# FIELD TESTING TRANSGENIC GRAPEVINE ROOTSTOCKS EXPRESSING CHIMERIC ANTIMICROBIAL PROTEIN (CAP) AND POLYGALACTURONASE-INHIBITING PROTEIN (PGIP)


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## ABSTRACT

The goal of this study was to conclude the first field trial and to initiate a second field trial to evaluate the rootstock-based expression of chimeric antimicrobial proteins (CAP) and polygalacturonase inhibitory protein (PGIP) to provide transgraft protection of the scion grapevine variety against PD. In the first field trial Thompson Seedless rootstocks (TS) expressing four CAP and four PGIP grafted to wild type TS scions was challenged with four yearly infections with the PD pathogen. These transgenic rootstocks performed better than control-untransformed rootstocks in trans-graft protecting the scion from developing and succumbing to PD. Since TS is not a rootstock this approach needed to be validated in a commercially relevant rootstock. Two commercially relevant rootstocks 101-14 and 1103 were successfully transformed and transgenic grapevine plants obtained for six different versions of CAP proteins: CAP-1, 2 the original NE-CB and an enhanced version of the same. CAP-3, 4 that expresses VsP14a, a plant version of the NE protein present in CAP-1. CAP-5, 6 proteins replace the CB (cecropin B) with plant versions HAT52 and PPC20 respectively in CAP-5 and CAP-6. These transgenic CAP-expressing rootstocks were tested in the greenhouse starting in fall 2016. The additional all of the CAP expressing transgenics that looked promising in the greenhouse evaluations were propagated for field introduction in 2018 to further evaluate different lines to identify those with good efficacy in protecting grafted, sensitive scion cultivar Chardonnay from developing PD.


## LAYPERSON SUMMARY

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 2016 and field evaluations began in 2018. Elite rootstock lines identified in this project will be good candidates for commercialization.

## 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 PD. Rootstocks (Thompson Seedless, TS) expressing CAP or PGIP proteins have been evaluated in the field, this part of the study was concluded in 2018. 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) and an enhanced version of the original (35s OM/RAMY/Flag CAP-2; Dandekar et al., 2012c; 2013; 2014) four additional CAP constructs were developed that contained VsP14a (CAP-3); VsP14a-CB (CAP-4); VsP14a-HAT52 (CAP-5), VsP14a-PPC20(CAP-6). These additional CAP constructs were developed to address the concern that the protein components of the present CAP-1 have a non-plant origin. Transformation of these 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.

