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Project Title: *In Planta* Testing of Signal peptides and Anti-Microbial Proteins for Rapid Clearance of *Xylella*.

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ABSTRACT

Xylella fastidiosa (X. fastidiosa), a Gram-negative bacterium, is the causative agent of Pierces's disease. Because *X. fastidiosa* is xylem-limited, any potential anti-*X. fastidiosa* gene product must be present in xylem at an effective concentration to provide disease control. Understanding how existing proteins are transported to xylem is necessary to target delivery of therapeutic proteins to this organ. We collected xylem exudate from *Vitis vinifera* cv. Chardonnay and analyzed its protein composition by two-dimensional gel electrophoresis, then purified and sequenced some of the abundant proteins to identify corresponding genes in the grapevine EST database. We identified the signal sequences present in these gene sequences and made vectors where these signals were fused to mature polygalcturonase inhibiting protein (mPGIP), which is secretion competent. Five different vectors were successfully constructed to test four signal sequences. These vectors were incorporated into *Agrobacterium* and used to transform grapevine. Callus and embryos were successfully selected and regenerated to give transformed grapevine lines for each construct. Plants have been obtained for all five constructs and protein inhibiting activity for five constructs has been tested. Based on PGIP activity 8 of 10 mPGIP, 2 of 5 Ramy, 3 of 11 XPS, 8 of 11 ChiPGIP and 6 of 10 NtPGIP in vitro lines have been transferred to the greenhouse and acclimated. Ten PGIP transgenic lines (4 NtPGIP and 6 ChiPGIP) have been manually inoculated with *X. fastidiosa* and are in early stages of evaluation for tolerance against Pierce's Disease. The remainder mPGIP, XPS and Ramy lines are in the process of acclimating and growing enough tissue to micropropagate.

Initial grafting experiments showed that Chi signal peptide mobilized PGIP from the Chi-PGIP transgenic plant used as rootstock to the TS wild plant used as scion. Next we will validate that other signal peptides are essential and sufficient to mobilize proteins into grapevine xylem. Such transgenic proteins, if synthesized in a rootstock, could confer resistance to xylem-specific infections such as Pierce's Disease and assist in control of *X. fastidiosa*.

As an alternative to various signal peptides fused to PGIP we designed a chimeric anti-microbial protein with two functional domains. One domain (the surface recognition domain, SRD) specifically binds to the bacterium outer-membrane and the other domain lyses the membrane and kills *X. fastidiosa*. In this chimera, human neutrophil elastase (HNE) is the SRD that recognizes MopB, the major outer membrane protein of *X. fastidiosa*. The second domain is cecropin B (CECB), a lytic peptide that targets and lyses gram-negative bacterial membranes. We have combined HNE and CECB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized in two versions, one with a mammalian signal peptide sequence designated HNE-CECB and the other with a signal peptide sequence from a plant *pgip* gene designated pgip-HNE-CECB. Both were individually incorporated into binary vectors, and transformed into grapevine (*Vitis vinifera* var 'Thompson Seedless') and SR1 tobacco using *Agrobacterium.* Plant transformation experiments with both HNE-CECB and pgip-HNE-CECB were successful and plants have been obtained.

Using PCR, the presence of HNE-CECB or pgipHNE-CECB was confirmed in 36 and 7 plants, respectively. 36 HNE-Cec and 7 pgip HNE-Cec *in vitro* lines have been transferred to the greenhouse and are in the process of acclimating and growing enough tissue to micropropagate for *Xylella* infection experiment. 16 of 36 HNE-CECB have been manually inoculated with *X. fastidiosa* and are in different stages of evaluation for tolerance against Pierce's Disease. Observation from first round which contains 5 HNE-CECB lines is very promising since lines HNE-CECB 41-151 and 40-41 showed 40% more resistance when compared with wild type TS control.

OBJECTIVES

1. Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with *X. fastidiosa*.

2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*X. fastidiosa* activity *in planta,* and test for graft transmissibility.

