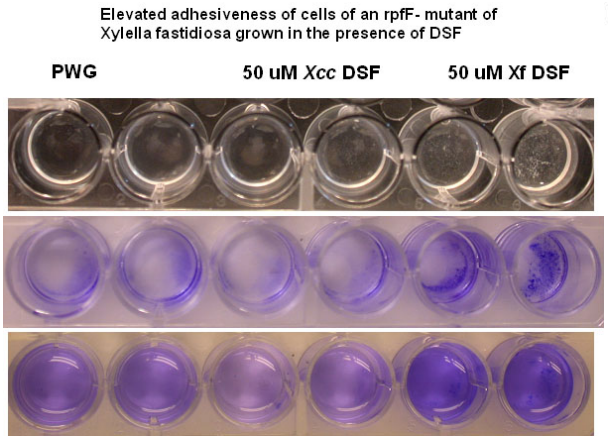

Figure 13

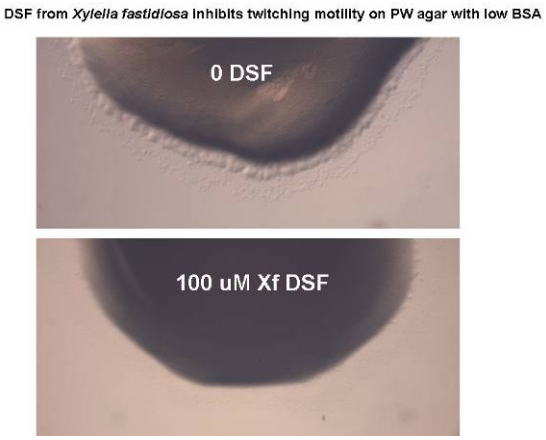

Figure 14

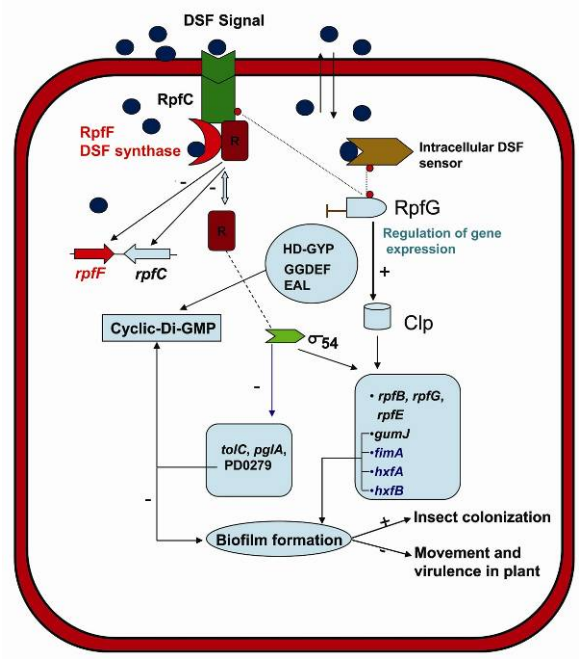

Figure 15. A proposed model for DFS-mediated cell-cell signaling regulation in *Xylella fastidiosa*.

Table 3. Relative quantification of gene expression regulated by *rpfF* and *rpfC* by real-time RT-PCR.

	Fold change ± SE*		
Gene name	*rpfF*⁻	*rpfC*⁻	*rpfF*⁻–*rpfC*⁻
fimA	0.4 ± 0.04	2.15 ± 0.18	0.73 ± 0.19
hxfA (*xadA*)	0.56 + 0.07	3.2 ± 0.1	0.7 ± 0.17
hxfB	0.15 ± 0.05	5.2 ± 0.52	0.49 ± 0.3
gumJ	0.56 ± 0.02	2.6 ± 0.2	0.4 ± 0.04
rpfF	n.d.	6.6 ± 0.71	n.d.
rpfC	4.9 ± 0.4	n.d.	n.d.
rpfE	0.73 ± 0.06	2.2 ± 0.17	0.7 ± 0.12
rpfB	0.6 ± 0.09	2.13 ± 0.07	0.50 ± 0.3
rpfG	0.7 ± 0.06	1.8 ± 0.04	1.13 ± 0.45
(PD0279)	5.3 ± 0.3	3.5 ± 0.23	0.62 ± 0.06
tolC	5.5 ± 0.7	3.8 ± 0.6	0.6 ± 0.09
pglA	1.9 ± 0.17	1.8 ± 0.04	0.7 ± 0.07

*Amount of RNA relative to that in the wild-type *X. fastidiosa* cells is equal to 1.0 and is normalized for cellular abundance by using 16S ribosomal RNA as an endogenous control. n.d. indicates not determined. Standard errors were calculated based on at least two independent experiments.

Objective 5. Plant regulation of Xf virulence factors.
Before investigating the effects of plant extracts on gene expression in *Xf,* we have further examined the complex pattern of gene regulation in *Xf* that is DSF dependent to better understand which virulence genes might be most informative to examine. Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins potentially involved in intracellular signaling are present. Gene expression of several genes was thus examined in both an *rpfF* and *rpfC* mutant background as well as a double mutant (**Table 3**). The results have enabled the production of a more complete model of DSF-dependent gene expression in *Xf* (**Figure 15**). The several genes identified in **Table 3** will be examined by RT-PCR in cultures of *Xf* to which plant extracts have been applied as proposed.

CONCLUSIONS
Several methods of altering DSF levels in plants, including direct introduction of DSF-producing bacteria into plants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. 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 plants; such grape plants produce at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Studies are underway using grafting experiments to determine if DSF produced by rootstocks can move to scions and confer disease control. Transgenic DSF-producing plants appear particularly promising and studies should soon indicate whether they could serve as a rootstock instead of a scion. Several genes encoding traits such as exoenzyme production, type IV pili involved in twitching motility, and a variety of fimbrial and non-fimbrial adhesins are most strongly regulated by the accumulation of DSF in bacterial cultures as well as in planta. The expression of these genes will be assessed when *Xf* is within different plant species to determine the host plant specificity of expression of such virulence genes. While the principle of disease control by altering DSF levels has been demonstrated, more work is needed to determine how to achieve this by the most practical means.

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.