**Final Report for CDFA Contract Number:** 2007-100

**Project Title**: Evaluation of Signal Sequences for the Delivery of Transgene Products into the Xylem of Grapevine.

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**Reporting period:** The results reported here are from work conducted July 2007 to June 2008

## ABSTRACT

*Xylella 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 accumulate there 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 proteins into the xylem. Our research has accomplished the first three objectives that include 1) obtain partial sequences of proteins found in grape xylem exudates and search cDNA databases for signal sequence identification and selection, 2) design and construct chimeric genes by fusing the selected signal sequences to a sequence coding for a mature secreted protein, in this case the mature pear polygalacturonase inhibiting protein (pPGIP) and 3) transform grapevines with the chimeric genes via *Agrobacterium tumefaciens*. To achieve the mentioned objectives we collected xylem exudates from grapevines and analyzed its protein composition. Further analysis showed that the proteins found in the exudates are secretion competent proteins that share functional similarities with signal peptide containing proteins found in xylem exudates of other plant species. Using the TGI *Vitis vinifera* gene index we found signal peptide gene sequences that corresponded with two of the grape xylem localized proteins found in our exudates. These gene sequences for xylem proteins, Chi1b and one similar to NtPRp27, were fused in-frame to the coding region of the mature pear PGIP (pPGIP) gene.

We also made three other constructs, one with just the mature pPGIP (lacking a signal peptide) as control, another incorporating a signal peptide from a cucumber xylem sap protein ( XSP30), along with one incorporating a signal peptide from the rice amylase protein (Ramy3D) that we have described in earlier publications. We successfully transformed *Vitis vinifera* 'Thompsons Seedless' (TS) grape with these five vectors.

We are currently working on the completion of our fourth objective; to evaluate the efficiency of the different signal sequences in targeting protein products to the xylem tissue of grapevine through the analysis of the expression and secretion of pPGIP in grapevines bearing roots and grafted to wild type grapevine scions. This latter objective is part of a new proposal that will conclude this effort. We have successfully obtained callus cultures, embryos and plants for all five vectors. Chi1b and NtPRp27 transformed TS plants that are PCR positive for the transgene and have PGIP activity are being replicated in culture before they are transferred to the greenhouse for the final stages of evaluation. Using the zone inhibition assay for polygalacturonase activity we have determined that 11 ChiPGIP an 10 NtPGIP plants were positive for polygalacturonase inhibiting activity, showing a range of inhibition of 6-62 % and 0-45%, respectively. Micro-propagation of promising transformants and grafting of transgenic rootstock to wild type scions are currently underway. The individual ChiPGIP and NtPGIP lines that meet the evaluation criteria are being acclimated to the soil and have been gradually moving to the greenhouse where they will be grown up to between 6" and 12" tall. They will be then inoculated with *X. fastidiosa* by hand and by insect and evaluated for symptoms of Pierce's disease (PD) after 3 months.

**OBJECTIVES**

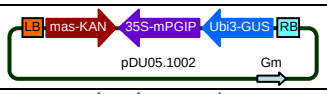
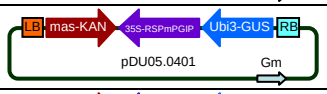
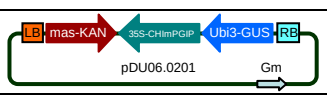
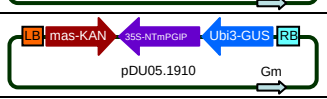
1. Obtain partial sequences of proteins found in grape xylem exudates and search cDNA databases for signal sequence identification and selection.
2. Design and construct chimeric genes by fusing the selected signal sequences to a sequence coding for a mature secreted protein (pPGIP).
3. Transform grapevines with the chimeric genes via Agrobacterium tumefaciens.
4. Evaluate the efficiency of the different signal sequences in targeting protein products to the xylem tissue of grapevine through the analysis of the expression and secretion of pPGIP in grapevines bearing roots and grafted to wild type grapevine scions.

**RESULTS**

Our first three research objectives have been successfully accomplished in our 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 plant 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 (Table 1). 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 designated pDU05.1002 (Table 1). We also incorporated signal peptides from the xylem sap protein XSP30 and the rice amylase protein Ramy3D that we have described in earlier reports. These binary vectors are designated pDA05.XSP and pDU05.0401, respectively (Table 1).