## OBJECTIVES

**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.

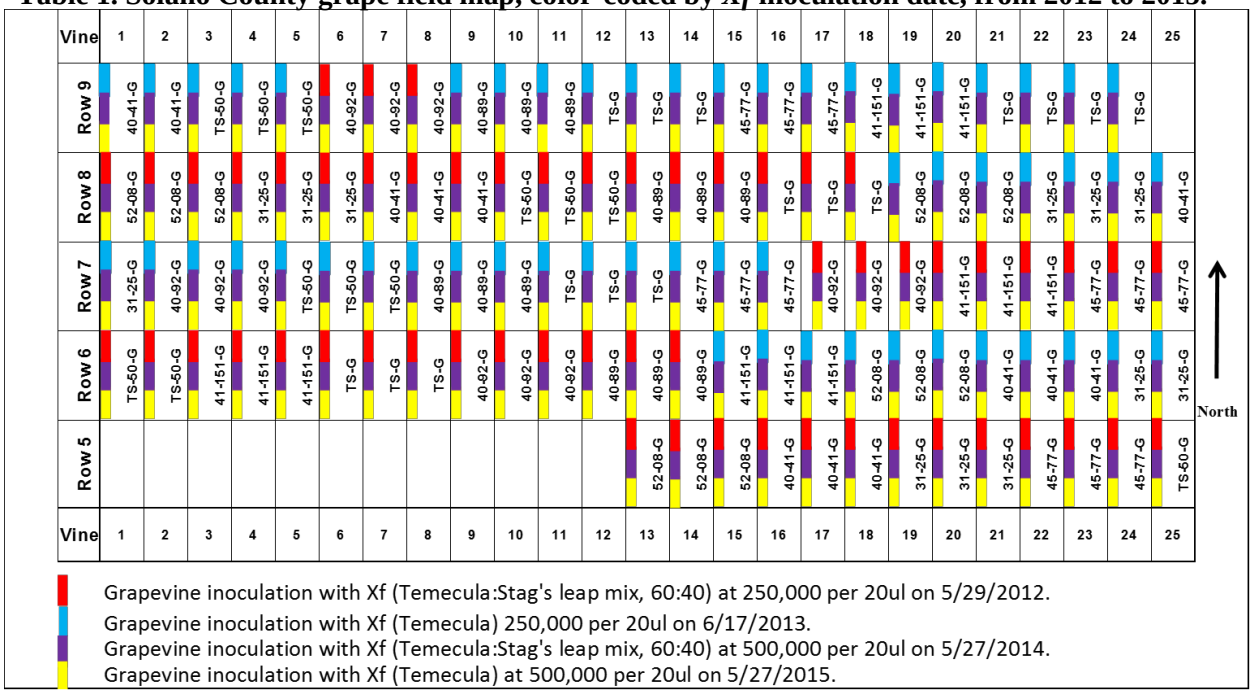
## RESULTS AND DISCUSSION

**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.**

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 2012 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.**



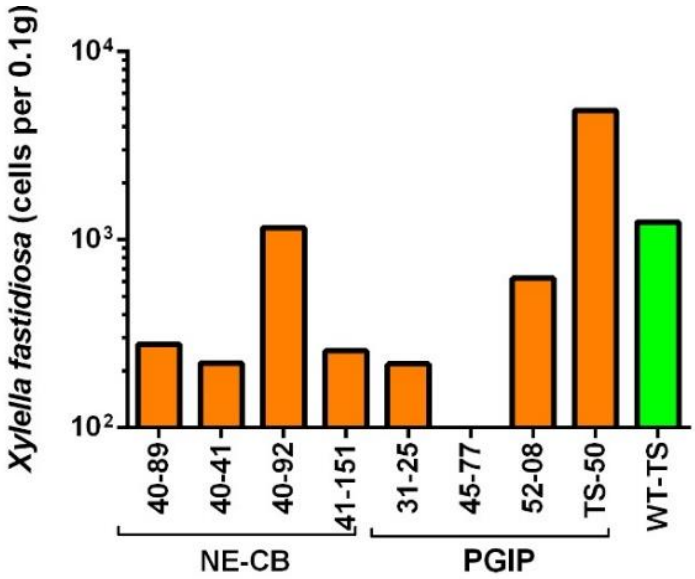



**Figure 1. *Xf* quantification by qPCR of Solano grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014 and fall 2015.**

**X*f* quantification by qPCR of inoculated grafted individual transgenic canes.**
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 (**Figure 1; see above**).

**Grapevine Survival after sequential years of *Xylella fastidiosa* inoculation**
Grapevine survival of wild-type Thompson Seedless scions that were grafted to protective transgenic Thompson Seedless rootstocks and inoculated with *Xylella fastidiosa* in 2013, 2014, and 2015 was assessed in the Fall of both 2015 and 2016 using a score of 1 to 5, where: 1 = Healthy vigorous grapevine; 2 = Slightly reduced growth. Symptoms on inoculated canes; 3 = Reduced growth. Symptoms on both inoculated and uninoculated canes; 4 = Highly symptomatic, ends of canes dying back and 5 = Dead plant. Shown in Figure 2 below is the assessment in the fall of 2015 , the transgenic lines expressing either CAP and PGIP were able to trans graft protect TS scions from disease development. By the fall of 2016 all of the TS were dead while the transgenics survived as shown in the Figure below.

