

**“Renewal Progress Report for CDEA Agreement Number 15-0578-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 March 2017

**Introduction**

This research is a continuation of the field evaluation of chimeric anti-microbial protein (CAP: Dandekar et al., 2012a) and polygalacturonase inhibitory protein (PGIP; (PGIP; Agüero et al., 2005, 2006) expressing rootstocks that enable trans-graft protection of scion varieties of grapevine from developing Pierce’s Disease (PD) after infection with *Xylella fastidiosa* (Xf). Rootstocks (Thompson Seedless, TS) expressing these proteins individually were evaluated in the field, this part of the study was concluded on winter 2017. TS rootstock lines expressing either CAP or PGIP show promise in their ability to transgraft protect a scion variety (also TS) against PD which is being validated with in-field inoculations. The lines expressing CAP showed the highest efficacy in protecting grafted transgenic grapevines from developing PD. Since 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 binding 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). In addition to the original NE-CB CAP (CAP-1) four additional CAP constructs were developed that contained VsP14a (CAP-2); VsP14a-CB (CAP-3); VsP14a-HAT52 (CAP-4) and VsP14a-PPC20 (Dandekar et al., 2012c; 2013; 2014). Transformation of these 5 CAP constructs into the 101-14 and 1103 rootstock backgrounds was initiated in 2015. These transgenic CAP-expressing rootstocks greenhouse and field testing was started in fall 2016. Some additional CAP constructs that will be tested here are aimed to address the concern that the protein components of the present CAP-1 have a non-plant origin. 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**

**Objective 1. Complete the efficacy of current round of *in planta* expressed chimeric NE-CB and PGIP proteins to inhibit and clear Xf infection in xylem tissue and through the graft union in grapevines grown under field conditions.**

This objective has two activities, the field evaluation of transgenic TS expressing either CAP or PGIP and to evaluate the best lines. The second to begin greenhouse- followed by field-testing of transgenic rootstocks in a commercially relevant background to identify lines that show resistance to PD.

**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 testing of the current round of plants in the field.

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**).

**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 5 |         |         |         |          |          |          |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| Row 6 | TS-50-G | TS-50-G | TS-50-G | 41-151-G | 41-151-G | 41-151-G | TS-G    | TS-G    | TS-G    | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G | 40-89-G |
| Row 7 | TS-50-G | 31-25-G | 40-92-G | 40-92-G  | 40-92-G  | 40-92-G  | TS-50-G | TS-50-G | TS-50-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G |
| Row 8 | TS-50-G | 52-08-G | 52-08-G | 52-08-G  | 52-08-G  | 52-08-G  | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G | 52-08-G |
| Row 9 | TS-50-G | 40-41-G | 40-41-G | 40-41-G  | 40-41-G  | 40-41-G  | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G | 40-41-G |

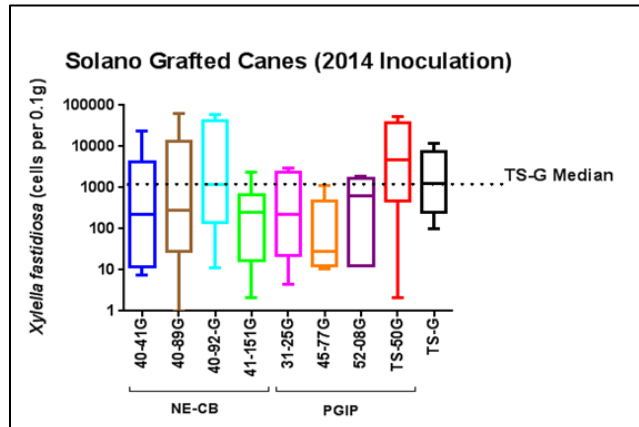
Grapevine inoculation with Xf (Temecula:Stag's leap mix, 60:40) at 250,000 per 20ul on 5/29/2012.  
 Grapevine inoculation with Xf (Temecula) 250,000 per 20ul on 6/17/2013.  
 Grapevine inoculation with Xf (Temecula:Stag's leap mix, 60:40) at 500,000 per 20ul on 5/27/2014.  
 Grapevine inoculation with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