RESULTS AND DISCUSSION

1. <u>Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with Xf</u>:

In previous research, peptide spectrum and BLAST analysis showed that the proteins found in grape xylem exudates are secreted and share function similarities with proteins found in xylem exudates of other species (Buhtz et al. 2004). cDNA sequences matching 2 of them found in the TGI *Vitis vinifera* gene index (http:compbio.dfci.harvard.edu/tgi/plant.html) were used to design primers that were used to amplify the predicted fragments from genomic DNA of 'Chardonnay' and 'Cabernet Sauvignon' (Aguero et al 2008). These fragments were annotated as Chi1b and NtPRp27. These fragments were then fused to DNA sequences that contained the mature polygalacturonase inhibiting protein (mPGIP) gene through gene splicing using a PCR-based overlap extension method (SOE) (Horton et al. 1990) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). These two chimeric genes were then ligated into a plant expression vector containing the 35S cauliflower mosaic virus promoter and the octopine synthase terminator and the resultant expression cassettes were then ligated into the binary vector pDU99.2215

(Escobar, et al. 2001) which contains an *nptII*-selectable marker gene and a *uidA* (β-glucuronidase, GUS) scorable marker gene. The mature PGIP sequences without any signal peptide sequences was also incorporated into pDU99.2215 to serve as a control and this vector is designated pDU05.1002. This mPGIP should be immobile although PGIP with its native signal peptide is secretion competent in grape. We also fused mature PGIP to the signal peptides from the xylem sap protein XSP30 and the rice amylase protein Ramy3D, which has been very effective in secretion of human α_1-antitrypsin in rice cell cultures (Trexler et al. 2002). These binary vectors are designated pDA05.XSP and pDU05.0401, respectively.

As a result of the above transformation plants have been obtained from the Parsons Plant Transformation Facility (Davis, CA) for all 5 vectors described above Plants from the all binary vectors have been analyzed using PCR (Table 1). DNA was isolated from *in vitro* leaves using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Primers for detection of PGIP were 5' Mature PGIP: 5' ATGGATCTCTGCAACCCCGACGAC and 3'PGIP: 5' TTACTTGCAGCTTGGGAGTG.

Tissues from the in vitro plants have been tested for PGIP activity using the zone inhibition assay with PG (Table 1) (Taylor and Secor 1988). PG preparations were obtained from *Botrytis cinerea* strain Del 11 isolated from grape (Aguero et al. 2005). Protein from leaf tissue (~100mg) was extracted in extraction buffer (Dandekar, et al. 1998) at a ratio of 1 ul/mg. Tissue was ground in a 2 ml microfuge tube containing a 5mm stainless steel bead in a TissueLyzer (Qiagen). The homogenate was centrifuged at 16000 g for 5 minutes and the supernatant was used for testing.

Table 1: Current status of testing transgenic lines *Vitis vinifera* var Thompson Seedlees grapevines with different signal peptides and PGIP gene

No.	Signal peptide	Binary Vector	Plant Lines	(+) PCR for PGIP	(+) PGIP Activity	Lines grafted	Moved to Greenhouse
1	None	pDU05.1002	12	10	9	1	8
2	Ramy	pDU05.0401	5	5	4	0	2
3	XSP	pDA05.XSP	11	11	5	0	3
4	Chi	pDU06.0201	11	11	10	1	8
5	Nt	pDU05.1910	10	10	5	1	6

The 12 mPGIP, 5 Ramy, 11 XSP, 11 ChiPGIP and 10 NtPGIP, plants assayed for polygalacturonase inhibiting activity had a range of inhibition from 0-22%, 0-44%, 0-28%, 0-57 % and 0-45 %, respectively. The ChiPGIP plants had a greater number of lines with strong inhibition than the other lines and all lines assayed showed polygalacturonase inhibiting activity. Also, compared to ChiPGIP there were more lines, 3 Ramy, 5 NtPGIP vs 1 ChiPGIP, which had no inhibition activity. Based on PGIP activity 8 mPGIP lines with none (expected) to medium, 2 Ramy with strong, 3 XPS with medium, 6 ChiPGIP with medium to strong and 8 NtPGIP with medium to strong PGIP activity have been transferred to the greenhouse and acclimated (Fig1 A, B).

Each acclimated transgenic line was propagated to obtain 4-6 plants (Fig 1C) to be used as mother plants for further propagation to provide cuttings for *Xylella* infection and grafting experiments. *Xylella* infection will consist of inoculation by hand (Almeida and Purcell 2003a) and later by insect (Almedia and Purcell 2003b). Both will be evaluated for symptoms of Pierce's disease (PD) after 3 months. Since we found in earlier research that pPGIP with its endogenous signal peptide is xylem competent we are using TS50, a grapevine successfully transformed with this construct and highly expressed as a positive control in the inoculation experiments (Aguero et al. 2005).