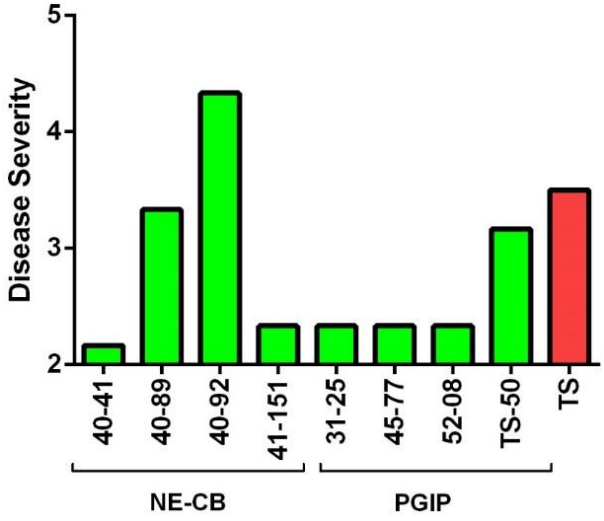


**Figure 2. Severity or absence of PD symptoms for all Solano grafted inoculated grapevines on fall 2015.**

**Spring Budbreak Evaluation**

During the course of the field trial, it was observed that buds on infected canes would fail to push after dormancy and a large portion of the buds that did break would dry up and die soon after. In the spring of 2015, this observation was measured by counting every bud on previously infected canes and monitored to see if it succeeded in developing into a new cane. Both transgenic and wild type plants were evaluated. Transgenic plants had a much higher success rate of buds developing into new canes than did wild-type Thompson seedless (**Figure 3**) had a low level of bud break much lower than that observed for the transgenic lines.

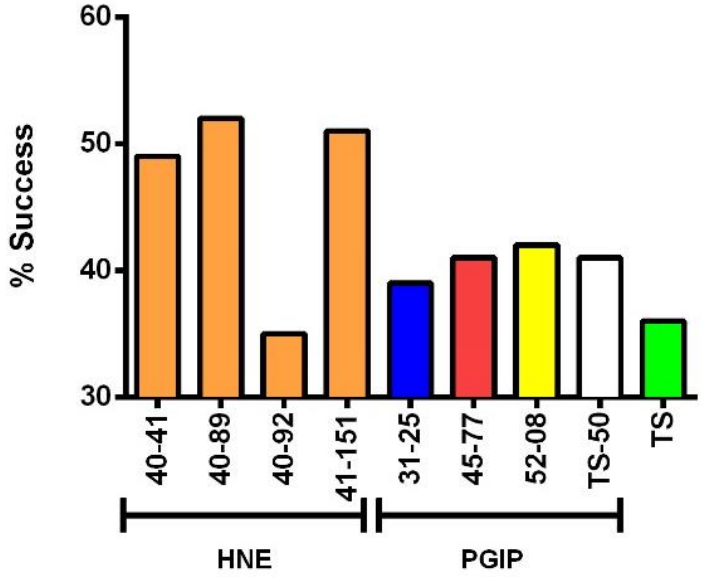



**Figure 3. Comparative analysis of spring bud break in control compared to transgenic lines inoculated with *Xf* in spring 2015.**

**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.**

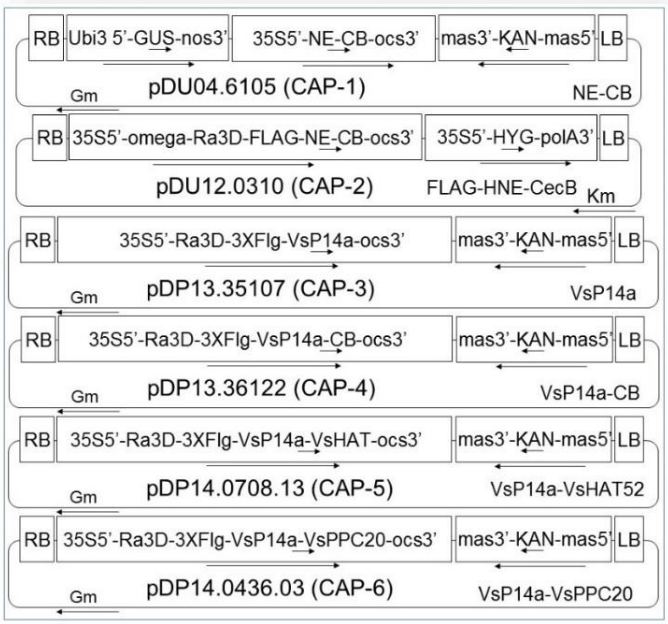


**Figure 4. CAP vectors designed to test the original and grapevine CAP components, and used to create transgenic 101-14 and 1103 rootstocks that will be tested in greenhouse and field.**

**Activity 2. Conduct greenhouse and field evaluation of CAP-expressing 101-14 and 1103 rootstocks.** 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 on two commercially relevant rootstocks, 101-14 and 1103 (Christensen, 2003).

The components present in these constructs are shown above in **Figure 4**. 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, CAP-5 and CAP-6 shown in **Figure 4** have been previously described (Chakraborty et al., 2014b, Dandekar et al.,2012c; Dandekar et al., 2013 and Dandekar et al., 2014). 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 4**. The transgenic plants obtained from the facility propagated for testing described in detail below. The transformation of the two rootstock species with all six CAP constructs was initiated in 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. Table 2 indicates the number of lines that are slated for field introduction in 2018.

