"Interim Progress Report for CDFA Agreement Number 17-0419-000-SA"
Project Title: Field testing transgenic grapevine rootstocks expressing CAP and PGIP proteins.

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Time period covered by the report: July 2016 to February 2018.

Introduction
The focus of this study is to evaluate the rootstock-based expression of chimeric antimicrobial proteins (CAP; Dandekar et al., 2012a) and polygalacturonase inhibitory protein (PGIP; Agüero et al., 2005, 2006) to provide transgraft protection of the scion grapevine variety against Pierce's disease (PD). Rootstocks (Thompson Seedless, TS) expressing these proteins individually were evaluated in the field, this part of the study was concluded in 2017. TS rootstock lines expressing either CAP or PGIP show promise in their ability to transgraft protect a scion variety (also TS) against PD, validated with in-field inoculations. The lines expressing CAP showed the highest efficacy in protecting grafted transgenic grapevines from developing PD. TS is not a rootstock, these genes must be tested in a commercially relevant rootstock. Methods to successfully transform two commercially relevant rootstocks 101-14 and 1103 (Christensen, 2003) was successfully developed (Dandekar et al., 2011; 2012b) and the method was further improved by David Tricoli in the plant transformation facility at UC Davis. The original NE-CB CAP construct (Dandekar 2012a) was improved by identifying grapevine-derived components (Chakraborty et al., 2013; 2014b). The surface interacting NE component (neutrophil elastase) was replaced with P14a protein from *Vitis shuttleworthii* that also displays serine protease activity (Chakraborty et al., 2013; Dandekar et al., 2012c; 2013). The antimicrobial component CB (cecropin B) was replaced with HAT52 and/or PPC20 that were identified using novel bioinformatics tools developed by us (Chakraborty et al., 2013; 2014a) and the efficacy of the selected peptides were verified for their ability to kill *Xf* cells (Chakraborty et al., 2014b). The ongoing testing involves evaluating novel CAP lines in commercially relevant rootstocks 101-14 and 1103 (Christensen, 2003). In addition to the original NE-CB CAP (CAP-1) five additional CAP constructs included in the current round of testing are 35s OM/RAMY/Flag CAP (CAP-2); VsP14a (CAP-3); VsP14a-CB (CAP-4), VsP14a-HAT52 (CAP-5) and VsP14a-PPC20 (CAP-6; Dandekar et al., 2012c; 2013; 2014). These transgenic CAP-expressing rootstocks testing in the greenhouse and field started in fall 2016. The additional CAP constructs that will be tested are aimed to address the concern that the protein components of the present CAP-1 have a non-plant origin. Transformation of these 5 CAP constructs into the 101-14 and 1103 rootstock backgrounds was initiated in 2014 greenhouse testing was initiated in 2016 with field testing 2018 onward. The field introduction of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting grafted, sensitive scion cultivar Chardonnay from developing PD.

List of objectives
The major goal is to field-test transgenic rootstocks to identify lines that can transgraft-protect a scion variety from Pierce's disease (PD) development.

Objective 1. Complete the current round of efficacy testing of *in planta*-expressed chimeric antimicrobial proteins for the ability to clear *Xf* infection in xylem tissue and through the graft union in grapevines grown under field conditions.

Activity 1. Complete and conclude current field tests.
Activity 2. Conduct greenhouse and field evaluation of CAP-expressing 110-14 and 1103 rootstocks.

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Activity 1. Complete and conclude current field tests.

At the Solano County site, half of the non-grafted transgenic lines were manually inoculated as described (Almeida et al. 2003) on July 13, 2011, and the rest on May 29, 2012. Half of the grafted transgenic lines were also manually inoculated on a later date. Nongrafted and grafted grapevines at the Solano site that were not previously inoculated were manually inoculated on June 17, 2013, completing the inoculations of all grapevines at this location. On May 27, 2014, and May 27, 2015, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four new canes per year from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf*. Inoculation dates from 2011 to 2015 are shown in a color-coded map (**Table 1, Figure 1**).

Figure 1. Solano County grafted transgenic grapevines inoculated in spring 2014 and spring 2015 (left, photo taken in fall 2016), terminated Solano field (right, photo taken in spring 2017).