From each line, 25-35 plants are propagated (from cuttings) at the same time (Fig 1D-F). *Xylella infection* experiments are done in multiple rounds. Each round consists of 5-6 transgenic lines and 2

controls (TS –Thompson Seedless- wild type as negative control and TS50 as positive control). Each round of experiments includes 30 plants from each line, 15 inoculated and 15 non-inoculated controls.

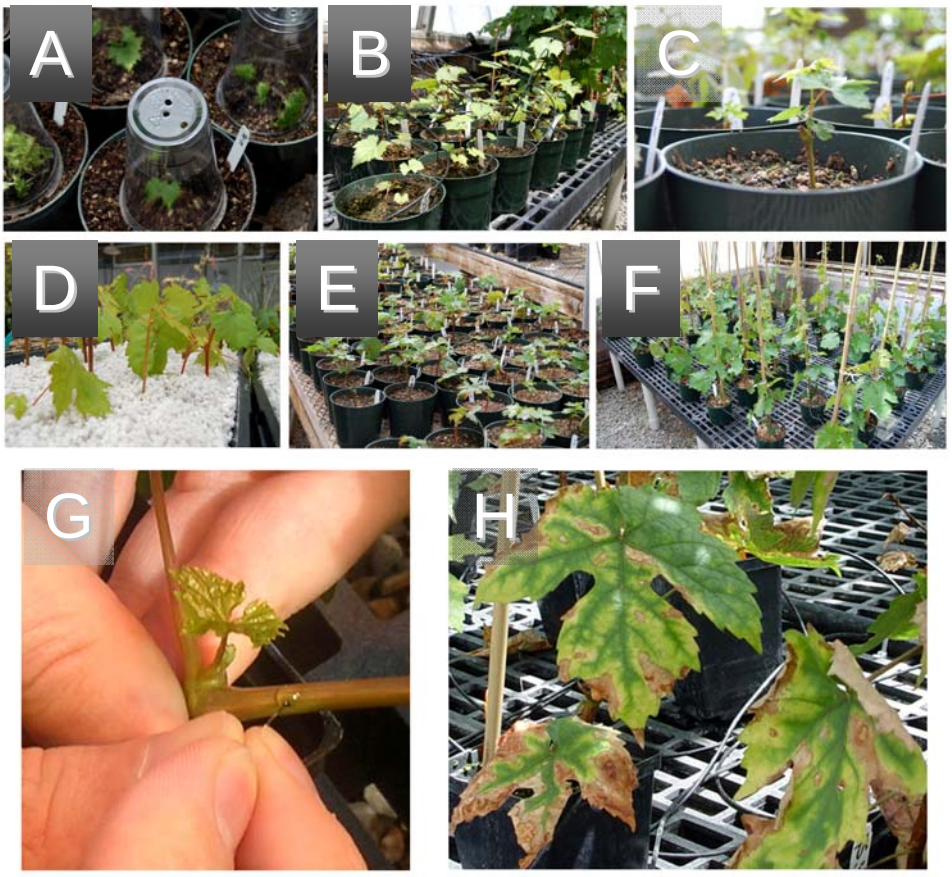

Figure 1. Grape plants acclimation to greenhouse to *Xf* infection time line A) Acclimating in vitro plants in the GH (3-4 weeks). B) Generating initial mother plants (6 weeks). C) Generating sufficient mother plants for propagation (7 weeks). D) Propagating 180-210 plants for each round of experiment (3 weeks). E) Transferring rooted plants to soil. F) Plants ready for inoculation after 8-10 weeks. G) Inoculation. H) First symptoms after inoculation (6-7 weeks).

Transgenic TS, wild type TS (negative control) and TS50 (positive control) plants are inoculated with 20µl of the GFP expressing *X. fastidiosa* (Newman et al., 2003, 3A2). The plants are inoculated with 10 µl the first day and re-inoculated with 10µl the second day, for each inoculation an independently grown *Xylella* culture was used. The *Xylella* is introduced to each plant approximately 3-4 inches above the soil using an insect pin number zero as shown in the Fig. 1G. Plants are pruned regularly and kept approximately 90-100cm tall until *Xylella* symptoms appears. The time required for each round of *Xylella* infection experiment go from 33 to 37 weeks, starting from in vitro plants transferred to greenhouse until first *Xylela* infection symptoms appears (Fig. 2).

