Renewal Progress Report for CDFA Agreement number 09-0748

Field Evaluation of DSF-producing grape for control of Pierce's disease

Principal Investigator:
Steven Lindow
University of California
Department of Plant and Microbial Biology
111 Koshland Hall
Berkeley, CA 94720-3102
icelab@berkeley.edu

Cooperators:
Renee Koutsoukis, Clelia Baccari
University of California
Department of Plant and Microbial Biology
111 Koshland Hall
Berkeley, CA 94720-3102

David Gilchrist
Dept. Plant Pathology
University of California
Davis, CA 95616

Reporting period: The results reported here are from work conducted March, 2010 to March, 2012

Abstract:
A cell density-dependent gene expression system in *X. fastidiosa* mediated by a small signal molecule called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) controls the behavior of *X. fastidiosa*. The accumulation of DSF attenuates the virulence of *Xf* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, XadA, and FimA (that make cells sticky and hence suppress its movement in the plant) while down-regulating the production of secreted enzymes such as polygalacturonase and endogluconase which are required for digestion of pit membranes and thus for movement through the plant. Artificially increasing DSF levels in plants in various ways increases the resistance of these plants to Pierce's disease. Disease control in the greenhouse can be conferred by production of DSF in transgenic plants expressing the gene for the DSF synthase from *X. fastidosa*; such plants exhibit high levels of disease resistance when used as scions and confer at least partial control of disease when used as rootstocks. This project is designed to test the robustness of disease control by pathogen confusion under field conditions where plants will be exposed to realistic conditions in the field and especially under conditions of natural inoculation with insect vectors. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties in two field sites. Plants were established in one field site in Solano County on August 2, 2010. Plants were planted at a Riverside County site on April 26, 2011. The plants established in the Solano County site have grown well and were in general in excess of 2 to 3 meters in length by July, 2011. All plants at the Solano County experimental site were needle-inoculated with a suspension of *X. fastidiosa* on July 22, 2011. From one to 4 vines per plant were inoculated, each at a given site with a 20 ul droplet of *X. fastidiosa* containing about 20,000 cells of *X. fastidiosa*. As of October, 2011 no visible signs of disease are apparent in any of the plants in Solano County. Plants at the Riverside County plot are subject to natural infection, and a low incidence of infection was observed by late October, 2011. While the disease incidence in the first year was low, transgenic Freedom plants which had exhibited the highest level of disease resistance in the greenhouse remain free of infection, unlike control plants.

Layperson Summary:
X. fastdiosa coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal molecule (DSF) which acts to suppress its virulence in plants. Artificially increasing DSF levels in grape by introducing the *rpfF* gene which encodes a DSF synthase reduces disease severity in greenhouse trials. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties. Trials have been successfully established in both Solano and Riverside counties. While no disease has yet been observed in the Solano County trial, disease resulting from natural infection has been assessed at the Riverside County trial. While the disease incidence in the first year was low, transgenic Freedom plants which had exhibited the highest level of disease resistance in the greenhouse remain free of infection, unlike control plants.

Introduction:
Our work has shown that *X. fastidiosa* uses DSF perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence cells do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape

to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion" in the greenhouse, more work is needed to understand how well this will translate into disease control under field conditions. That is, the methods of inoculation of plants in the greenhouse may be considered quite aggressive compared to the low levels of inoculum that might be delivered by insect vectors. Likewise, plants in the greenhouse have undetermined levels of stress that might contribute to Pierce's disease symptoms compared to that in the field. Thus we need to test the relative susceptibility of DSF-producing plants in the field both under conditions where they will be inoculated with the pathogen as well as received "natural" inoculation with infested sharpshooter vectors. We also have recently developed several new sensitive biosensors that enable us to measure *X. fastdiosa* DSF both in culture and within plants. We could gain considerable insight into the process of disease control by assessing the levels of DSF produced by transgenic *rpfF*-transformed grape under field conditions.

Objectives:

1) Determine the susceptibility of DSF-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties for Pierce's disease.
2) Determine population size of the pathogen in DSF-producing plants under field conditions.
3) Determine the levels of DSF in transgenic *rpfF*-expressing grape under field conditions as a means of determining their susceptibility to Pierce's disease.

Results and Discussion:

Disease susceptibility of transgenic DSF-producing grape in field trials.

Field tests are being performed with two different genetic constructs of the *rpfF* gene in grape and assessed in two different plant contexts. The *rpfF* has been introduced into Freedom (a rootstock variety) in a way that does not cause it to be directed to any subcellular location (non-targeted). The *rpfF* gene has also been modified to harbor a 5' sequence encoding the leader peptide introduced into grape (Thompson seedless) as a translational fusion protein with a small peptide sequence from RUBISCO that presumably causes this RpfF fusion gene product to be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). These two transgenic grape varieties are thus being tested as both own-rooted plants as well as rootstocks to which susceptible grape varieties will be grafted. The following treatments are thus being examined in field trials:

Treatment 1	Non-targeted RpfF Freedom
Treatment 2	Chloroplast-targeted RpfF Thompson
Treatment 3	Non-targeted RpfF Freedom as rootstock with normal Thompson scion
Treatment 4	Chloroplast-targeted RpfF Thompson as rootstock with normal Thompson scion
Treatment 5	Normal Freedom rootstock with normal Thompson scion
Treatment 6	Normal Thompson rootstock with normal Thompson scion
Treatment 7	Normal Freedom
Treatment 8	Normal Thompson

Figure 1. Overview of research plot in Solano County in soon after DSF-producing plants were established (top). Close-up of transgenic Freedom vines in mid-September 2010 (bottom).

Treatments 5-8 serve as appropriate control to allow direct assessment of the effect of DSF expression on disease in own rooted plants as well as to account for the effects of grafting per se on disease susceptibility of the scions grafted onto DSF-producing rootstocks.

One field trial was established in Solano County on August 2, 2010. Twelve plants of each treatment were established in randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about 3 cm long) from mature vines of plants grown in the greenhouse at UC Berkeley. Cuttings were placed in a sand/perlite/peatmoss mixture and subjected to frequent misting for about 4 weeks, after which point roots of about 10 appeared. Plants were then be transferred to 1 gallon pots and propagated to a height of about 1 m before transplanting into the field. Grafted plants were produced in a similar manner. 20 cm stem segments from a susceptible grape variety were grafted onto 20 cm segments of an appropriate rootstock variety and the graft union wrapped with grafting tape. The distal end of the rootstock variety (harboring the grafted scion) was then be placed in rooting soil mix and tooted as described above. After emergence of roots, the grafted plant were then transplanted and grown to a size of about 1 m as above before transplanting into the field site.

The plants all survived transplanting and are growing well (Figure 1). The plants were too small to inoculate in the 2010 growing season and hence were inoculated on July 26, 2011 (no natural inoculum of *X. fastidiosa* occurs in this plot area and so manual inoculation of the vines with the pathogen will be performed. The plants established in the Solano County site have grown well and were, in general, in excess of 2 to 3 meters in length by July, 2011 (Figure 2). All plants at the Solano County experimental site were needle-inoculated with a suspension of *X. fastidiosa*. From one to 4 vines per plant were inoculated, depending on the size and number of vines per plant. Each inoculation site received a 20 ul droplet of *X. fastidiosa* containing about 20,000 cells of *X. fastidiosa* (Figure 3). Because researchers from both UC-Berkeley and UC-Davis will be contributing treatment to each plot, and since the controls for some researchers will be the same, some control plants are being shared between research groups. All plants at UC-Davis were inoculated by needle puncture through drops of a common inoculum source of *X. fastidiosa* of about 10^6 cells/ml. As of early October, 2011 no symptoms were apparent, although the plants continued to grow very well (Figure 4). Assays of leaves chosen randomly near the points of inoculation revealed that about 10% of the leaves had detectable (but low (<1000 cells/g)) populations of *X. fastidiosa* when sampled about 6 weeks after inoculation. No *X. fastidiosa* was detected in samples of leaves of control plants collected in late October, suggesting that the low concentrations of cells used to inoculate plants had not resulted in infection events, even in control plants. The plants will thus be re-inoculated in early May, 2012.

Figure 2. Images of Thompson Seedless grape (left) and Freedom grape (right) at the Solano County field trial in July 2011.

Figure 3. Process of inoculation of grape at the Solano County field trial in July, 2011. A needle was inserted through a vine and a droplet of inoculum applied to the needle tip. After withdrawal of the needle, the bacterial inoculum is drawn into the vine due to the tension of the water in the xylem vessels.

Some of the plants needed to establish the trial at Riverside county were damaged in the greenhouse at UC Berkeley in 2010 due to pesticide applications, and since plants from other researchers at UC Davis were also not ready for transplanting to the field in 2010, a decision was made to establish all of the plants from the UCB and UCD research groups together in early 2011. The plants for the Riverside County were generated and were transferred to a lath house at UC Davis on March 23, 2011 to harden off for about 3 weeks. The plants were then transported to Riverside County for establishment in the field experiment together with plants from researchers at UC Davis and were planted on April 26, 2011 (Figure 5). The plants at the Riverside County trial will not be artificially inoculated, but instead will be subjected to natural infection from infested sharpshooter vectors having access to *X. fastidiosa* from surrounding infected grape vines.

Figure 4. Appearance of transgenic Freedom grape in the Solano Country trial in early October, 2011.

Figure 5. Establishment of grape trial in Riverside County.

Leaves exhibiting scorching symptoms characteristic of Pierce's disease at the Riverside County trial were assessed in late October, 2012. Even though the plants were not established at this trial until late April, they grown rapidly, and disease symptoms were apparent by late October (Figure 6). An individual leaf with symptoms of Pierce's disease was collected from each vine which had exhibited symptoms and returned to Berkeley to assay for the presence of *X. fastidiosa* in the petioles to verify that the symptoms were due to infection with *X. fastidiosa*. In all cases symptomatic leaves yielded high populations of *X. fastidiosa*.

Figure 6. Appearance of symptoms of Pierce's disease on Thompson seedless grape in a trial in Riverside county.

The incidence of disease on each vine from each plant in the Riverside County trial was assessed visually. In addition, the severity of disease symptoms on those leaves that appeared disease was also assessed for each leaf. This enabled an assessment of the incidence

of disease (the proportion of vines that had any infected leaves, as well as the total number of leaves per plant that were symptomatic) as well as the severity of disease on those leaves that exhibited symptoms. Overall, the incidence of infection plants in the trial in Riverside County was relatively low (Fig. 7). Generally only 10 to 20% of the vines on a given plant were infected. It is noteworthy, however, that no infections of the transgenic Freedom plants were observed, as these plants for the most resistant and greenhouse trials.

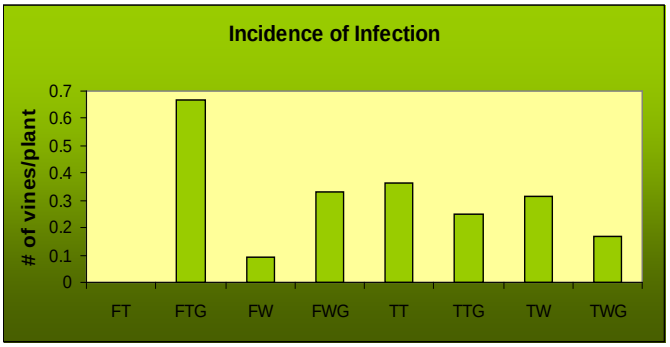

Figure 7. The incidence of infection of grapevines in different treatments. The number of vines from a given plant that exhibited at least one symptomatic leaf is shown. FT denotes transgenic Freedom grape transformed with the *Xylella fastidiosa rpfF* gene. FTG denotes grafted plants having a rootstock of transgenic Freedom but with a normal Thompson seedless scion. FW denotes normal Freedom grape. FWG denotes grafted plants having a normal Freedom rootstock and a normal Thompson seedless scion. TT denotes transgenic Thompson seedless grape transformed with the chloroplast targeted *rpfF* gene of *Xylella fastidiosa*. TTG denotes grafted plants having a transgenic Thompson seedless rootstock with a normal Thompson seedless scion. TW denotes normal Thompson seedless plants. TWG denotes grafted plants having a normal Thompson seedless rootstock and a normal Thompson seedless scion.

The extent to which *Xylella fastidiosa* infection spread throughout the plant after initial infection from natural sharpshooter inoculations was assessed by measuring the number of infected leaves on those vines which had exhibited any symptoms. Because the number of infected vines was relatively low, it was difficult to demonstrate differences in the extent of movement. Similarly extensive movement were observed in all those plants which had exhibited any infection (Fig. 8). Again, no infection was observed in transgenic Freedom grape.

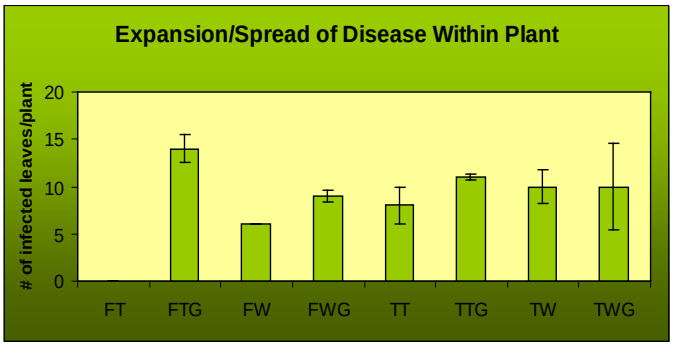

Figure 8. Expansion and spread of symptoms of Pierces disease in infected grapevines in a Riverside County trial. The number of infected leaves on a given vine that had exhibited any symptoms of Pierces disease is shown. Treatment codes are the same as denoted in figure 7.

The severity of Pierce's disease on fine food become infected was assessed using a qualitative scale to denote disease severity. Because of the relatively small number of infected vines, the severity of disease appeared similar in the different treatments (Fig. 9).

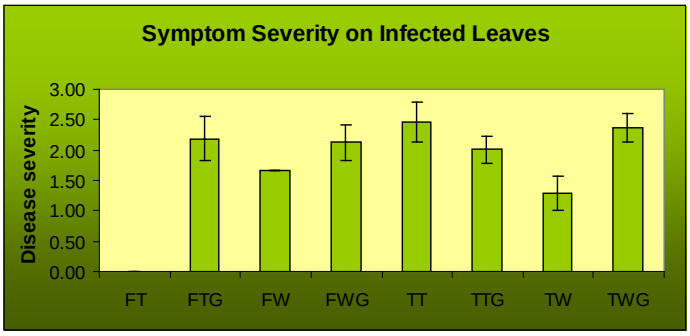

Figure 9. Severity of Pierce's disease symptoms on grapes in a trial in Riverside County. Disease severity was scored as: 0= no symptoms, 1= mild symptoms, 2= moderate symptoms (<50% of leaf blade scorched), 3= severe symptoms (> 50% of leaf blade scorched). Treatment codes are the same as indicated in figure 7.

Research Relevance Statement:
Extensive laboratory and greenhouse studies had revealed that cell density signaling by *Xylella fastidiosa* leads to a suppression of traits that enable growth and movement within plants upon the accumulation of DSF signaling molecule. Greenhouse studies have shown that various methods to augment DSF within plants in advance of infection by *Xylella fastidiosa* with suppress the ability of the bacteria to move away from the point of inoculation. These field trials are a culmination of a test of this strategy of pathogen confusion as a means for disease control. While the transgenic plants being evaluated are unlikely to be directly commercially applicable, they are realistic tests of this strategy that will enable decisions to be made as to whether transgenic strategies will be fruitful for disease control. In addition, transgenic plants are being evaluated both as scions and as rootstocks, to determine whether rootstock deployment of transgenic plants will be a successful strategy for disease control.

Status of funds:
The funds allocated to date for the study are nearly fully exhausted since the extensive fieldwork that has been undertaken has required frequent trips to both Solano and Riverside counties, as well as extensive field sampling of diseased leaves and pruning of the plants.

Summary and status of intellectual property associated with the project:
A patent application (12/422,825) entitled “biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules” had been submitted March 13, 2009. While most of the claims had been rejected earlier, the University of California patent office has filed on March 13, 2012 a motion requesting reconsideration of the application with clarification of, and justification for, claims related to the production of transgenic plants transformed with the *rpfF* gene from Xylella fastidiosa.