

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

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ABSTRACT

This research is a continuation of the field evaluation of chimeric anti-microbial protein (CAP; Dandekar et al., 2012a) and polygalacturonase inhibitory protein (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 in 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, validated with in-field inoculations. The lines expressing CAP showed the highest efficacy in protecting grafted transgenic grapevines from developing PD. The ongoing testing involves evaluating novel CAP lines in commercially relevant rootstocks 101-14 and 1103 (Christensen, 2003). 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 (OM/RAMY/Flag CAP; CAP-2; Dandekar et al., 2012c; 2013; 2014) four additional CAP constructs included in the current round of testing are VsP14a (CAP-3); VsP14a-CB (CAP-4); VsP14a-HAT52 (CAP-5) and VsP14a-PPC20 (CAP-6). Transformation of these six CAP constructs into the 101-14 and 1103 rootstock backgrounds was initiated in 2015, greenhouse testing was started in fall 2016 with field introductions planned for summer of 2018. 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.

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 2018 and field evaluations will begin in spring of 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

these proteins individually are currently being evaluated in the field, this part of the study was concluded this year. 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 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 1. Complete and conclude testing of the current round of plants in the field

Activity 2. Conduct greenhouse and field evaluation of CAP-expressing 110-14 and 1103 rootstocks.

RESULTS AND DISCUSSION

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**).



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																									
Row 8																									
Row 7																									
Row 6																									
Row 5																									
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

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

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 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 (**Figure 3**).

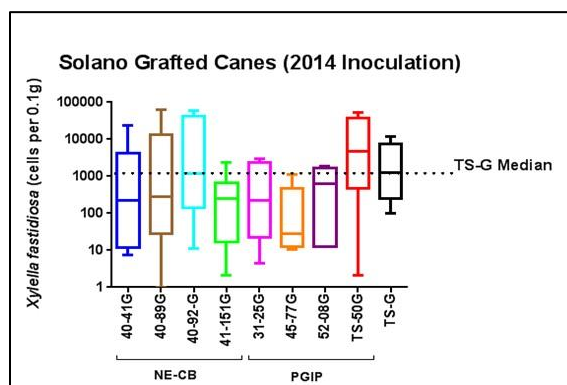


Figure 2. *Xf* quantification by qPCR of Solano grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014 and fall 2015.

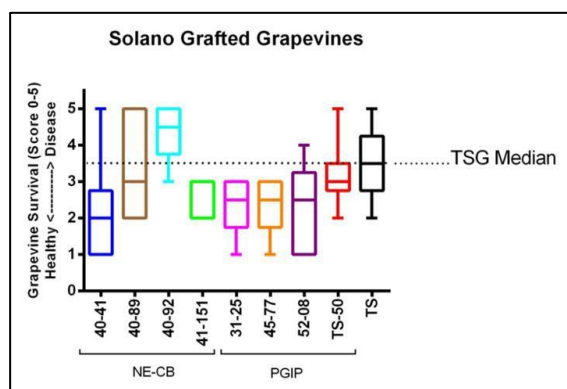


Figure 3. Severity or absence of PD symptoms for all Solano grafted inoculated grapevines on fall 2015.

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 (**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 summer of 2017.