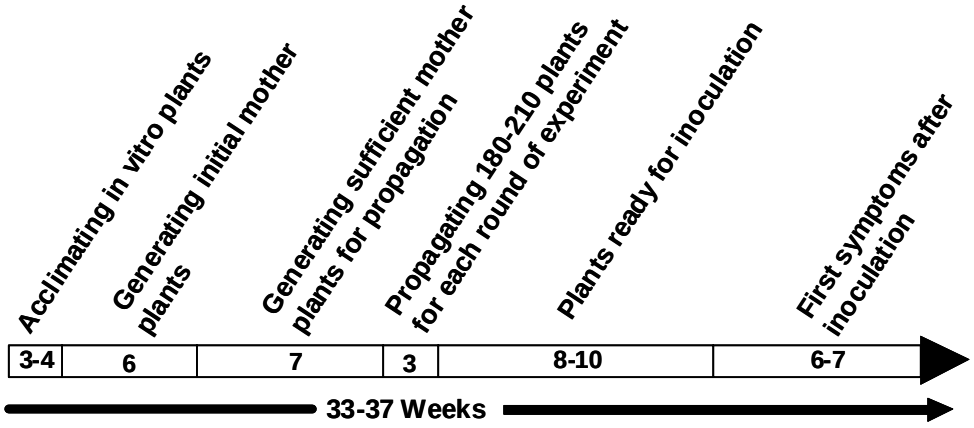

Figure. 2 *Xylella* Infection Experiment Timeline (Weeks)

Ten PGIP transgenic lines (4 NtPGIP and 6 ChiPGIP) are in early stages of evaluation for tolerance against Pierce's Disease. *Xylella infection* experiments are done in multiple rounds, in each round 5-6 transgenic plants, TS50 (as positive control) and wild type TS (as negative control) are included. Round 3 includes 4 NtPGIP lines, TS50 (positive control) and wild type TS (negative control) which have been inoculated. Round 4 includes 6 ChiPGIP lines, T50 and wild type TS is in early stage of growth.

Transgenic grapevines resistance to Pierces's Disease will be characterized by *Xylella* population in petiole and the using the standardized system based on percentage of leaf area scorching , a characteristic symptom of PD, (Krivanek et al 2005a, 2005b). Bacterial population count is performed after 10-12 weeks post inoculation. Petiole sections from a specific leaf number above inoculation is collected and surface sterilized using 30% bleach for 2 minutes, next 95% ethanol for 2 minutes followed by rinsing using sterile water. The tissue is chopped and homogenized using the Tissue Lyser for 2 minutes. From each lyzed sample dilutions of 1:10, 1:100 and 1:1000 are made and plated on freshly made PD3 media (Almeida and Purcell, 2003a) and incubated at 28°C. After approximately 2 weeks, bacterial colonies are visible and ready to be counted.

To validate the efficiency of xylem specific signal sequences for targeting PGIP from grafted transgenic rootstocks to scion xylem sap, we have initiated grafting experiments where selected transformed lines (rootstocks) were grafted with wild type TS (scion). The movement of the PGIP protein from the rootstock up into the xylem of the wild type scion was evaluated using the radial assay (Aguero et al 2005). Preliminary testing of PGIP activity using leaf extracts and xylem sap from non-grafted TS50 (positive control), ChiPGIP 45-35 and ChiPGIP 45-83 showed PG inhibiting activity. The same lines when grafted also showed inhibiting activity from leaf extract and xylem sap. TS50 showed the highest activity in grafted and non-grafted leaf and non-grafted xylem sap. Interestingly xylem sap from Chi45-35 and Chi45-83 showed a greater inhibition when they were grafted with wild type TS as compared to non-grafted, indicating that the PGIP is moving from the rootstock to the scion (Fig.3).