Table 1. Solano County grape field map, color-coded by *Xf* inoculation date, from 2012 to 2015.

Vine	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Row 9	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	TS-G	
Row 8	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G
Row 7	31-25-G	40-92-G	40-92-G	40-92-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	40-92-G	40-92-G	40-92-G	41-151-G	41-151-G	41-151-G	45-77-G	45-77-G	45-77-G
Row 6	TS-50-G	TS-50-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	41-151-G	41-151-G	41-151-G	52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G
Row 5													52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G	31-25-G	45-77-G	45-77-G	45-77-G	TS-50-G
Vine	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25

North

Grapevine inoculation with Xf (Temecula:Stag's leap mix, 60:40) at 250,000 per 20ul on 5/29/2012.
Grapevine inoculation with Xf (Temecula) 250,000 per 20ul on 6/17/2013.
Grapevine inoculation with Xf (Temecula:Stag's leap mix, 60:40) at 500,000 per 20ul on 5/27/2014.
Grapevine inoculation with Xf (Temecula) at 500,000 per 20ul on 5/27/2015.

On July 22, 2014 and September 15, 2015, one 2014-inoculated cane from each grafted transgenic plant was harvested for quantification of *Xf* by qPCR using an Applied Biosystems SYBR green fluorescence detection system. *Xf* DNA was extracted using a modified CTAB (hexadecyltrimethyl-ammonium- bromide) method that allowed us to obtain DNA of a quantity and quality suitable for qPCR. The *Xf* 16s primer pair (forward 5'-AATAAATCATAAAAAAATCGCCAACATAAACCCA-3' and (reverse 5'-AATAAATCATAACCAGGCGTCCTCACAAGTTAC-3') was used for *Xf* quantification. qPCR standard curves were obtained using concentrations of *Xf* ranging from 10^2 to 10^6 cells per 0.1 g tissue. *Xf* was detected in grafted transgenic vines, but *Xf* titers were lower than in grafted control grapevines (Fig. 2).

Severity or absence of PD symptoms was assessed for all Solano County grafted transgenic grapevines inoculated from 2012 to 2015 in fall 2015 using the PD disease symptom severity rating system 0 to 5, where 0 = healthy vine, all leaves green with no scorching; 1= first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and is declining and 5 = the cane is dead. PD symptom severity scores were lower in most grafted inoculated transgenic lines from each strategy (CAP or PGIP) than in grafted untransformed controls (Fig. 3).

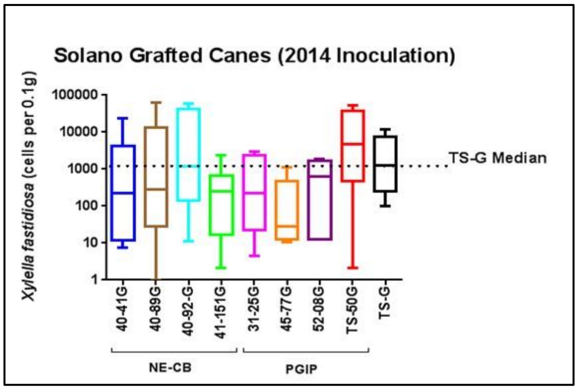

Figure 2. *Xf* quantification by qPCR of Solano grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014 and fall 2015.

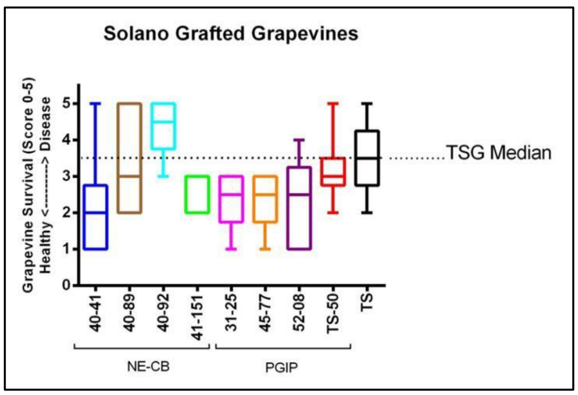

Figure 3. Severity or absence of PD symptoms for all Solano grafted inoculated grapevines on fall 2015.

