INTERIM PROGRESS 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

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Ms. Koutsoukis will inoculate and assay plants for presence of the pathogen in the field study.

REPORTING PERIOD: The results reported here are from work conducted July 1, 2016 to August, 2018

INTRODUCTION

This is a continuing project that exploits results we have 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 nearly complete. The work of this new continuing project is to establish field trials at UC-Davis in 2019 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 is a continuing project that exploits results we have 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 getting closer to completion. The work of this new continuing project is to establish field trials at UC-Davis in 2019 where these lines can be compared with each other for PD control when used as both scions and rootstocks.

Objective 1. Disease susceptibility of transgenic DSF-producing grape in field trials.

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 is being 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 has been 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 is 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) are being inoculated with *Xf* by droplet needle puncture as in earlier studies. Disease severity is being assessed visually weekly after inoculation. In this process, we are able to identify the transformant from each variety/construct combination that is 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. Nearly all have been successfully propagated and vegetative clones produced to enable testing for disease susceptibility. Disease susceptibility has been completed from the majority of the transgenic lines, although a few of the lines have been inoculated but disease assessments are still being made under greenhouse conditions at Berkeley.

Variety	Gene introduced	
	Untargeted RpfF	Chloroplast-targeted RpfF
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 have proven 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 has yielded relatively few transformants, with none being recovered for three of the varieties being investigated. These transformations will again be repeated with a fresh Agrobacterium/vector combination. A modification of this vector is also being developed to determine if it will be more successful. As noted above, screening for disease resistance of the non-targeted RpfF plants already delivered is underway. Unfortunately, there were major greenhouse malfunctions in August, 2017 and April, 2018 which blocked watering of the plants for a day. This malfunction unfortunately also happened during a relatively warm. In Berkeley, and the plants suffered substantial damage. The plants had 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 have now been re-inoculated. This unfortunate setback will delay the final assessment of the disease resistance of these plants until late 2018. The disease resistance of several of these different transgenic lines has now been completed and is illustrated as an example of the variation and disease-resistant seen in various transgenic lines (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).

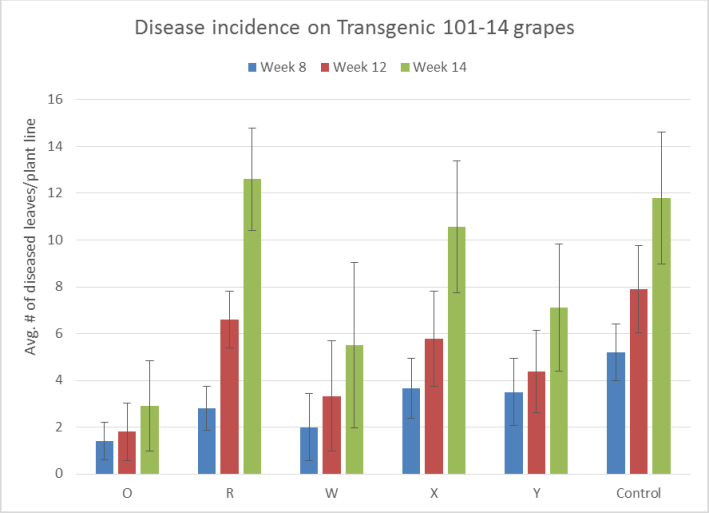

Figure 1. Disease severity exhibited by different transgenic lines of the grape rootstock 101-14 measured 8, 12, and 14 weeks after inoculation with *X. fastidiosa*. The vertical lines represent the standard error of the mean disease incidence.

