EVALUATION OF SIGNAL SEQUENCES FOR THE DELIVERY OF TRANSGENE PRODUCTS INTO THE XYLEM OF GRAPEVINE

Project Leader:
Abhaya M. Dandekar
Dept. of Plant Science
University of California
Davis, CA 95616
amdandeka@ucdavis.edu

Cooperators:

John Labavitch
Dept. of Plant Science
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Ana Maria Ibanez
Dept. of Plant Science
University of California
Davis, CA 95616
amibanez@ucdavis.edu

Cecilia Aguero
Dept. of Plant Science
University of California
Davis, CA 95616
cbaguero@ucdavis.edu

Sarah McFarland
Dept. of Plant Science
University of California
Davis, CA 95616
sbmcfarland@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2007 to September 2007.

ABSTRACT

Xylella fastidiosa (*Xf*), a gram-negative bacterium, is the causative agent of Pierce's disease (PD) in grapevines. Because *Xf* 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. This is a continuing project that began in late 2005. In our earlier research we collected xylem exudates from grapevines and analyzed its protein composition by two-dimensional gel electrophoresis. Peptide spectrum and Blast analysis showed that the proteins found in the exudates are secreted proteins that share function similarities with proteins found in xylem exudates of other species. The corresponding cDNA sequences of five of them were found in the TIGR *Vitis vinifera* gene index. Two of these sequences for xylem proteins Chi1b and one similar to NtPRp27 were fused to the mature pear polygalacturonase inhibiting protein (pPGIP)-encoding gene. We also made three other constructs incorporating signal peptides from the xylem sap protein XSP30, the rice amylase protein Ramy3D that we have described in earlier reports along with pPGIP lacking a signal peptide as control. We have successfully transformed *Vitis vinifera* 'Thompson Seedless' (TS) grape with the five vectors and callus and embryo cultures for all five vectors have been obtained. The expression of these chimeric genes is being evaluated in permanent transformations in order to evaluate their ability to target pPGIP to the xylem. We have successfully obtained Chi1b and Nt transformed TS plants that are PCR positive for the transgene. Using a zone inhibition assay, we have determined that seven out of 15 plants tested so far are positive for polygalacturonase inhibiting activity. Micropropagation of promising transformants and bench grafting of transgenic rootstock to wildtype scions are currently underway. 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 *Xf* and GWSS genes to the xylem.

INTRODUCTION

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 (1). 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 (2). 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 (3).

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 (4). 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. Then if pPGIP is secretion competent in grapes, it constitutes a good alternative to inactivate *Xf* genes products like polygalacturonase (PG), the enzyme required for *Xf* to successfully infect grapevines and as critical virulence factor for *Xf* pathogenesis in grapevines (5). In previous experiments we found GFP, when fused with the signal peptide XSP30, a xylem specific protein from cucumber (6) to either not be recognized or not be secretion competent. We intend to use the mature pPGIP as a secretion competent product fused to the signal peptides to be analyzed.

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 (7). cDNA sequences of five of them were found in the TIGR *Vitis vinifera* gene index. However, it was possible to predict the signal peptide in two contigs only (TC 39929 and TC 45857, annotated as Chi1b and similar to NtPRp27 respectively). Based on their sequences, we designed primers that were used to amplify the predicted fragments from genomic DNA of 'Chardonnay' and 'Cabernet Sauvignon'. These fragments were then fused to DNA sequences that contained the mature pPGIP gene through gene splicing using a PCR-based overlap extension method (SOE) (8) 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 (9). 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 (10).

The proposed work described in this proposal 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* 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 *Xf* and GWSS genes to the xylem.

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:
   - **a.** analysis of the expression and secretion of pPGIP in embryo callus cultures of transformed grapevines.
   - **b.** analysis of the expression and secretion of pPGIP in grapevines bearing roots and grafted to wild type grapevine scions.

RESULTS

The first three objectives have been accomplished and summarized in the introduction. These results pertain to our progress in accomplishing objective 4. The permanent transformation of *Vitis vinifera* 'Thompson Seedless' (TS) has been initiated

for all five vectors. The methods for *Agrobacterium*-mediated transformation have been reported earlier by us (10). We have obtained transformed callus cultures for all five vectors (Table 2) and analysis of the expression and secretion of pPGIP in embryo callus cultures of transformed grapevines is in progress. All of the callus lines will be tested for the presence of the transforming DNA gene segments using PCR, transcripts will be quantitated using TaqMan RT-PCR to identify high expressing callus lines. Five individual lines of callus cultures from each of the five vectors have been initiated into liquid cultures to evaluate the secretion of PGIP into the culture medium as would be expected of the apoplast targeted protein. PGIP secretion and activity will be evaluated using the zone inhibition assay with PG (Figure 2) (11). Select callii that have been transformed from each of the five constructs have been induced to undergo embryogenesis. These callus cultures that are embryogenic will be selected on kanamycin and these embryos will be used to obtain shoots for individual plants. All the methods that we will be using for the analysis of the callus or the plants have been described by us (4).