North



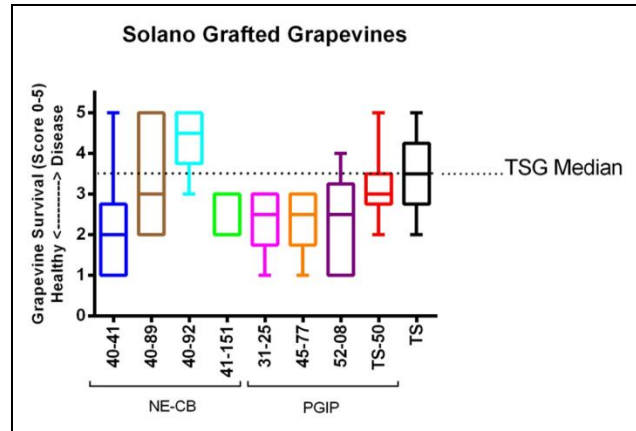
**Figure 1.** Solano County grafted transgenic grapevines inoculated in spring 2014 and spring 2015 (left, photo taken in fall 2016), terminated Solano field (right, photo taken in winter 2017).

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 at *Xf* counts that were lower than in grafted control grapevines (**Figure 2**).



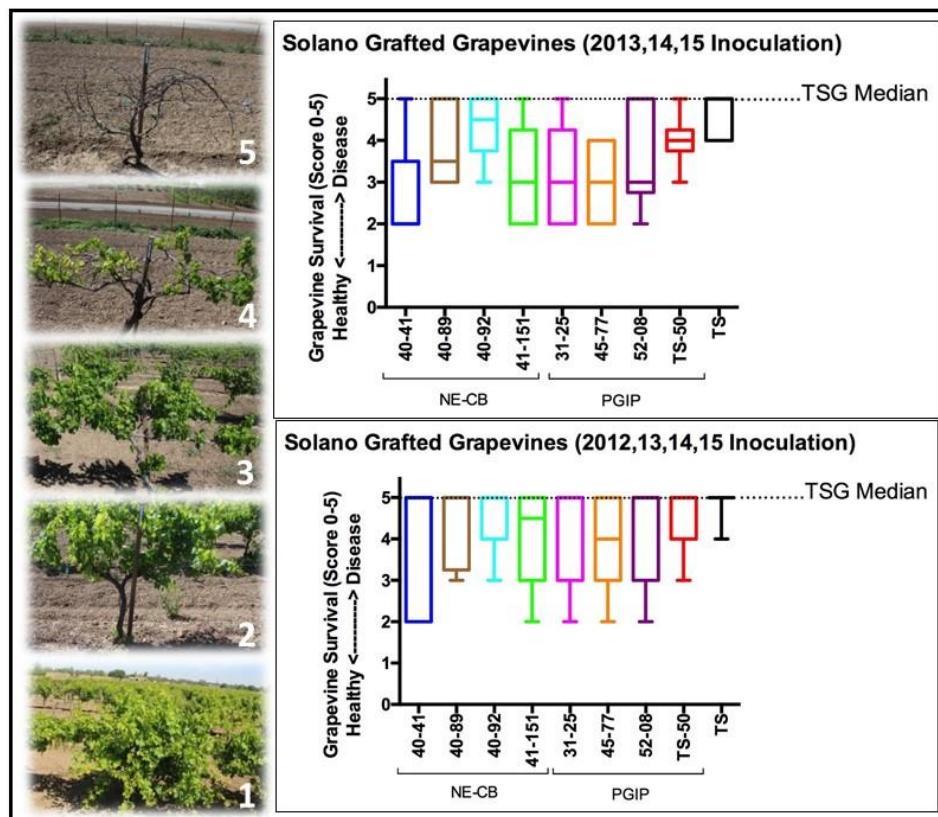
**Figure 2.** *Xf* quantification by qPCR of Solano grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014 and fall 2015.

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 (**Figure 3**).