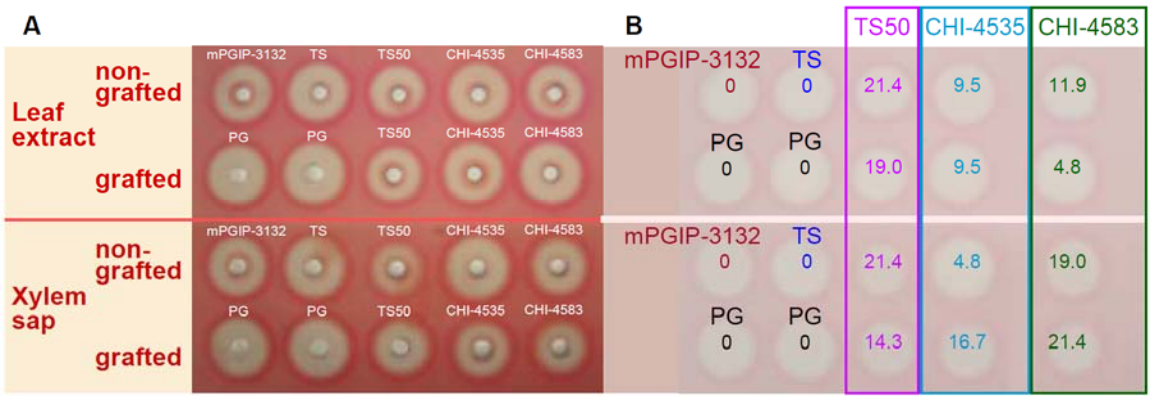

Figure 3. Zone inhibition assay for PG inhibition activity. **A,** assay plate image; **B,** percent inhibition measured in the assay. Leaf extracts and xylem sap from non-grafted and grafted transformed TS50, CHI 45-35 and CHI 45-83 lines were positive for PGIP activity. Transgenic mPGIP 31-32 and wild type TS show no inhibitory activity. PG is the negative control.

2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*X. fastidiosa* activity *in planta*, and test for graft transmissibility:

In vitro grape plants transformed with the constructs, pDU04.6105 (Elastase-Cecropin = HNE-CECB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CECB) (Table 2), have been received from the Parsons Plant Transformation Facility. 69 HNE-CECB plants and 18 pgipSP-HNE-CECB plants have been screened by PCR to verify the individual transformation events. PCR was performed on DNA isolated from *in vitro* leaves using the Qiagen DNeasy Plant Mini Kit. Primers used for detection of elastase were: CaMV355-2: 5' GACGTAAGGGATGACGCACAAT and 3HNEb: 5' TTACTAGAGTGCTTTTGCTTCTCCCAG.

The resulting 36 positive HNE-CECB and 7 positive pgipHNE plants (Table 2) have been micropropagated and transferred to the greenhouse for *Xylella* infection experiments as for the above PGIP lines

Table 2: Current status of testing transgenic lines *Vitis vinifera* var Thompson Seedlees grapevines with HNE-CECB and pgipHNE-CECB genes

No.	Signal peptide	Plasmid	Plants Lines	(+) PCR	(-) PCR	Moved to Greenhouse
1	HNE	pDU04.6105	69	36	0	36
2	PGIP-HNE	pDA05.0525	10	7	3	7

Ten lines have been analyzed via RT-PCR for elastase expression yielding results between 0.61-0.89 % of actin. RT-PCR was performed on RNA isolated from greenhouse grown grape leaves (fourth fully opened leaf) using Qiagen RNeasy Plant Mini kit (Valencia, CA). 50 ng of RNA was/were analyzed using Promega Access RT-PCR kit (Madison, WI). Primers used for amplification of elastase were HNE: HGCb-1, CGTCTCGCTTGCCTTTTCCTAGCCTGTGTGTTGC and HGCb-2, GGCTTTAACTATTCCGTTTCGAATATTCC. Actin primers were used as internal standards to normalize the elastase expression levels. ActinF sequence was: TACAATGAGCTTCGGGTTGC and for ActinR: GCTCTTTGCAGTTTCCAGCT (Santos-Rosa et al., 2008).

Sixteen HNE-CECB lines are under evaluation for tolerance to Pierce's Disease. Each acclimated transgenic line was propagated to obtain mother plants, then further propagated and used for *Xylella* infection experiments as explained above for PGIP transgenic lines. *Xylella* infection experiments are done in multiple rounds; in each round 5-6 transgenic lines and wild type TS (as negative control) are included.