**Table 2. Pierce disease resistance CAP-expressing transgenic rootstocks advancing to field trial.**

| CAP Designation | Binary Vector | Gene | Field Test (Lines) | |
|---|---|---|---|---|
| | | | 101-14 | 1103 |
| CAP-1 | pDU04.6105 | HNE-CB | 6 | 0 |
| CAP-2 | pDU12.0310 | New HNE-CB | 0 | 8 |
| CAP-3 | pDP13.35107 | VsP14a | 8 | 0 |
| CAP-4 | pDp13.36122 | VsP14a-CB | 2 | 0 |
| CAP-5 | pDP14.0708.13 | VsP14a-VsHat52 | 1 | 3 |
| CAP-6 | pDP14.0436.03 | VsP14a-PPC20 | 7 | 4 |

To determine the best potential candidates for challenging in the new field trial, a series of tests were performed. Each transformed plantlet received from the UC Davis Plant Transformation Facility is initially screened by PCR to confirm the presence of the transgene (**Figure 5**: see below).

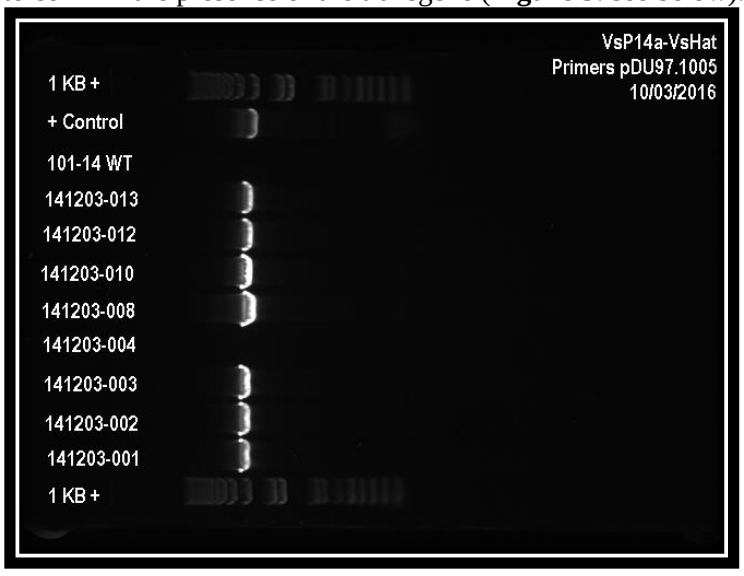



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**Figure 5. Validation of the transgenic 101-14 and 1103 grapevine rootstocks for the expression of CAP constructs. Shown here is the amplification and detection of the CAP gene sequences.**

Next, performed is a protein extraction on each plant that has been confirmed to have the transgene via PCR and two SDS gels are run to observe the total protein and the level of protein being expressed by the transgene. Shown below (**Figure 6**) on the left is an SDS gel of the total proteins isolated from CAP-6 expressing lines and on the right is a western blot generated using an antibody for the detection of the CAP-6 lines which show a single reactive protein corresponding to CAP-6.