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

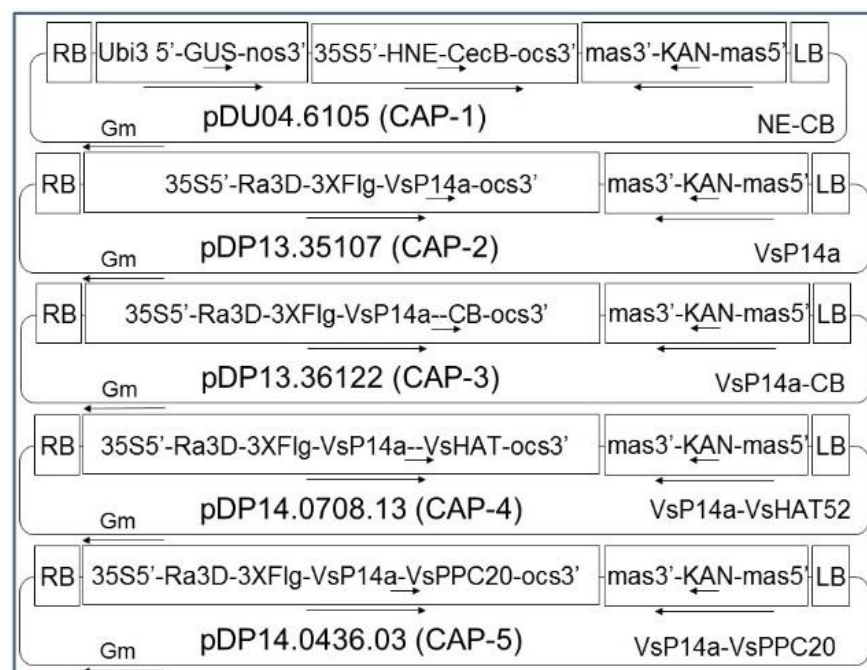
Grapevine survival of grafted transgenic grapevines that were inoculated in 2014/2015 was assessed on October 6, 2016, using a 1 to 5 score, where 1 = very healthy and vigorous grapevine; 2 = healthy grapevine and slightly reduced vigor; 3 = slightly reduced spring growth; 4 = much reduced spring growth and 5 = dead grapevine (**Figure 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 winter of 2017 (**Figure 1**).



**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 green house and field testing of five vector constructs that are in the plant transformation pipeline on two commercially relevant rootstocks, 101-14 and 1103 (Christensen, 2003). The components present in these constructs are shown in **Figure 5** below. The construction of CAP-1 was described earlier (Dandekar et al., 2012a) and the components mostly from grapevine and construction of CAP-2, CAP-3, CAP-4 and CAP-5 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 **Figure 5**. The transgenic plants obtained from the facility propagated for testing described in detail below. The transformation of the two rootstock species with all five CAP constructs was initiated in 2014 and the selection and regeneration of plants is ongoing.



**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.

Transformation of the first construct (CAP-1) yielded 30, 101-14 and three 1103 derived transgenic lines. The most progress was made in the analysis (described below) of these CAP-1 lines this summer as can be seen in **Table 2**. Since the yield for 1103 lined transformed with CAP-1 was low a new transformation was initiated back in Aug 2015. In addition, on summer 2016, we began receiving 110-14 and 1103 lines transformed with the other constructs (CAP-2 to 5) and the numbers and distribution of these lines is indicated in **Table 2**.

A propagation/testing pipeline has been successfully developed to test the efficacy of both 101-14 and 1103 grapevines and the transgenic lines will be tested for PD resistance in the greenhouse as they emerge from the transformation and after propagation. The testing of the 101-14 and 1103 transformed rootstocks transformed with CAP-1 has already been completed in the greenhouse and field testing of the promising lines was initiated in fall of 2016. The field introduction of these rootstocks is aimed at evaluating their efficacy in protecting grafted sensitive Chardonnay grapevine variety from developing PD.