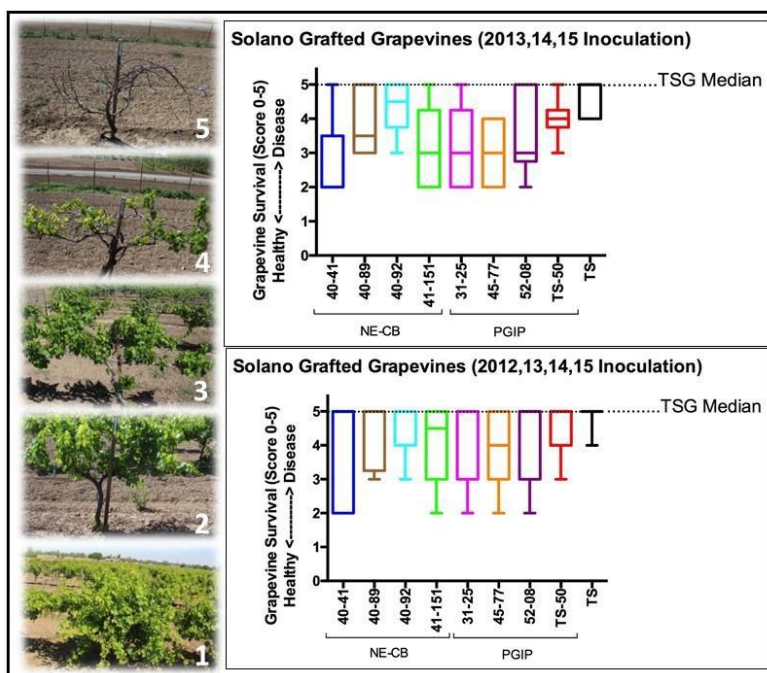


Figure 4. Grapevine survival of Solano grafted transgenic grapevine inoculated in 2013-2015 (upper right) and all inoculated grafted transgenic grapevines (lower right), scored in fall 2016 using a scale of 1 to 5 (left).

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 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), CAP-2 is an enhanced version of CAP-1 and CAP-3, CAP-4, CAP-5, CAP-6 are modification of the original CAP that contain grapevine derived components shown in **Figure 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 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.

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, on 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**.

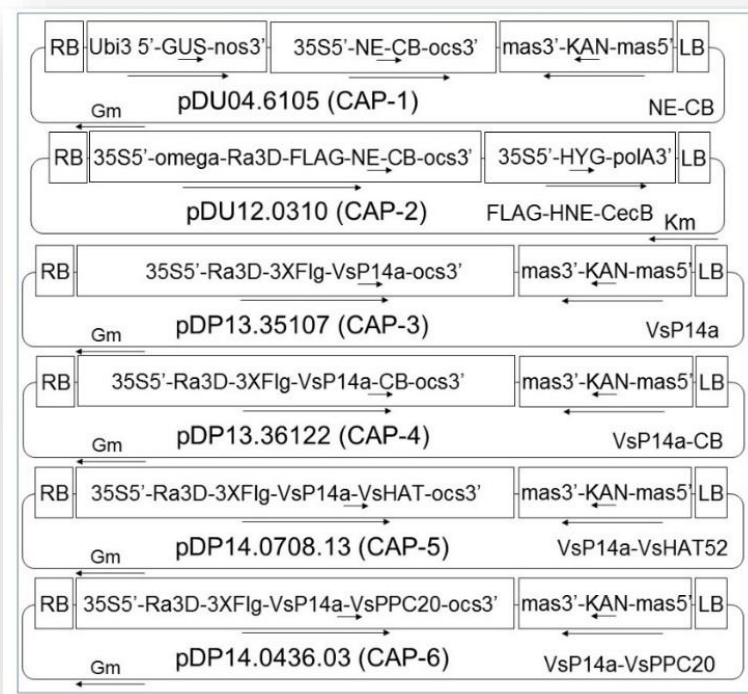


Figure 5. CAP vectors testing of the original and grapevine components, used to create transgenic 101-14 and 1103 rootstocks that will be verified in greenhouse and field.

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

CAP	Binary Vector	Transgenic Plants Received		Greenhouse Testing(In Progress)		Advancing For Field Testing	
Designation		101-14	1103	101-14	1103	101-14	1103
CAP-1	pDU04.6105	30	4	30	4	6	0
CAP-2	pDU12.0310		10		10	0	8
CAP-3	pDP13.35107	20	3	8 (12)		8	0
CAP-4	pDP13.36122	20	1	6 (14)		2	0
CAP-5	pDP14.0708.13	11	6	10	(3)	1	3
CAP-6	pDP14.0436.03	8	6	8		7	4

A successful propagation/testing pipeline was developed to test the efficacy of both 101-14 and 1103 grapevines and the transgenic lines for PD resistance in the greenhouse. The 101-14 and 1103 transgenic rootstocks lines were validated for the presence of CAP transgenes using PCR. Those 101-14 and 1103 plants that were PCR-positive were clonally propagated to conduct greenhouse testing to determine resistance to PD. All of the clonally propagated transgenic lines were trained in a two-cane system and inoculated on one of the canes with *Xf*. Plants were inoculated with 20uL of *Xf* containing 20 million cells at roughly three nodes above the fork in the canes and eight leaves below the top of the cane. Then the plant was turned over and inoculated with another 20 million cells

(20uL) of *Xf* directly behind the first inoculation. The *Xf* inoculum was prepared as described earlier (Dandekar et al., 2012a).