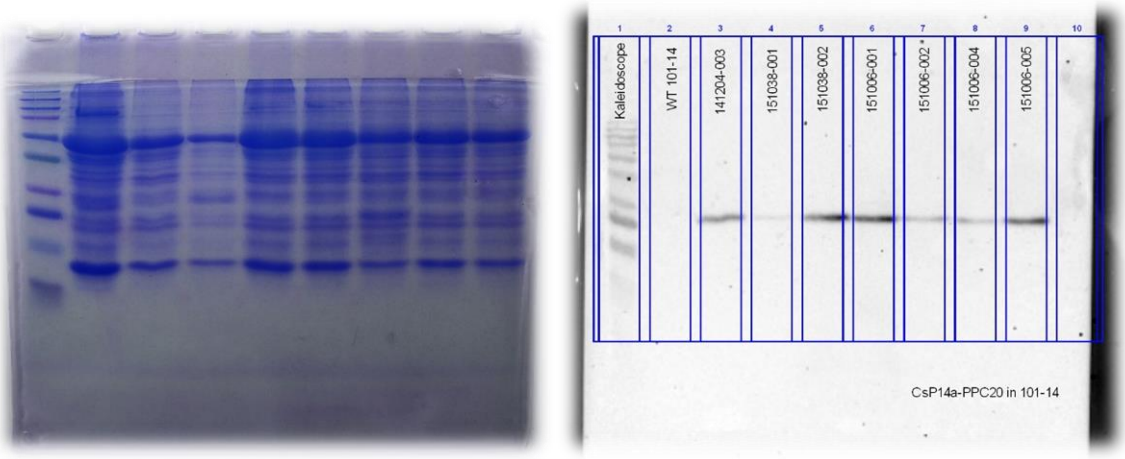


**Figure 6. Validation of the transgenic 101-14 and 1103 grapevine rootstocks for the expression of CAP constructs containing grapevine components. Shown here is the detection of the VsP14a protein present CAP transgenic lines.**

**Greenhouse Screening of Next Generation Plants (Activity 2 Cont.).**
As plants are received from the UC Davis Plant Transformation Facility and undergo laboratory testing, they also undergo a disease screening in the greenhouse. Once a plant is received from the Transformation Facility it is allowed to grow in the greenhouse until it reaches a size where it can be clonally propagated to produce enough replicates for a disease screening by manual inoculation of the bacterium *Xylella fastidiosa* (**Figure 7**).


**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*.**

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). This scoring method worked for observing the spread of PD symptoms but did not account for the severity of symptoms so a new more detailed scoring system was 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 with values of: 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 (**Figure 8).** Pierce's disease symptoms were scored using the detailed scoring system. Results of the screening process of CAP-5 plants in the 101-14 background is shown in **Figure 9.**