**Table 2.** Progress on the analysis of transgenic lines obtained from different CAP constructs in two rootstock backgrounds

| CAP Designation | Binary Vector | Greenhouse propagation |      | Greenhouse Testing |      | Field Testing |      |
|-----------------|---------------|------------------------|------|--------------------|------|---------------|------|
|                 |               | 101-14                 | 1103 | 101-14             | 1103 | 101-14        | 1103 |
| CAP-1           | pDU04.6105    | 30                     | 4    | 30                 | 4    | 6             | 0    |
| CAP-2           | pDP13.35107   | 8                      | 2    |                    |      |               |      |
| CAP-3           | pDP13.36122   | 6                      | 1    |                    |      |               |      |
| CAP-4           | pDP14.0708.   | 10                     | 5    | 7                  |      |               |      |
| CAP-5           | pDP14.0436.03 | 8                      | 5    |                    |      |               |      |

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 33 CAP1 transgenic lines have been analyzed and six have been identified for field testing. All six were 110-14 transgenic. Of the six 110-14 transgenics 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 (**Figure 6**). All lines 1103 scored bad and received a score of 5. The six 101-14 transgenic rootstocks expressing CAP1 that scored a 1 or a 2 have been clonally propagated from the uninfected mother plants. Well-established 101-14 transgenic rootstocks plants were planted (**Figure 7**) in the Solano field on September 22, 2016 and will be grafted with the scion Chardonnay in the spring of 2017.



**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).



**Figure 7.** CAP1 transgenic rootstocks planted at Solano field in fall 2016.  
Photo taken in winter 2017.

Seven out of ten transgenic events expressing VsP14a-VsHat22 of the 101-14 background that screened PCR positive have been clonally propagated and are currently infected with *Xylella fastidiosa*. All other plants in the 101-14 background that have been confirmed PCR positive are in the cloning/growing/inoculating pipeline for inoculation with *Xf*. Plants of the 1103 background that have been confirmed PCR positive have been cloned and are in the growing stage prior to inoculation (**Figure 8**). Plants of each background continue to be produced at the transformation facility, as plants emerge they are propagated for greenhouse and field testing.



**Figure 8:** Transgenic 110-14 and 1103 lines expressing (CAP-2 to 5) are in the cloning/growing/inoculating pipeline for greenhouse inoculation with *Xf*. Photos taken in winter 2017.

**Publications produced and pending, and presentations made related to the funded project.**

Dandekar, A.M., A.M. Ibanez, and A. Jacobson. 2016. Field testing transgenic grapevine rootstocks expressing chimeric antimicrobial protein and polygalacturonase-inhibiting protein. Proceedings of Pierce's Disease Research Symposium held at December 12-14, 2016 at the Marriot Courtyard Hotel San Diego California. pp. 35-42.

Dandekar, A.M. 2016. Field testing transgenic grapevine rootstocks expressing chimeric antimicrobial protein and polygalacturonase-inhibiting protein. Oral presentation at the Pierce's Disease Research Symposium. December 13, 2016, San Diego California.

Dandekar, A.M., D. Gilchrist, P. Rolshausen, A.M. Ibanez, A. Jacobson D. Dolan, R. Just and H. Gouran. 2015. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2015. pp. 18-26.

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Dandekar, A.M., H. Gouran, A.M. Ibáñez, S.L. Uratsu, C.B. Agüero, S. McFarland, Y. Borhani, P.A. Feldstein, G. Bruening, R. Nascimento, L.R. Goulart, P.E. Pardington, A. Chaudhary, M. Norvell, E. Civerelo and G. Gupta. 2012. An engineered innate defense protects grapevines from Pierce's disease. Proc. Nat. Acad. Sci. USA 109: 3721-3725.

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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 are currently being 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 is being 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 this research. This 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. The technology to transform these two rootstocks developed in an earlier project is being implemented to develop transgenic 110-14 and 1103 rootstocks expressing different versions of the CAP protein. We have implemented a 2-cane PD disease screen to test these transgenic rootstocks. We evaluated 33 transgenic rootstock lines expressing CAP-1 and identified six good lines that we will test in the field for their ability to protect the sensitive scion cultivar Chardonnay from developing PD. Transgenic rootstocks lines expressing Cap2 to Cap5 are being developed and as they emerge will be tested in the greenhouse and field, a process that is currently ongoing. 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, 2016 to March 31, 2017.

#### **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.

#### **Literature cited.**

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