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

CDFA contract number: 2007-100

Reporting period: The results reported here are from work conducted from April-June 2008

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Objectives of Proposed Research
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.

Research accomplishments and results
The first three objectives have been accomplished

Objective 1. Obtain partial sequences of proteins found in grape xylem exudates and search cDNA databases for signal sequence identification and selection.
Objective 2. Design and construct chimeric genes by fusing the selected signal sequences to a sequence coding for a mature secreted protein (pPGIP).
Objective 3. Transform grapevines with the chimeric genes via Agrobacterium tumefaciens.

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 TIGR *Vitis vinifera* gene index were used to design primers that were used to amplify the predicted fragments from genomic DNA of 'Chardonnay' and 'Cabernet Sauvignon'. These fragments were annotated as Chi1b and similar to NtPRp27 respectively (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. 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, 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 (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 although PGIP with its native signal peptide 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 very effective in secretion of human α1-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 have been transformed into the disarmed *A. tumefaciens* strain EHA 105 by electroporation (Aguero et al. 2006). The next step, the permanent transformation of *Vitis vinifera* 'Thompson Seedless' (TS) has been completed in all 5 vectors.

Objective 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.

Last quarter 24 of the plants transformed with Nt and ChiPGIP were tested for PGIP activity using the zone inhibition assay with PG (Table 2, Figure 1). This quarter we repeated the assay

with most of the clones and did a more quantitative analysis. 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 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 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 expressive as a positive control in the inoculation experiments (Aguero et al. 2005). Bacterial populations in the plants will be initially evaluated using a RT-PCR system that was designed to detect and quantify *X. fastidiosa* in grape vine tissue very specifically using the highly conserved target gene Eftu (Gambetta et al 2007).

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

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						
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

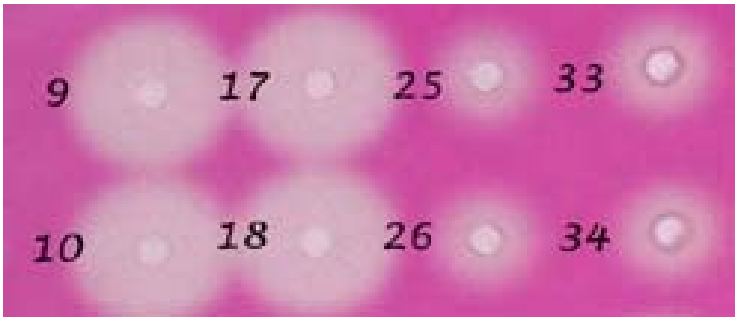

Figure 1. Zone inhibition assay results. Wells 9, 10, 17 and 18 are showing no polygalacturonase inhibition activity and wells 25, 26, 33 and 34 are positive for polygalacturonase inhibition activity.

The screening of mature-PGIP and XSP-PGIP plants has been initiated. We are also in the intial stages of attempting to graft ChiPGIP and NtPGIP promising lines to wild type scions 'TS' in the greenhouse. An additional year of work may be required to accomplish this last evaluation that requires grafted plants.

Literature Cited

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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 will be essential that any anti-Xylella gene product be present in the xylem in an effective concentration. Work on understanding the mechanism of how proteins are targeted to this plant compartment will be relevant for the delivery of therapeutic proteins into the xylem. In addition, it will be a useful tool for Xylella and glassy-wing sharpshooter (GWSS) gene function studies.

Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes. 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 that allows the entry into the vesicular transport system (Vitale and Denecke 1999). 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).

In previous research, we have found that the product of the pPGIP encoding gene, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al. 2005). pPGIP 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). If pPGIP is secretion competent in grapes and can be optimally targeted to the xylem, it may be used to inactivate the *X. fastidiosa* gene product PG. We intend to use the mature pPGIP, as a secretion competent product, fused to the signal peptides to be analyzed.

The proposed work described in this report carefully 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 that are intended to target the products of *X. fastidiosa* and GWSS genes to the xylem.

Summary

Our first three research objectives have been accomplished. Those objectives were to 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. In our earlier research 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 proteins found in xylem exudates of other species. Using the TIGR *Vitis vinifera* gene index we found signal peptide gene sequences that corresponded with two of the grape xylem proteins found in our exudates. These gene sequences for xylem proteins Chi1b and one similar to NtPRp27 were fused to the (pPGIP)-encoding gene. We also made three other constructs, one with 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 reports. We have successfully transformed *Vitis vinifera* 'Thompsons Seedless' (TS) grape with the five vectors and callus cultures, embryos and plants for all five vectors have been obtained.

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. We have successfully obtained transformed plants for 4 vectors. Chi1b and NtPRp27 transformed TS plants that are PCR positive for the

transgene are being replicated in culture and undergo screening evaluations before they are transferred to the greenhouse for the final stages of evaluation. These evaluations include a zone inhibition assay for polygalacturonase inhibiting activity and quantitative RT-PCR to identify lines that are strongly expressing the transgene. 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" and12" tall. They will be then inoculated with *X. fastidiosa* by hand and by insect. Bacterial populations in the plants will be initially evaluated using a RT-PCR system that was designed to detect and quantify *X. fastidiosa* in grape vine tissue very specifically using the highly conserved target gene Eftu. The screening of mature-PGIP and XSP-PGIP plants has been initiated.

Status of Intellectual Property

No disclosures have been made.