Table 1: Construction of vectors for the expression of mature PGIP with various signal peptide sequences

| No | Signal Peptide | | Reporter Gene | Promoter | Marker Genes | Vector |
|---|---|---|---|---|---|---|
| 1 | None |  | Mature PGIP | CaMV35S | GUS and Kan | pDU05.1002 |
| 2 | Rice amylase-Ramy3Dsp |  | Mature PGIP | CaMV35S | GUS and Kan | pDU05.0401 |
| 3 | Xylem sap protein 30-XSP30sp |  | Mature PGIP | CaMV35S | GUS and Kan | pDA05.XSP |
| 4 | Chi1b signal peptide |  | Mature PGIP | CaMV35S | GUS and Kan | pDU06.0201 |
| 5 | NtPRp27 signal peptide |  | Mature PGIP | CaMV35S | GUS and Kan | pDU05.1910 |

Binary vector # 1 is the control and should be immobile as we have deleted the natural signal peptide fron the pear PGIP sequence with its native signal peptide intact the pear PGIP is secretion competent in grape. In binary vector #2, mature PGIP has been fused to the signal sequence of rice amylase 3 (Ramy3D), which has been previoualy shown to be very effective in secretion of human $\alpha_1$-antitrypsin in rice cell cultures (Trexler et al. 2002). In binary vector # 3 mature PGIP has been fused to the signal sequence of cucumber XSP30, which is a xylem-specific protein. Constructs 4 and 5 have been described above. All five binary vectors were transformed into the disarmed *A. tumefaciens* strain EHA 105 pCH32 (Hamilton, 1997) by electroporation (Wen-jun and Forde 1989) and the stable transformation of *Vitis vinifera* 'Thompson Seedless' (TS) has been completed in all 5 vectors. The methods for Agrobacterium-mediated transformation developed by us for this and previous projects has been published by us (Aguero et al. 2006).

As a result of the above transformation callus, embryos and plants have been obtained for all 5 vectors (Table 2). Callus cultures that were embryogenic were selected on kanamycin and grown into plants. We have tested all the plants containing vectors 4 and 5 using PCR (Table 3). DNA was isolated from leaves using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Primers used for detection of nptII were Aph3: 5' ATGATTGAACAAGATGGATTGCACGCA and Aph4: 5' GAAGAACTCGTCAAGAAGGCGATAGA. Primers for detection of PGIP were 5' Mature PGIP: 5' ATGGATCTCTGCAACCCCGACGAC and 3'PGIP: 5' TTACTTGCAGCTTGGGAGTG. Tissue from these plants has been tested individually for PGIP activity using the zone inhibition assay with PG (Table 3, Figure 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 tube containing a 5mm stainless steel bead in a TissueLyzer (Qiagen). The homogenate was centrifuged at 16000 g for 5 minutes. Protein concentration of the supernatant was determined according to Bradford (1976). The inhibition of endo-PG activity from culture filtrates of *B. cinerea* was determined by zone inhibition assay in a 1% agarose gel in a 0.1M sodium acetate buffer pH 5 supplemented with 100mg/L pectin (modified from Taylor and Secor, 1988).

Table 2: Status of *Vitis vinifera* 'Thompsons seedless' transformants

| No. | Signal peptide | Plasmid | Callus | Embryos | Plants |
|---|---|---|---|---|---|
| 1 | mature | pDU05.1002 | yes | Yes | yes |
| 2 | Ramy | pDU05.0401 | yes | Yes | yes |
| 3 | XSP | pDA05.XSP | yes | Yes | yes |
| 4 | Chi | pDU06.0201 | yes | Yes | Yes |
| 5 | Nt | pDU05.1910 | yes | Yes | Yes |

24 of the plants transformed with Nt and ChiPGIP were tested for PGIP activity using the zone inhibition assay with PG (Table 3, Figure 1). Select lines were looked at quantitatively. All 11 ChiPGIP plants were assayed and had a range of inhibition from 6-62 %. The ten remaining NtPGIP plants were also assayed and had a range of 0-45 % inhibition. The ChiPGIP plants had a greater number of lines with strong inhibition than the NtPGIP plants, 6 vs 2, respectively. Also, there were more, 3 NtPGIP vs 0 ChiPGIP, that had no inhibition activity. The 6 ChiPGIP plants with strong inhibition and 5 of the NtPGIP with strong to medium inhibition are being micropropagated to obtain 40 clones of each line for testing with Xylella to determine efficacy of the PGIP protein. In the meantime individual clones are being acclimated to the soil for transfer to the greenhouse for initial challenge experiments with Xylella. The vines will be allowed to grow up to 6"-12" (about 10 nodes long), then inoculated with *X. fastidiosa* by hand and by insect and 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 a grapevine successfully transformed with this construct and highly expressed as a positive control in the inoculation experiments (Aguero et al. 2005).