Grapevine survival of grafted transgenic grapevines that were inoculated in 2014/2015 was assessed on October 6, 2016, using a 1 to 5 score, where 1 = very healthy and vigorous grapevine; 2 = healthy grapevine and slightly reduced vigor; 3 = slightly reduced spring growth; 4 = much reduced spring growth and 5 = dead grapevine (Fig. 4). The grapevine survival rate was greater in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls, with the greater efficacy seen in CAP lines. The Solano field was terminated in the summer of 2017.

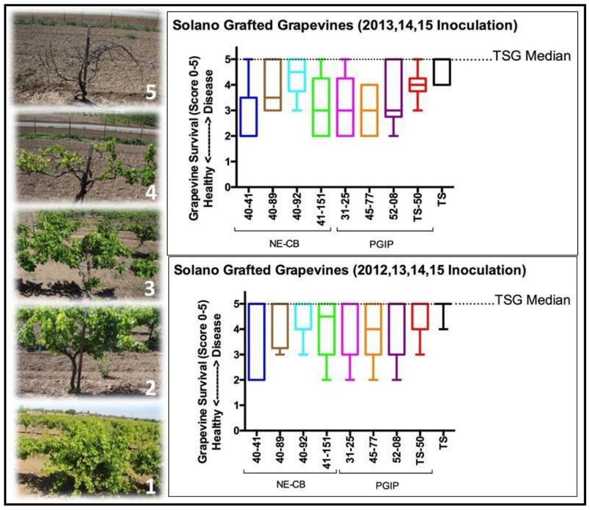

Figure 4. Grapevine survival of Solano grafted transgenic grapevine inoculated in 2013-2015 (upper right) and all inoculated grafted transgenic grapevines (lower right), scored in fall 2016 using a scale of 1 to 5 (left).

Activity 2. Conduct greenhouse and field evaluation of CAP-expressing 101-14 and 1103 rootstocks.

This activity focused on greenhouse and field testing of six vector constructs that are in the plant transformation pipeline using two commercially relevant rootstocks, 101-14 and 1103 (Christensen, 2003). The components present in these constructs are shown in Fig. 5 below. The construction of CAP-1 was described earlier (Dandekar et al., 2012a) and the construction of CAP-2, CAP-3, CAP-4, CAP-5 and CAP-6 shown in Fig. 5 have been previously described (Chakraborty et al., 2014b, Dandekar et al.,2012c; Dandekar et al., 2013 and Dandekar et al., 2014a). The grapevine transformation methods for the 101-14 and 1103 rootstocks have been described previously (Dandekar et al., 2011 and Dandekar et al., 2012b) but were further improved by David Tricoli in the UC Davis Plant Transformation Facility who did the transformation of all of the binary vector constructs shown in Fig. 5. The transgenic plants obtained from the facility were propagated for testing described in detail below. The transformation of the two rootstock species with all six CAP constructs was initiated some time back (2014) and the selection and regeneration of plants is ongoing. The field introduction of these rootstocks is aimed at evaluating their efficacy in protecting grafted sensitive Chardonnay grapevine variety from developing PD.

Transformation of the first construct (CAP-1) yielded 30, 101-14 and four 1103 derived transgenic lines. Since the yield for 1103 lines transformed with CAP-1 was low, a new transformation was initiated back in Aug 2015. In addition, in summer 2016, we began receiving 110-14 and 1103 lines transformed with the other constructs (CAP-2 to 6) and the numbers and distribution of these lines is indicated in Table 2 (below).

Table 2. Pierce disease resistance greenhouse testing of CAP-expressing transgenic rootstocks

CAP Designation	**Binary Vector**	**Greenhouse Propagation**		**Greenhouse Testing**		**Advance for Field Testing**	
		101-14	**1103**	**101-14**	**1103**	**101-14**	**1103**
CAP-1	**pDU04.6105**	**30**	**4**	**30**	**4**	**6**	**0**
CAP-2	**pDU12.0310**	**0**	**11**	**0**	**10**	**0**	**8**
CAP-3	**pDP13.35107**	**11**	**3**	**8**	**2**	**7**	**0**
CAP-4	**pDP13.36122**	**13**	**1**	**6**	**0**	**2**	**0**
CAP-5	**pDP14.0708.13**	**11**	**6**	**10**	**6**	**1**	**3**
CAP-6	**pDP14.0436.03**	**8**	**6**	**8**	**6**	**7**	**4**

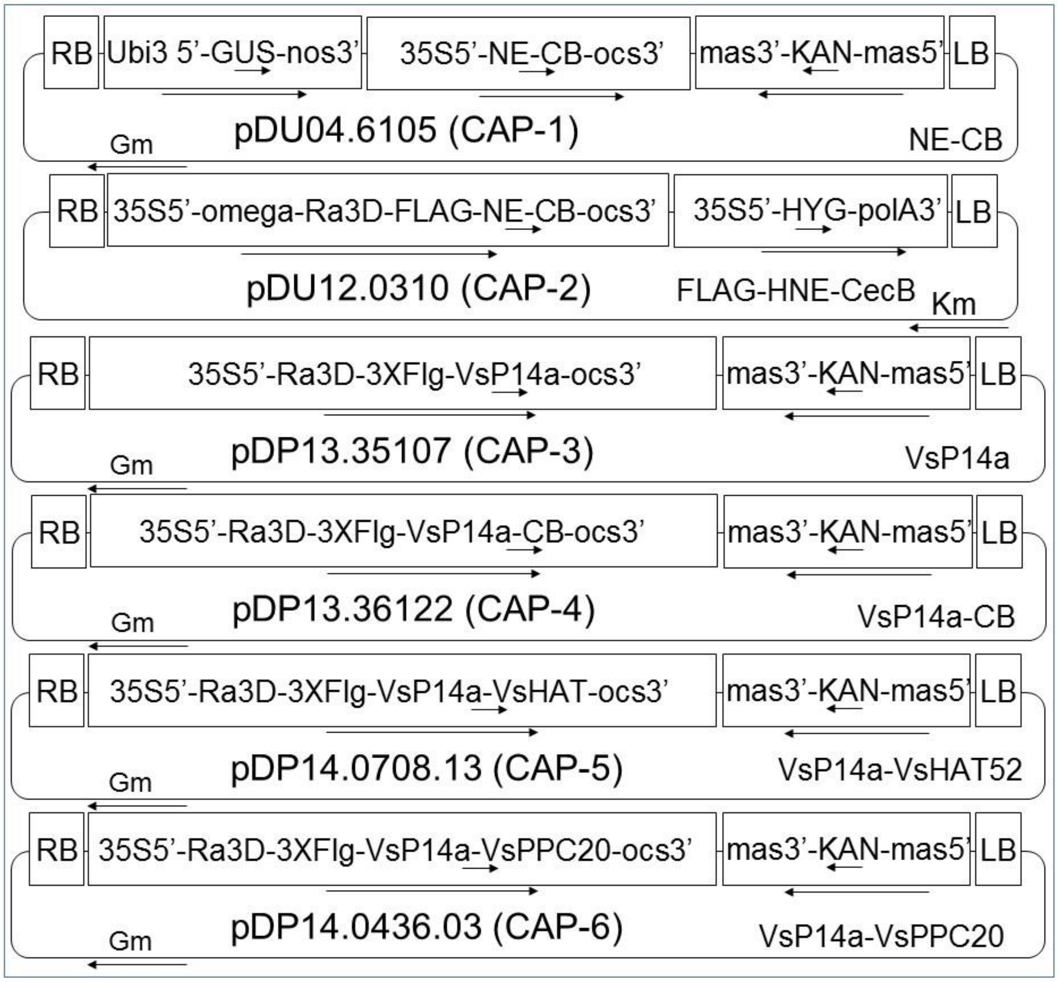

Figure 5. CAP vectors testing of the original and grapevine components, used to create transgenic 101-14 and 1103 rootstocks that will be verified in greenhouse and field.