The transgenic rootstocks successfully inoculated as described above were evaluated for PD symptoms 12 weeks post inoculation when the first disease symptoms appeared 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 were analyzed and six showed good resistance and propagated 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 (**Figure 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-4 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*. (**Figure 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 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 disease symptoms for the CAP-4 plants in the 101-14 background were scored using the detailed score system. Result of the screening process of CAP-5 and CAP-6 plants in the 1103 background is shown in **Figure 9**.



Figure 8. Pierce'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.

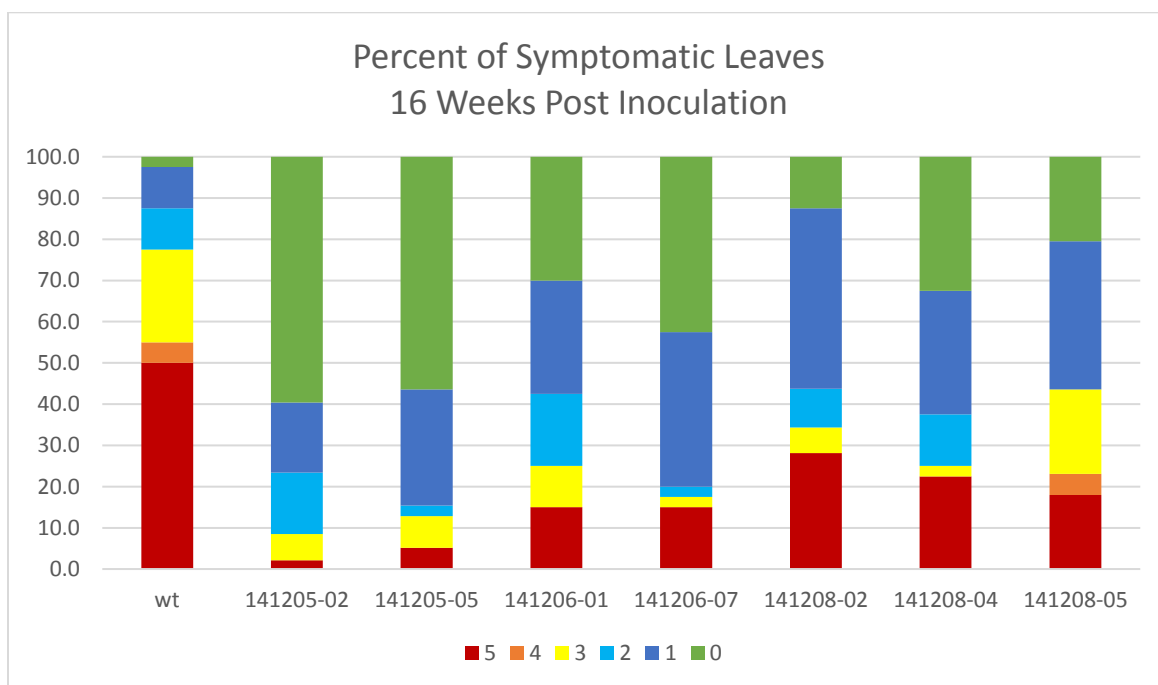


Figure 9. Last data point collected while screening the 101-14 transgenic rootstocks expressing CAP-4. Plants are scored weekly after the Pierce's Disease symptoms begin to show.

CONCLUSIONS

We have successfully concluded field-testing of TS as a rootstock expressing CAP or PGIP. Grapevine survival of grafted transgenic grapevines inoculated between 2012 and 2015 was assessed and survival rate of most grafted inoculated transgenic TS lines using both strategies was greater than in untransformed controls, with the CAP lines most efficient in protecting against PD. The phenotypic disease data corresponded to the bacterial titer estimations using qPCR revealed lower bacterial titers in transgenic plants as compared to the wild type

susceptible TS plants. Severity or absence of PD symptoms on all Solano County grafted transgenic grapevines inoculated between 2012 and 2015 was also assessed and PD disease symptom severity scores were lower in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls. The field-testing data confirm that TS rootstock lines expressing either CAP or PGIP are able to provide protection against PD. We have developed a successful propagation and two cane testing pipeline to evaluate 62 101-14 and 14 1103 transgenic rootstocks expressing various CAP constructs. Field testing will be initiated in fall of 2018.

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