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 from September 2007-March 2008

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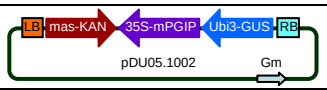
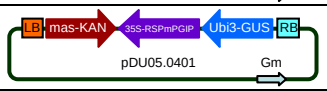
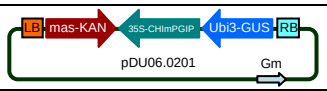
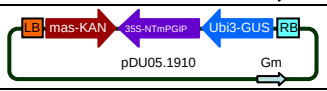
Objectives of Proposed Research
1. Obtain partial sequences of proteins found in grape xylem exudates and search cDNA databases for signal sequence identification and selection.
2. Design and construct chimeric genes by fusing the selected signal sequences to a sequence coding for a mature secreted protein (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 we 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 then used to amplify the predicted fragments from genomic DNA of *V. vinifera* '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 (pPGIP) 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 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	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 endogenous 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 (Masuda et al. 1999). 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. The next step, the permanent transformation of *V. 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.

As mentioned above the transformation of TS is complete and transformed callus, embryos and plants have been obtained. The methods for Agrobacterium-mediated transformation have been reported earlier by us (Aguero et al. 2006). Callus cultures that are embryogenic were selected on kanamycin and grown into plants. We have tested all the plants containing vectors 4 and 5 using PCR and as plants from the other three lines come in, all will be tested for the presence of the transforming DNA gene segments using PCR. Transcripts will be quantified using TaqMan reverse transcription PCR (RT-qPCR) to identify high expressing lines. Tissue from these plants has been tested individually for PGIP activity using the zone inhibition assay with polygalacturonase (PG; Figure 2; Aguero et al. 2005). PG preparations were obtained from *Botrytis cinerea* strain Del 11 isolated from grape. The fungus was cultured in a modified Pratts Medium (Fergus, 1952) supplemented with 1g/l Yeast extract and 3 g/l glucose on a rotary shaker in dark. 3 day old cultures were filtered out through 4.25 nm glass fiber filter and 0.22 µm filter and then were subjected to dialysis through 6-8000 molecular porous membrane (Spectra/Por). Finely crushed tissue (0.15g) was homogenized in a protein extraction buffer. The homogenate was centrifuged at 14000 rpm for 5 minutes. The inhibition of endo-PG activity from culture filtrates of *B. cinerea* was determined by zone inhibition assay in a 1% agarose gel supplemented with 100mg/L pectin in a 0.1M sodium acetate buffer pH 5 (Tylor and Secor, 1988) using equal amounts of total protein. We have already initiated grafting experiments where selected transformed lines will be bench grafted (micro-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

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

Vector insert		Callus	Embryo	Plant	Positive PCR for NPT II	Positive for PGIP Activity	Lines cloned	Lines micro-grafted
1	pDU05.1002	yes	yes	yes	To be completed			
2	pDU05.0401	yes	yes	yes	To be completed			
3	XSP	yes	yes	yes	To be completed			
4	pDU06.0201	no	yes	yes	10/11	9	10	2
5	pDU05.1910	no	yes	yes	17/22	5	12	

We have now obtained plants containing all five vectors (Table 2). Ten out of 11 plants transformed with vector #4 and 17 of the 22 plants transformed with vector #5 have tested positive via PCR for the transgene using nptII primers (Table 2). PCR tests for vectors #1-3 are still in progress. We have tested 24 of the plants transformed with vector #4 and #5 for PGIP activity using the zone inhibition assay with PG. Five out of the 17 PCR positive plants transformed with vector #5 and 9 out of the 9 PCR positive plants transformed with vector #4 were positively showing PG inhibition activity (Table 2). Micro-propagation of the more promising plants is already underway. Multiple clones have been generated for 10 out 10 PCR positive lines with vector #4 and 12 out of 17 with vector #5 (Table 2). Initially the micro-propagated plants will be evaluated for high expression levels using Quantitative PCR. High expressing lines showing positive PG inhibition will then be transferred to soil and transferred to the green house for growth. The vines will be allowed to grow up to 6"-12" (about 10 nodes long), then inoculated with *X. fastidiosa* both by hand and by insect. Inoculated plants will be evaluated at 1 and 2 months time points using RT-qPCR and evaluated for symptoms of Pierce's disease (PD) after 3 months. The qPCR method we use 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). 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). We have successfully micro-grafted transformed rootstock containing pPGIP with its endogenous signal peptide to wild type 'TS' scion (Figure 1). We have successfully micro-grafted 2 lines transformed with vector 4 to wild type 'TS' scion and more micro-grafts using plants transformed with vector 4 and 5 are in progress (Table 2). An additional year of work may be required to accomplish this last evaluation that requires grafted plants.