Table 3: Evaluation of *Vitis vinifera* 'Thompsons seedless' transformed plants

| No. | Signal peptide | Plasmid | Plant | Positive PCR for PGIP | Positive for PGIP Activity | Lines cloned | Lines grafted | Moved greenhouse |
|---|---|---|---|---|---|---|---|---|
| 1 | mature | pDU05.1002 | yes | In progress | In progress | | | |
| 2 | Ramy | pDU05.0401 | yes | To be tested | To be tested | | | |
| 3 | XSP | pDA05.XSP | yes | In progress | In progress | | | |
| 4 | Chi | pDU06.0201 | yes | 11/11 | 11 | 6 | 2 | In progress |
| 5 | Nt | pDU05.1910 | yes | 17/22 | 7 | 5 | | In progress |

Using the zone inhibition assay for polygalacturonase activity, we have determined that 11 ChiPGIP and 10 NtPGIP plants were positive for polygalacturonase inhibiting activity, showing a range of inhibition of 6-62 % and 0-45%, respectively. Micro-propagation of promising transformants and grafting of transgenic rootstock to wild type scions are currently underway.

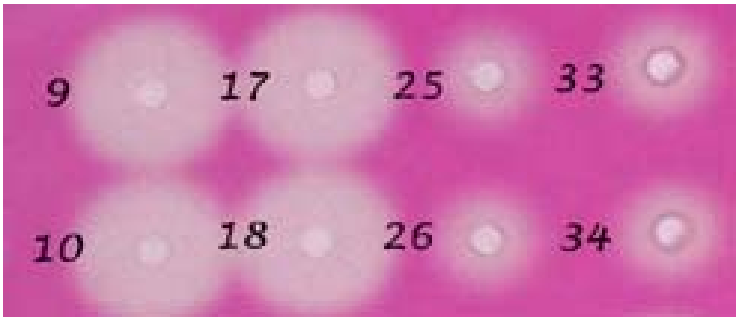



**Figure 1**. Zone inhibition assay results. Wells 9, 10, 17 and 18 are showing no polygalacturonase inhibition activity and wells 25, 26, 33 and 34 are positive for polygalacturonase inhibition activity.

The screening of mature-PGIP and XSP-PGIP plants has been initiated. We have also initiated grafting experiments where selected transformed lines will be grafted with wild type TS scion, we have done this type of experiment previously to evaluate the movement of the PGIP protein from the rootstock up into the xylem of the wild type scion (Aguero et al. 2005). An additional year of work may be required to accomplish this last evaluation that requires grafted plants.

## CONCLUSIONS

Over the course of the past year callus cultures, embryos and plants for all five vectors have been obtained. We have finished our *in vitro* analysis of 15 ChiPGIP and 22 NtPGIP plants. These analysis include PCR and PGIP activity assays. Based on these results we have selected 6 chiPGIP and 5 ntPGIP plants to micropropagate with the purpose of providing multiple clones of these plants to transfer to soil and then to the greenhouse. Once established in the greenhouse the plants will be inoculated with Xylella and evaluated for appearance of symptoms of Pierce's disease. Sufficient negative control plants (non transformed) are also being propagated to provide scion material for grafting to the transgenic rootstocks. We are also in the process of micropropagating the, mature-PGIP- and XSP-PGIP lines to provide enough material for testing by PCR and for the PGIP activity assay.

## PUBLICATIONS RESULTING FROM THIS WORK

Dandekar, A.M., Labavitch, J., Ibanez A.M., Aguero, Cecilia., and McFarland, S. 2007. Evaluation oof signal sequences for the delivery of transgene products into the xylem of grapevines. Syposium Proceedings for Pierce Disease Research Symposium. San Diego, CA USA,

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## STATUS OF INTELECTUAL PROPERTY

No disclosures have been made.

## 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 proteins that could 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 choise 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 ddetermine 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.