We continue to encounter complications with assessing disease symptoms during winter months, as we typically have the best results for disease assessment studies during spring and summer months. Overall, the process of evaluating the various lines for disease resistance has 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 has lengthened the time needed to obtain the vegetative clones required for disease susceptibility testing. We have however now obtained sufficient number of plants from each of the 4 newly transformed grape varieties to evaluate the relative efficacy of expression of RpfF, and thus DSF production to achieve disease resistance in these various varieties. Not only will this provide us evidence for the relative effectiveness of DSF production as a disease control strategy and the different grape varieties, but it will allow us to identify the most highly resistant variety for a given variety. Our goal is still to produce enough self-rooted plants of the most resistant lines for field testing as well as to generate grafted plants with these plants serving as rootstocks for field testing that will begin in 2019. We feel it is best to establish all of the pants at the same time so sa to better make direct comparisons of their susceptibility to Pierce's disease. The grafting

process will add an additional three months to the process of generating plants for use in field studies, but we hope to be able to complete this for these grafted plants by early 2019.

Field tests with at least some of the transgenic lines will be initiated beginning in 2019 with the various grape variety/genetic construct combinations discussed above. Given the difficulty of producing chloroplast-targeted *rpfF* constructs of certain of the varieties, it is however unlikely that they will be available for planting before 2019. We will continue to evaluate such transformed lines as success in their transformation is achieved at the UC Davis transformation facility.

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

These transgenic grape varieties will be tested as both own-rooted plants as well as rootstocks to which the susceptible grape variety Cabernet Sauvignon will be grafted. Thus, a maximum of 14 different treatments will assess each grape variety/gene construct on own-rooted plants. An additional up to 14 treatments will evaluate each grape variety/gene construct as a rootstock onto which Cabernet Sauvignon will be grafted as a scion.

12 plants of each treatment will be established in a randomized complete block design with 4 blocks of three plants each for each treatment that will be inoculated with *Xf* after establishment. In addition, 4 plants in each treatment (one plant per block) will be left un-inoculated with *Xf* as a control to observe plant development and yield to determine whether DSF production had any effect on plant development under field conditions. No such effects have been observed in field studies conducted to date or in greenhouse studies however. Half of the plants will be own-rooted plants and the other half will be grafted plants with a normal Cabernet Sauvignon scion. Half of the plants will be inoculated with *Xf*. 12 of the plants from each treatment will be inoculated 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 (inoculation will be done about May 1). 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. This scale will be most important in the third year of the study (two years after inoculation) when spread through the plant will be assessed. 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. As noted above, the majority of the plants are anticipated to be available for planting by early 2019, and inoculation and disease assessment will be initiated only in 2020.

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. As only a few plants are available to establish in the field plot in 2017, and most will be available only by early 2019, inoculation and disease assessment will be initiated only in 2020.

PUBLICATIONS AND PRESENTATIONS:

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.

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". These field trials are direct demonstration projects 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. The current ongoing studies 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 are generating and testing 4 different DSF-producing grape varieties both as own-rooted plants as well as rootstocks for susceptibility to Pierce's disease. The majority of these transgenic grape varieties have now been produced at the plant transformation facility at UC Davis and the majority have been evaluated under greenhouse conditions at Berkeley to determine those particular transgenic lines that have highest disease resistance. Additional gene constructs will be made to generate transgenic plants in which the DSF synthase is directed to a cellular environment in which higher levels of DSF production can be expected in those few grape varieties in which such expression has not yet been successful. The transgenic varieties will be available for establishment in the field plot as own rooted plants or as rootstocks of plants with a normal Cabernet Sauvignon scion in 2018. Disease severity and population size of the pathogen will be assessed in the plants after their establishment in the field as a means of determining their susceptibility to Pierce's disease after artificial 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. For that reason, we have spent little of the funding available on this new project. Project 14-0143-SA has now ended and some additional work is needed to finish greenhouse evaluations of the transformed plants, to make vegetative clones of the most highly resistant plant lines, and especially to produce the grafted plants for the field testing. This work will be supported by this current project. Fieldwork will be initiated only in 2019. Inoculation and disease assessment will begin in 2020. We therefore expect to request a no-cost extension at the time the current project period ends to be able to be able to extend the time available to evaluate the transgenic plants in the field.

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/.