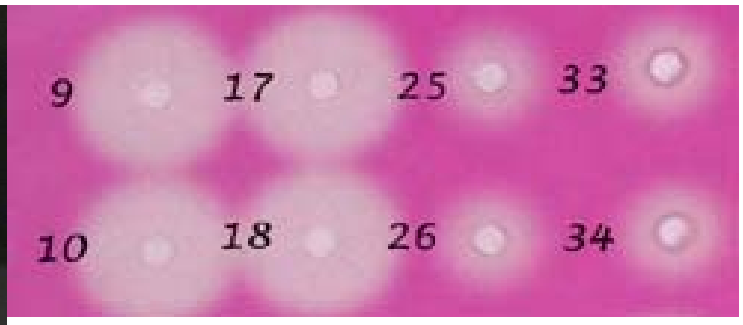


Figure 1.left: Photograph of a TS root stock and scion successfully grafted together. The red arrow indicates the graft point. **Figure 2**.above: Zone inhibition assay results. Wells 9, 10, 17 and 18 are showing no polygalacturonase inhibition activity and wells 25, 26, 33 and 34 are positive for polygalacturonase inhibition activity.

Publications, reports and presentations

Dandekar, A.M., Labavitch, J., Ibañez A.M., Aguero, C. and McFarland S. Evaluation of signal sequences for the delivery of transgene products into the xylem of grapevine. 2007 Pierce's Disease Research Symposium.

Dandekar, A.M., Labavitch, J., Ibañez A.M., Aguero, C. and McFarland S. Evaluation of signal sequences for the delivery of transgene products into the xylem of grapevine. December 2007 Final Report.

Dandekar, A.M., Labavitch, J., Ibañez A.M., Aguero, C. and McFarland S. Evaluation of signal sequences for the delivery of transgene products into the xylem of grapevine. July 2007 to December 2007.

Dandekar, A.M., Labavitch, J., Ibañez A.M., Aguero, C. and McFarland S. Evaluation of signal sequences for the delivery of transgene products into the xylem of grapevine. June 2007 Progress Report.

Aguero, C.B., C.P. Meredith and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis (In Press).

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Dandekar, A.M., Labavitch, J., Ibañez A.M., Aguero, C. Evaluation of signal sequences for the delivery of transgenic products into the xylem. September 2005 to September 2006 Continuing Report for the project funded by CFP-PD Final. PI. Dr. Abhaya Dandekar. Department of Plant Sciences. UCDavis.

Dandekar, A.M., Gilchrist , D., Labavitch, J., Guimaraes, R., and Aguero, C. Evaluation of signal sequences for the delivery of transgene products into the xylem. 2005 Pierce's Disease Research Symposium.

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).

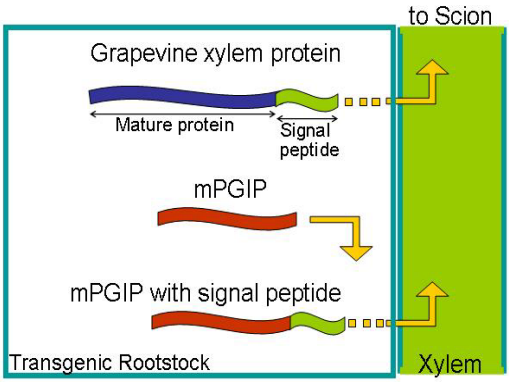

Figure 3. Theory: Identification of signal peptides of grapevine xylem proteins, modification of mPGIP with these peptides to test their secretion competence.

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 are using the mature pPGIP, as a secretion competent product, fused to the different 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 *Xf* polygalacturonase (PG) [which include] identification of PGIPs with high activity against *Xf* PG, delivery of PGIP to grape plant scions from transgenic rootstocks, and development of small molecule inhibitors of *Xf* PG". 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 we 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 (Nt) were fused to the (pPGIP)-encoding gene. We also made three other constructs, one with pPGIP lacking a signal peptide as control (mPGIP), 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. All five constructs were individually placed in identical vectors containing the CaMV35S promoter and two marker genes, Kan for antibiotic selection and GUS for scoring. We have successfully transformed *V. 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 all 5 vectors. Ten out of 11 plants transformed with Chi1b and 17 of the 22 plants transformed with Nt have tested positive via PCR for the transgene using nptII primers. Chi1b and Nt transformed TS plants that are PCR positive for the transgene are being replicated in culture and will 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 (PG) inhibiting activity and quantitative RT-PCR to identify lines that are strongly expressing the transgene. Using the zone inhibition assay, we have determined that 14

out of 24 plants tested so far are positive for PG inhibiting activity. Micro-propagation of promising transformants and micro-grafting of transgenic rootstock to wild type scions are currently underway. We have successfully micro-grafted the positive control vector with pPGIP and its endogenous signal peptide and 2 of the lines containing the Chi1b vector. The lines that meet the evaluation criteria will be transferred to soil then moved into a 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 evaluated using a quantitative RT-PCR system that was designed to detect and quantify *X. fastidiosa* in grape vine tissue very specifically using a highly conserved *X. fastidiosa* target gene.

Status of Intellectual Property

No disclosures have been made, so no activity in this area.

Literature Cited

Aguero, C.B., C.P. Meredith and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis (In Press).

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