Table 2. Status of *Vitis vinifera* 'Thompson seedless' transformants

	Vector insert	Callus	Embryo	Plant	Positive PCR for NPT II
1	pDU05.1002	yes	yes	no	To be tested
2	pDU05.0401	yes	yes	no	To be tested
3	XSP	yes	yes	no	To be tested
4	pDU06.0201	yes	yes	yes	10/10
5	pDU05.1910	yes	yes	yes	17/22

We have already obtained plants containing vectors #4 and #5 (Table 2). Seventeen of the 22 plants transformed with vector #5 and 10 out of 10 plants transformed with vector 4 have tested positive via PCR for the transgene using nptII primers (Table 2). Plants will be regenerated and the same detailed testing that is used for the callus and embryo cultures will be done with the plants. We have tested 15 of the plants transformed with vector #4 and #5 for PGIP activity using the zone inhibition assay with PG and seven plants were showing PG inhibition activity. Micropropagation of the more promising plants is already underway. Initially the micropropagated plants will be evaluated; these will then be transferred to soil and transferred to the green house for growth. The vines will be allowed to grow in the green house for four to six months and the xylem fluid will be extracted with a pressure bomb. We also plan to do some grafting experiments where selected transformed lines will be bench 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 (4). Since we found in this earlier research that pPGIP with its endogenous signal peptide is xylem competent we are using it as a positive control (4). We have successfully bench grafted transformed rootstock containing pPGIP with its endogenous signal peptide to wild type 'TS' scion (Figure 1) and micrografts using plants transformed with vectors four and five are in progress. An additional year of work may be required to accomplish this last evaluation that requires grafted plants.

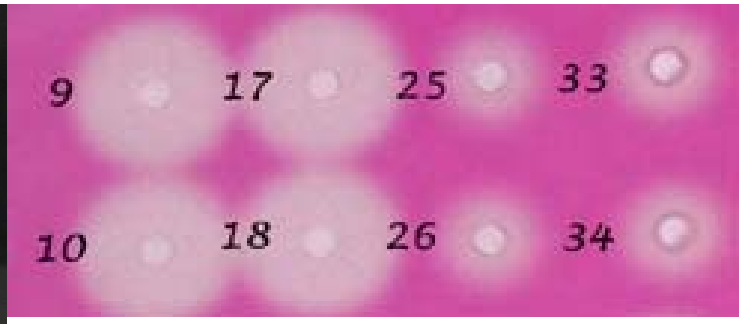

Figure 1 (left). Photograph of a TS root stock and scion successfully grafted together. The red arrow indicates the graft point. **Figure 2 (above).** Zone inhibition assay results. Wells 9, 10, 17 and 18 are showing no polygalacturonic inhibition activity and wells 25, 26, 33 and 34 are positive for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

CONCLUSION

We have accomplished the first three objectives and we have already made significant progress toward achieving our fourth objective. We have successfully transformed TS with the five PGIP vectors and obtain both callus and embryo cultures. In the case of vectors #4 and #5 we have obtained plants as well. The analysis of the expression and secretion of pPGIP in

embryo callus cultures of transformed grapevines is underway. Results from our initial analysis look promising. Micropropagation and bench grafting of transformed TS has been initiated. 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 *Xf* and GWSS genes to the xylem.

REFERENCES

1. Nielsen, H., Engelbrecht, J, Brunak, S, and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10:1–6.
2. Vitale, A., and Denecke, J. 1999. The endoplasmic reticulum: Gateway of the secretory pathway. Plant Cell. 11:615-628.
3. Yoshida K., Matsui, T., and Shinmyo, A. 2004. The plant vesicular transport engineering for production of useful recombinant proteins. J. Mol. Catal. B: Enzym. 28:167–171.
4. Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pat. 6:43-51.
5. Roper, M.C., Greve C.L., Warren JG., Labavitch J.M., and Kirkpatrick B.C. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* garapevines. MPMI 20:411-419
6. Masuda, S., Saskuta, C., and Satoh, S. 1999. cDNA cloning of a novel lectin-like xylem sap protein and its root-specific expression in cucumber. Plant Cell Physiol. 40:1177-1181.
7. