COMPREHENSIVE FINAL REPORT FOR CDFA AGREEMENT NUMBER 16-0513-SA

FIELD EVALUATION OF PIERCE'S DISEASE RESISTANCE OF VARIOUS DSF-PRODUCING GRAPE VARIETIES AS SCIONS AND ROOTSTOCKS

PRINCIPAL INVESTIGATOR:
Steven Lindow
University of California
Department of Plant and Microbial Biology
111 Koshland Hall
Berkeley, CA 94720-3102
icelab@berkeley.edu
510-642-4174

COOPERATORS:
Renee Koutsoukis
University of California
Department of Plant and Microbial Biology
111 Koshland Hall
Berkeley, CA 94720-3102
510-643 6498
Ms. Koutsoukis inoculated and assayed plants for presence of the pathogen in the field study as well as prepared grafted plants for transplanting and assisted in the planting process.

REPORTING PERIOD: The results reported here are from work conducted July 1, 2016 to August 30, 2019

INTRODUCTION

This was a continuing project that exploits results we obtained in the project 14-0143-SA entitled "Comparison and optimization of different methods to alter DSF-mediated signaling in *Xylella fastidiosa* in plants to achieve Pierce's disease control" which was funded by the CDFA PD program. One of the major objectives of that project was to "Compare DSF production and level of disease control conferred by transformation of *Xf* RpfF into several different grape cultivars". This and other projects in the previous 8 years had described a cell density-dependent gene expression system in *X. fastidiosa* (*Xf*) mediated by a family of small signal molecules called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis). 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 pits and thus for movement through the plant. Artificially increasing DSF levels in transgenic plants expressing the gene for the DSF synthase from *Xf* was found to be highly effective in reducing disease severity of inoculated plants when used as scions and to confer at least partial control of disease when used as rootstocks. Nearly all of the work had been done in the Freedom rootstock variety, and the goal of project 14-0143-SA was to transform a variety of other wine grape and rootstock varieties to determine the robustness of this strategy of disease control. The majority of these transgenic plants have now been generated and extensive greenhouse testing to identify the most resistant lines of those that could be transformed is complete. The work of this new continuing project was to establish field trials at UC-Davis where these lines can be compared with each other for PD control when used as both scions and rootstocks.

OBJECTIVES:

1) Determine the susceptibility of DSF-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties to Pierce's disease.
2) Determine population size of the pathogen in DSF-producing plants under field conditions.

RESULTS AND DISCUSSION:

This was a continuing project that exploited results we obtained in the project 14-0143-SA entitled "Comparison and optimization of different methods to alter DSF-mediated signaling in *Xylella fastidiosa* in plants to achieve Pierce's disease control" which was funded by the CDFA PD program. One of the major objectives of that project was to "Compare DSF production and level of disease control conferred by transformation of *Xf* RpfF into several different grape cultivars". This and other projects in the previous 8 years had described a cell density-dependent gene expression system in *X. fastidiosa* (*Xf*) mediated by a family of small signal molecules called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis). 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 pits and thus for movement through the plant. Artificially increasing DSF levels in transgenic plants expressing the gene for the DSF synthase from *Xf* was found to be highly effective in reducing disease severity of inoculated plants when used as scions and to confer at least partial control of disease when used as rootstocks. Nearly all of the work had been done in the Freeedom rootstock variety, and the goal of project 14-0143-SA was to transform a variety of other wine grape and rootstock varieties to determine the robustness of this strategy of disease control. The majority of these transgenic plants have now been generated and extensive greenhouse testing to identify the most persistent lines is complete. The work of this new continuing project was to establish field trials at UC-Davis where these lines can be compared with each other for PD control when used as both scions and rootstocks.

<u>Objective 1. Disease susceptibility of transgenic DSF-producing grape in field trials.</u>

As part of a continuing part of project 14-0143-SA grape varieties Thompson seedless as well as the advanced rootstock varieties 1103, 101-14 and Richter were transformed with the *rpfF* gene from *Xf*. In addition to un-targetted expression of RpfF, we produced plants in which RpfF is targeted to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose bisphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. This RpfF fusion gene product should be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). While the genetic constructs were made at UCB, transformation of the various grape varieties were conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis.

Our goal was to obtain between 5 and 10 individual transformants for each variety/construct combination. As will be summarized below, it was both slow and difficult to obtain sufficient numbers of transformants for certain of these combinations. Because the expression of *rpfF* in a given transformant of a given plant line will vary due to the chromosomal location of the randomly inserted DNA, it was necessary to identify those lines with the highest levels of expression. To determine the disease susceptibility of each line they were grown to a sufficiently large size that vegetative clones could be produced (3 months) and then each cloned plant was propagated and assessed for disease susceptibility (5 additional months). At least 12 vegetative clones each of the lines were produced from green cuttings of plants developing from each transgenic plant selected in the assays above. These plants as well as an untransformed control plant of a given variety (ca. 30 cm high) were inoculated with *Xf* by droplet needle puncture as in earlier studies. Disease severity was assessed visually weekly after inoculation. In this process, we were able to identify the transformant from each variety/construct combination that were most highly resistant to PD, and thus suitable for field evaluation. The following table indicates the number of individual independently transformed plants of each combination that have been delivered to Berkeley. All of the varieties that could be transformed have been successfully propagated and sufficient numbers of vegetative clones were produced to enable testing for disease susceptibility for nearly all transformants. Only a couple of the lines exhibited defective growth, making it impossible to produce clonal plants to assess disease resistance. Disease susceptibility has been completed from all of the transformed lines forward sufficient numbers of vegetative progeny could be produced.

Variety	Gene introduced	
	Untargeted RpfF	Chloroplast-targeted RpfF
	Number of transformed plants	
Thompson seedless	23	2
Richter 110	6	none
Paulsen 1103	6	none
Milardet et de Grasset 101-14	13	none

Certain of the varieties such as Chardonnay could not successfully be transformed at UC Davis. Furthermore, others such as Richter 110 and Paulsen 1103 proved to be somewhat more difficult to transform than other varieties, yielding fewer transformants than other grape varieties. Although the reason is unclear, the kanamycin resistance determining construct in which the chloroplast targeted RpfF is being delivered yielded relatively few transformants, with none being recovered for three of the varieties being investigated. Unfortunately, there were major greenhouse malfunctions in August, 2017 and April, 2018 which blocked watering of the plants for a day. This malfunction also happened during a relatively warm period in Berkeley, and the plants suffered substantial damage. The plants had both been inoculated for period of about 10 weeks at that point, and were on the verge of being assessed for visual symptoms of disease severity. Because the plants were so severely damaged they had to be cut back to the soil level and the newly emerging tissues had to be re-inoculated. This unfortunate setback delayed the final assessment of the disease resistance of these plants. The disease resistance of these different transgenic lines has now been completed. An example of the variation in disease-resistant seen in various transgenic lines is illustrated in Figure 1. As is typical of the various transgenic lines of a given grape variety, the disease resistance very substantially between the different lines; some transgenic lines exhibited much higher levels of disease resistance than others (Figure 1).

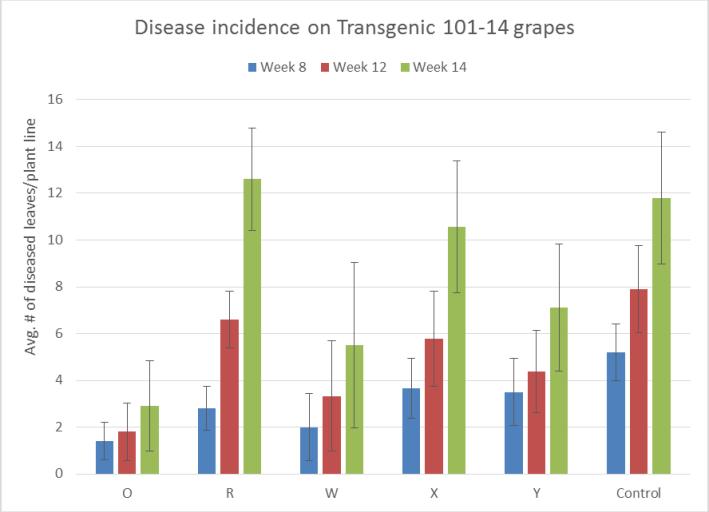

Figure 1. Disease severity exhibited by different transgenic lines of the grape rootstock 101-14 measured 8, 12, and 14 weeks after inoculation with *X. fastidiosa*. The vertical lines represent the standard error of the mean disease incidence.

We encountered complications with assessing disease symptoms during winter months. Not only do plants grow slowly in the winter, even when supplemental lighting was supplied, but the plants also apparently undergo less water stress, and therefore do not exhibit typical symptoms of Pierce's disease. As we typically have the best results for disease assessment studies during spring and summer months nearly all of our screening for disease susceptibility in the greenhouse was restricted to these months, thereby slowing progress on project. Overall, the process of evaluating the various lines for disease resistance proved to be slower than expected because the plants obtained from Davis often arrived during winter months and thus were both very small and very slow to grow under these winter growing conditions. This lengthened the time needed to obtain the vegetative clones required for disease susceptibility testing. Thus while we were ultimately successful in obtaining sufficient

number of vegetative clones for testing and robust assessments of their disease susceptibility, this process was substantially slower than we had anticipated.

Field tests with most of the transgenic lines was initiated in 2019. Plants produced in the greenhouse at UC Berkeley were transported to the field site at the Plant Pathology research farm and planted on August 20, 2019. The various grape variety/genetic construct combinations discussed listed below were planted on this date. These transgenic grape varieties as well as untransformed control plants of each variety were tested as both own-rooted plants as well as rootstocks to which the susceptible grape variety Cabernet Sauvignon was grafted.

Variety	Gene introduced		
	Untargeted RpfF	Chloroplast-targeted RpfF	Untransformed plants
Thompson seedless	+	+	+
Richter 110	+		+
Paulsen 1103	+		+
101-14	+		+
Freedom	+		+

10 plants of each treatment (a particular variety serving as either as own-rooted rootstock or as a rootstock to which Cabernet Sauvignon was grafted) were established in a randomized complete block design. Some difficulty was encountered in getting sufficient numbers of grafted transgenic Richter 110 and Paulsen 1103 plants as of August, 2019, an additional grafted plants are currently being produced and will be planted in April 2020. These plants will all be inoculated with *Xf* in May, 2020. The plants will be inoculated with *Xf* by needle puncture through drops of *Xf* of about 10^9 cells/ml as in previous studies. Disease symptoms in continuing studies will be measured bi-weekly starting at 8 weeks after inoculation on about May 1, 2020. Leaves exhibiting scorching symptoms characteristic of Pierce's disease will be counted on each occasion, and the number of infected leaves for each vine noted as in our other studies. An additional 0 to 5 rating scale will also be applied which accounts for both the number of vines on a plant that are symptomatic as well as the degree of symptoms on a given plant. ANOVA will be employed to determine differences in severity of disease (quantified as the number of infected leaves per vine) that are associated with treatment.

Objective 2. Assess population size of *Xf* in transgenic plants. To ensure that the symptoms of Pierce's disease in Objective 1 above are associated with *Xf* infection and to document the limited extent of excess colonization in transgenic DSF-producing vines inoculated with *Xf* compared to that of the corresponding non-transgenic vines, five petioles from each inoculated vine will be harvested (at approximately 40 cm intervals depending on the length of the vine for a given variety) at monthly intervals starting eight weeks after inoculation. Petioles will be surface sterilized and then macerated and appropriate dilutions of the macerate applied to PWG plates containing the fungicide natamycin. Colonies characteristic for *Xf* will then be counted and the population size of *Xf* determined. While this method is a bit more work than the method of PCR, it provides a more sensitive assay method and avoids some issues with false negative discovery rates associated with field sampling of grape tissues. ANOVA will be employed to determine differences in population size of *Xf* (quantified as log cells/petiole) that are associated with treatment. The non-parametric Sign test will also be performed to determine differences in the incidence with which any detectable *Xf* occurs in these petioles at a given sampling distance from the point of inoculation. This strategy will quantify disease to test the assumption that many petioles, especially on DSF-producing plants and at the distal ends of vines will be free of any detectable cells of *Xf*. Inoculation and disease assessment will be initiated in 2020.

PRESENTATIONS MADE:

Presentation entitled "The many density -dependent traits of *Xylella fastidiosa*: achieving disease control via pathogen confusion" presented at the University of Arizona, 2016.

Presentation at the 3rd International Conference on Biological Control of Plant Pathogenic Bacteria, Belgrade, Serbia, entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". 2016.

Presentation at University of Barcelona entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". 2016.

Presentation at Microbe 2016 - the Annual Meeting of the American Society for Microbiology, Boston, entitled "The biology of *Xylella fastidiosa* in plants and insects", 2016.

Presentation at the 17th international Congress on Molecular Plant-Microbe Interactions, Portland Oregon, entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion". July, 2016.

Presentation at the University of California, Davis entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". October, 2016.

Presentation at the 2016 Pierce's disease research symposium entitled "Biological control of Pierce's disease with an endophytic bacterium" presented December 14, 2016, San Diego California.

Presentation at the University of Iowa entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". February, 2017.

Presentation made at the annual meeting of the International Society for Extracellular Vesicles entitled "novel roles of quorum sensing regulated extracellular vesicles produced by *Xylella fastidiosa* and their role in virulence to plants". May, 2017.

Presentation made at the Department of Plant and Microbial Biology, the University of Zürich, November, 2017.

Presentation at the Department of Plant pathology, Auburn University entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion", March, 2018.

Presentation made at the 6th Xanthomonas genetics conference, Halle, Germany entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion", July, 2018.

Keynote presentation made at the 11th International Congress of Plant Pathology, Boston Massachusetts, entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion", July, 2018.

Presentation made at the 2018 Pierce's disease symposium, San Diego California, December, 2018.

Presentation made at the 21st annual IPM seminar for Lake and Mendocino County. Research and Extension Ctr., November 16th, 2018 entitled "Novel approaches to controlling Pierces disease and when grapevines".

Presentation made at the AG Unlimited 23rd annual grower meeting entitled "Control of frost damage and Pierce's disease in grapes", February 28, 2019.

Presentation made at the Fifth International Meeting of the International Committee on Huanglongbing disease entitled "detailed studies of plant pathogens can leave to novel methods of disease control: the case of *Xylella fastidiosa* in grapes in citrus", Riverside California, March, 2019.

RESEARCH RELEVANT STATEMENT:

Since we have shown that DSF accumulation within plants is a major signal used by *X. fastidiosa* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this

pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion". This field trial is a direct demonstration project to test the field efficacy of plants producing DSF to alter pathogen behavior in a way that symptom development is minimized. Results from earlier field trials in which only a limited number of grape varieties were evaluated in Solano County and Riverside County provided strong evidence that pathogen confusion can confer high levels of disease control - both to plants artificially inoculated had Solano County, and especially to plants infected naturally with infested sharpshooter vectors. The earlier work therefore has provided support that this strategy is a useful one for managing Pierces disease. This ongoing study is therefore are designed primarily to evaluate the robustness and general applicability of this strategy of disease control in a wide variety of grape varieties.

LAYPERSON SUMMARY:

Xf 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 generated and are testing 4 different DSF-producing grape varieties both as own-rooted plants as well as rootstocks for susceptibility varieties to Pierce's disease. These transgenic grape varieties have now been produced at the plant transformation facility at UC Davis and evaluated under greenhouse conditions at Berkeley to determine those particular transgenic lines that have the highest disease resistance. The transgenic varieties were established in a field plot as own rooted plants or as rootstocks of plants with a normal Cabernet Sauvignon scion in August, 2019. Disease severity and population size of the pathogen will be assessed in the plants in 2020 after their establishment in the field as a means of determining their susceptibility to Pierce's disease after inoculation.

STATUS OF FUNDS:

Because of the delay in obtaining the transgenic plants from the plant transformation facility at UC Davis, and the extensive time needed for evaluation of the transgenic plants that have been received, nearly all of the work to date has been in preparation for the field trial and has been funded by residual funds available from a no-cost extension of project 14-0143-SA as well as the funds available for this current project. The funds for this current project were exhausted by June 30, 2019. By that time nearly all of the plants were produced in the greenhouse for field planting in spring 2019, with only a few additional grafted plants still being made. We will finish the establishment and evaluation of the transgenic lines at the UC Davis field plot using discretionary funds available to me due to my prior duties as Executive Associate Dean of the College of Natural Resources.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY:

A US patent 8,247,648 B2 entitled "Biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules" was approved in June, 2012 and was issued on August 21, 2012. This patent should facilitate the commercial adoption of disease control methods to be further developed in this project. Information regarding UC-Berkeley IP policies can be found at: http://otl.berkeley.edu/.