Round 1 includes 5 HNE-CECB and wild type TS control which have been inoculated: the experiment is close to completion. First leaf scorch symptoms were visible within 6-7 weeks post inoculation which consists of cane color change to red and scorching around outer edge of the lower leaves (leaves 1-5). Observation from this round is very promising since lines HNE-CECB 41-151 and 40-41 showed 40% more resistance when compared with wild type TS control (Fig. 4 and 5). Round 2 includes 6 HNE-CECB lines and the wild type TS control included in Round 2 have been inoculated. Round 5, which includes 4 transgenic lines and the control is in early stage of growth.

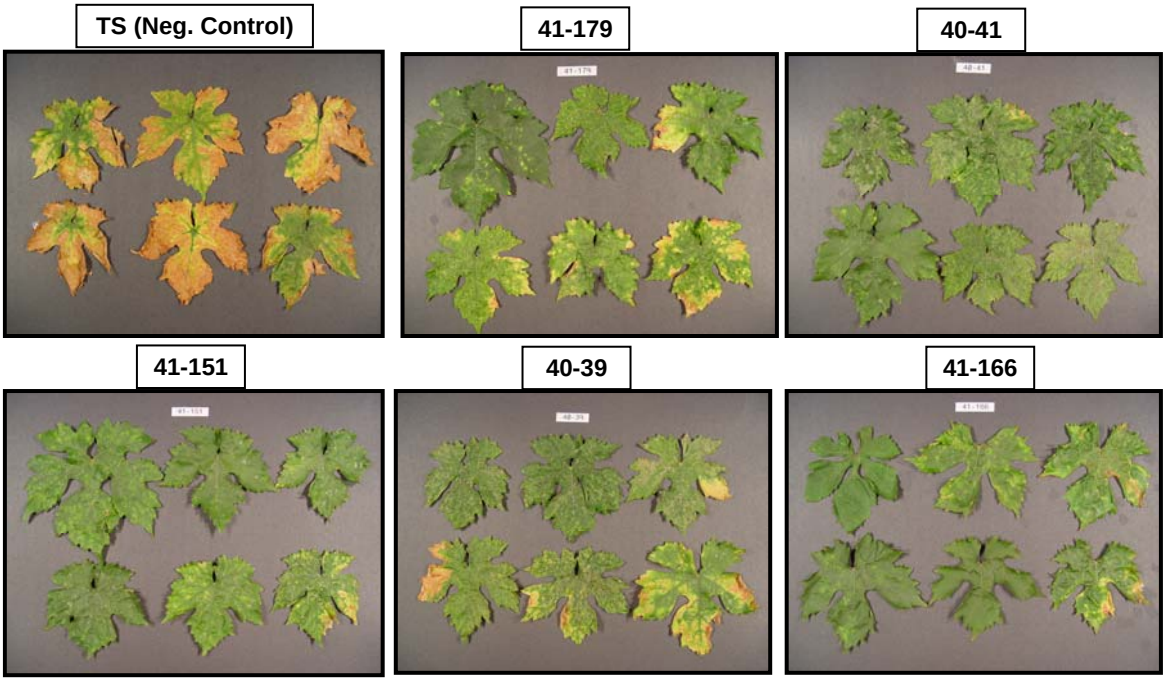

Figure 4: Leaf scorch symptoms in leave number 8 above point of inoculation from 6 different plants within each HNE-CECB line after 74 days post-inoculation.

CONCLUSIONS

The main objective of this project is to develop a potent therapy against *X. fastidiosa* by utilizing the principles of innate immunity by which plants counteract virulence factors like PG with PGIP or that recognize pathogens using their surface characteristics and then rapidly clear them by cell lysis. Because *X. fastidiosa* is xylem-limited, xylem-targeted expression of transgenic therapeutic proteins, such as PGIP and the antimicrobial chimeric proteins, may be used to prevent and control PD. Five different vectors were successfully constructed to test four signal sequences to target PGIP to the xylem of grapevine. Plants have been obtained for all five constructs and PG inhibiting activity for all constructs has been tested. mPGIP, XPS and Ramy lines are in the process of acclimating and growing enough tissue to

micropropagate. NtPGIP and ChiPGIP lines have been inoculated to test resistance for *X. fastidiosa.* Initial grafting experiments showed that Chi signal peptide mobilized PGIP from the Chi transgenic plant used as rootstock to the TS wild plant used as scion.