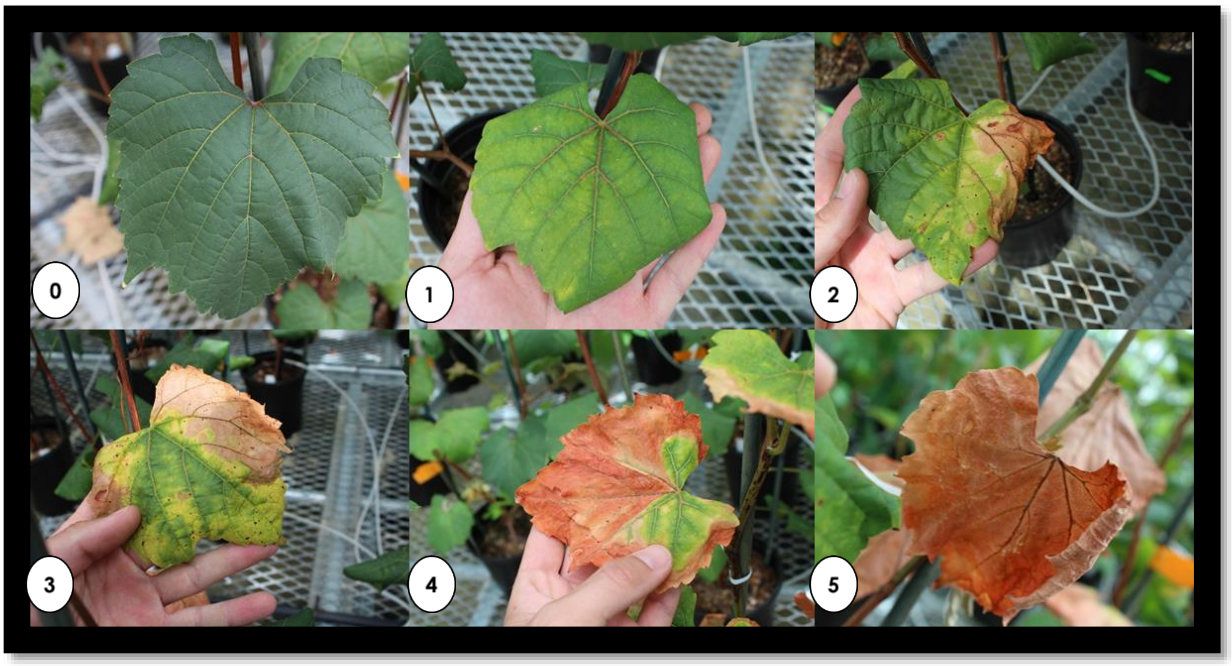


**Figure 8: Development of a modified 1-5 disease scoring system.**

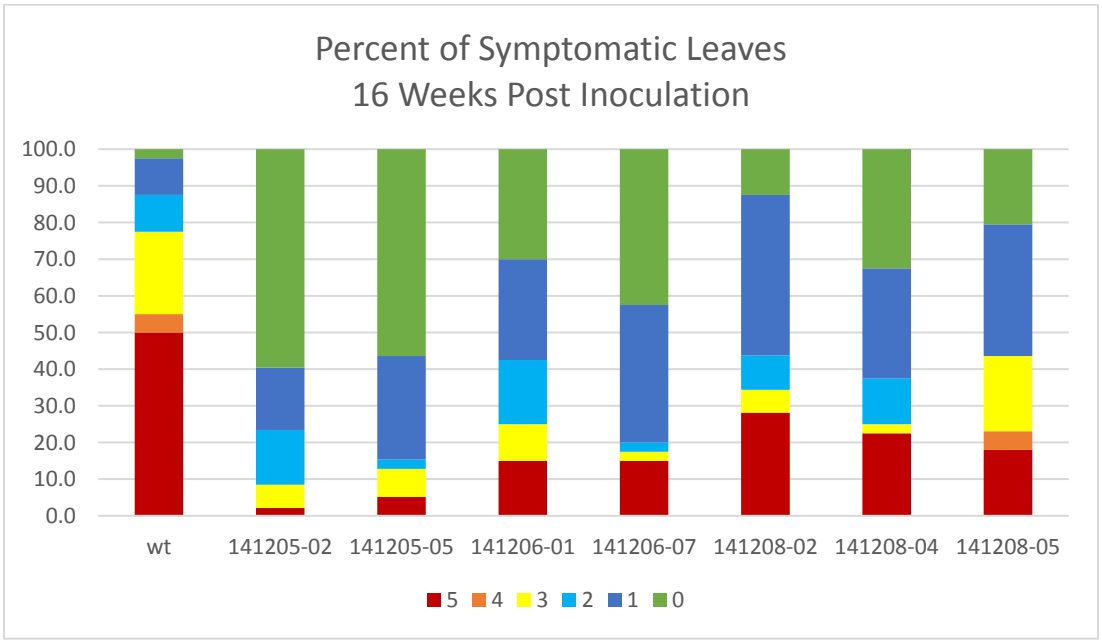

**Figure 9: The last data point collected while screening 1103 transgenic rootstocks expressing either CAP-5 or CAP-6.**

**Preparation of the New Field Trial.**
All proper permitting has been acquired from the regulatory agencies for the new field trial. Field irrigation and trellising were established in 2018. The first and second planting was done in the Summer and Fall 2018, respectively.

**Table 3 details the number of lines per construct that were introduced into the field on 2018, with additional plants continuing to work their way through the pipeline for this year's planting.**.

| Pedigree | Gene | CAP# | # of Lines | Scion Type | Replicates per Line |
|---|---|---|---|---|---|
| 101-14 | OldHNE | 1 | 6 | Char04 | 6 |
| 1103 | New HNE | 2 | 8 | Char04 | 6 |
| 101-14 | VsP14a | 3 | 8 | Char04 | 6 |
| 101-14 | VsP14a-CB | 4 | 2 | Char04 | 6 |
| 101-14 | VsP14a-VsHAT | 5 | 1 | Char04 | 6 |
| 1103 | VsP14a-VsHAT | 5 | 3 | Char04 | 6 |
| 101-14 | VsP14a-PPC20 | 6 | 7 | Char04 | 6 |
| 1103 | VsP14a-PPC20 | 6 | 4 | Char04 | 6 |
| TS | ntPGIP | | 4 | Char04 | 6 |
| TS | chiPGIP | | 4 | Char04 | 6 |
| TS | mPGIP | | 4 | Char04 | 6 |
| TS | ramyPGIP | | 4 | Char04 | 6 |
| TS | xspPGIP | | 4 | Char04 | 6 |

Plants to be introduced to the field that have successfully made it through our testing pipeline. All plants will be grafted with commercially relevant and PD sensitive scion Chardonnay 04.

**Shown above in Figure 10. 1) Sticks collected during dormancy. 2) Individual stick that has been de-budded, tagged, dipped in wax ready for rooting hormone and rooting. 3) Sticks rooting. 4) Sticks rooted and breaking dormancy. 5) Rooted clone. 6) Cones potted up awaiting grafting before being planted in field.**

## CONCLUSIONS

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 2016 and field evaluations began in 2018. Elite rootstock lines identified in this project will be good candidates for commercialization.

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