A greenhouse propagation/testing protocol was successfully developed to test for PD resistance in transgenic lines of 101-14 and 1103 rootstocks. The 101-14 and 1103 transgenic rootstocks lines are first screened for the presence of CAP transgene using PCR. Those 101-14 and 1103 plants that are PCR-positive are clonally propagated for greenhouse testing. The clones are trained into a two-cane system and inoculated on one of the canes with *Xf*. Plants are inoculated with 20uL of *Xf* at roughly three nodes above the fork in the canes and eight leaves below the top of the cane. Then the plant is turned over and inoculated with another 20uL of *Xf* directly behind the first inoculation. The *Xf* inoculum is prepared as described earlier (Dandekar et al., 2012a).

The transgenic rootstocks successfully inoculated as described above are evaluated for PD symptoms 12 weeks post inoculation when the first disease symptoms appear, and subsequently every two weeks thereafter until 18 weeks post inoculation. A scoring system of 1 to 5 was used with values of: 1 = No visible disease symptoms (Good); 2 = Disease symptoms on less than 4 leaves (Good/OK), 3 = Disease symptoms exhibited on 50 percent the cane (4 leaves, OK); 4 = Disease symptoms exhibited on 75 percent of the cane (6 leaves, OK/Bad) and 5 = Symptoms stretching the entire length of inoculate cane (8 leaves, Bad).

All 34 CAP-1 transgenic lines have been analyzed and six have been identified for field-testing. All six were 110-14 transgenic. Of the six 110-14 transgenic lines selected, one was an elite line and presented no PD symptoms and got a score of 1. The remaining five 101-14 plant lines got a score of 2, which look very promising and were considerably less sick than the untransformed 101-14 control which was scored a 5 (Fig. 6). All lines 1103 scored bad and received a score of 5. The six 101-14 transgenic rootstocks expressing CAP-1 that scored a 1 or a 2 have been clonally propagated from the uninfected mother plants.

Figure 6: Infected two cane vines with the left uninfected and right infected WT 101-14 grapevines with disease symptoms running the entire length of the infected cane (A). The elite CAP-1 transgenic line of 110-14 that showed no symptoms 18 weeks post inoculation (B).

Nine out of ten CAP-5 transgenic events expressing VsP14a-VsHat22 in the 101-14 background that screened PCR positive were clonally propagated and infected with *Xylella fastidiosa* and two have been identified for field-testing. All other plants in the 101-14 and 1103 backgrounds that have been confirmed PCR positive are in the cloning/growing/inoculating pipeline for inoculation with *Xf*. (Fig. 7). Plants of each background continue to be produced at the transformation facility, as plants emerge they are propagated for greenhouse and field-testing.

Figure 7: Transgenic 110-14 and 1103 lines expressing (CAP-2 to 6) are in the cloning/growing/inoculating pipeline for greenhouse inoculation with *Xf*.

A more detailed scoring system was recently developed for the analysis of Pierce's Disease symptoms during greenhouse screening. A scoring system of 0 to 5 was used to score each leaf as follows: 0 = No visible disease symptoms; 1 = Disease symptoms just appearing with < 10% leaf scorch, 2 = 10-25% of leaf scorched; 3 = 25-50% of leaf scorched, 4 = 50-75% of leaf scorched and 5 = 75-100% of leaf scorched or only petiole remaining (Fig. 8**).** Pierce disease symptoms for the CAP-5 plants in the 101-14 background were scored using the detailed score system. Result of the screening process of CAP-5 plants in the 101-14 background is shown in Fig. 9.

Figure 8. Pierces's disease symptoms scoring system of 0 to 5. Top, left to right 0, 1 and 2, bottom, left to right, 3, 4 and 5.

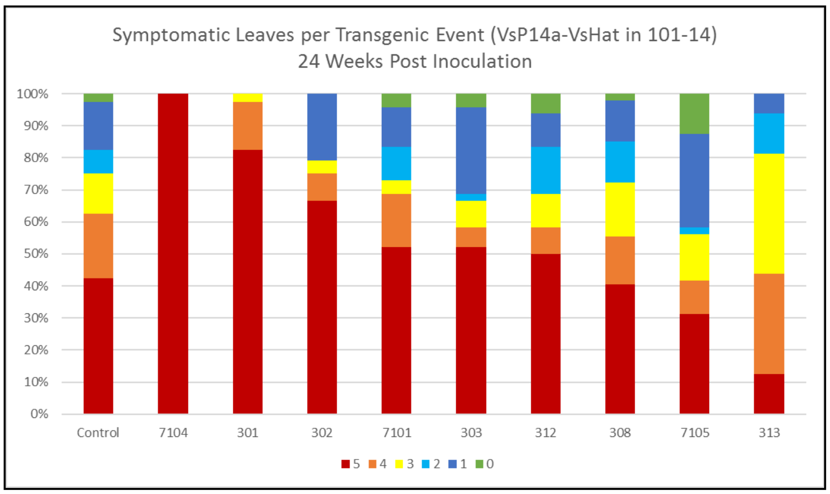

Figure 9. Last data point collected while screening the101-14 transgenic rootstocks expressing CAP-5. Plants are scored weekly after the Pierce's Disease symptoms begin to show.

<u>Publications produced and pending, and presentations made related to the funded project.</u>

Dandekar, A.M., A.M. Ibanez and A. Jacobson. 2017. Field testing transgenic grapevine rootstocks expressing chimeric antimicrobial protein and polygalacturonase-inhibiting protein. Research Progress Reports: Pierce's Disease and other Designated Pests and Diseases of Grapevines. December 2017. California Department of Food and Agriculture. pp. 20-28.

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Research relevance statement, indicating how this research will contribute toward finding solutions to Pierce's disease in California.

This proposal is a continuation of a project to test expression of a chimeric anti-microbial protein (CAP) and polygalacturonase inhibitory protein (PGIP) as a means to clear and block the movement of *Xylella fastidiosa* and provide resistance to Pierce's disease (PD). Rootstocks (Thompson Seedless, TS) expressing these proteins individually were successfully evaluated in the field, this study will build on this important research. TS rootstock lines expressing either CAP or PGIP are showing promise in protecting against PD that was validated with in-field inoculations. Since TS is not a rootstock these genes must be tested in a commercially relevant rootstock that is what will be accomplished in the ongoing continuation of this research. The ongoing research will test transgenic rootstocks developed in two previously funded projects (11- 0240-SA; 2011-2013 and project 12-130-SA; 2012-2014) for providing trans-graft protection against PD. The greenhouse and field testing of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting grafted, sensitive scion cultivar Chardonnay from developing PD. Elite rootstock lines will be good candidates for commercialization.

Layperson summary of project accomplishments.

This project is a continuation to evaluate the field efficacy of transgenic grapevine rootstocks expressing a chimeric anti-microbial protein (CAP) or a polygalacturonase inhibitory protein (PGIP) to provide protection to the grafted scion variety from developing Pierce's Disease (PD). We concluded a field evaluation where four CAP and four PGIP expressing Thompson Seedless (TS) were tested as rootstocks to protect grafted wild type TS scions. These plants were infected with *Xylella fastidiosa* (*Xf*) in 2012, 2013, 2014 and 2015 and evaluated each year for their ability to provide resistance to PD. Our conclusion is that the transgenic rootstocks were able to provide transgraft protection to the scion; they showed less symptoms, higher survival and harbored a lower titer of the pathogen than grafted untransformed controls. Since TS is not a commercially relevant rootstock we have now begun testing the field efficacy of this strategy by expressing different CAP proteins in commercially relevant rootstocks 110-14 and 1103. Green house evaluations were initiated in 2018 and field evaluations will begin in spring of 2018. Elite rootstock lines identified in this project will be good candidates for commercialization.

Status of funds.

We have expended all the funds available for the period July 1, 2017 to Feb. 28, 2018. Remaining funds will be spent in the period March 1, 2018 to June 30 2018.

Summary and status of intellectual property associated with the project.
An invention disclosure will be made for a plant patent once an elite transgenic rootstock line demonstrates excellent field efficacy in protecting a grafted sensitive scion from coming down with PD.

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