We are also testing two constructs containing two versions of the chimeric protein Elastase-Cecropin and have transformed tobacco and grapevine. Expression in tobacco indicates that protection against *X. fastidiosa* looks promising. Transgenic grapevines expressing these two constructs have been obtained and inoculated to test resistance for *X. fastidiosa*, Several *Xylella* infection experiments are underway to validate the efficacy of these two types of proteins to ensure that our signal sequences are essential and sufficient to mobilize proteins into grapevine xylem and that the targeted chimeric proteins control *X. fastidiosa* in grapevine tissues. Results looks promising since 2 HNE-CECB lines showed 40% more resistance when compared with wild type TS control.

STATUS OF INTELECTUAL PROPERTY

No disclosures have been made.

PUBLICATIONS RESULTING FORM THIS WORK

Dandekar, A.M., Labavitch, J., Ibanez A.M., Aguero, C.B. and McFarland, S. 2008. *In Planta* Testing of Signal peptides and Anti-Microbial Proteins for Rapid Clearance of *Xylella*. Syposium Proceedings for Pierce Disease Research Symposium. San Diego, CA USA,

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RESEARCH RELEVANCE

X. fastidiosa, a gram-negative bacterium, is the causative agent of Pierce's Disease (PD) in grapevines. Because *X. fastidiosa* is xylem-limited, it is essential that any anti-*Xylella* gene product be targeted to the xylem and have the ability to accumulate in this compartment to an effective concentration. Work on understanding the mechanism of how proteins are targeted to this plant compartment is relevant for the delivery of therapeutic and or effector proteins like PGIP to the xylem. In addition, targeting proteins to the xylem could also be used to influence or disrupt the *Xylella* and glassy-wing sharpshooter (GWSS) interaction as GWSS feeds on the xylem fluid.

Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes. In eukaryotes like plants they comprise the N-terminal part of the amino acid chain and are cleaved off while the protein is translocated through the membrane of the endoplasmic reticulum (Nielsen et al. 1997). Generally, signal peptides are interchangeable and secretion of non-secreted proteins becomes possible by the attachment of a signal peptide at the N-terminus of the mature protein (Vitale and Denecke 1999). We have previously demonstrated that expression of a secretory protein was sufficient to allow its entry into the vesicular transport system (Aguero et al., 2005). Numerous reports of successful recombinant protein production using signal peptides in transgenic plants have been reported; however, changing the signal sequence of recombinant proteins can affect the degree of protein production. For example, the efficiency of secretion of heterologous proteins in transgenic tobacco was improved by replacing the heterologous endogenous signal peptide with a signal peptide from a tobacco

protein (Yoshida et al. 2004). Clearly the choice of the type of signal peptide could influence the efficiency of secretion and protein accumulation in the xylem.

In previous research we have found that the product of the pear PGIP encoding gene, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al 2005). Pear PGIP has a peptide sequence that directs its secretion to the apoplast and its presence in xylem vessels may represent protein secreted into the vessels through pit membranes that serve as transfer pathways from neighboring parenchyma cells. Polygalacturonase (PG) is an enzyme required for X. *fastidiosa* to successfully infect grapevines and is a critical virulence factor for *X. fastidiosa* pathogenesis in grapevines (Roper et al. 2007). The pear PGIP would neutralize Xf PG activity, is secretion competent in grapes and can be further enhanced by optimizing its targeting mechanism to the xylem. We have used the mature pPGIP, as a secretion competent product, fusing it to various signal peptides that can be used to determine the one that is most efficient.

The work described in this report corresponds to research priorities developed by the National Academies in their publication, "California Agriculture Research Priorities: Pierce's Disease" as outlined in Chapter 4, Recommendations 4.3, 4.4 and 4.5 and Chapter 3, Recommendation 3.3. Additionally, the objectives of this research project are relevant to the research recommendations from the CDFA PD/GWSS research scientific review final report from August 2007 as described on page 21 section F.1 by the CDFA Research Scientific Advisory Panel, specifically Inhibition of X. fastidiosa Polygalacturonase (PG) enzyme. The results of this research will not only be applied in projects that test anti-*Xylella* gene products that should be delivered into the xylem but also in functional studies of proteins that are intended to target the *X. fastidiosa* and GWSS interaction.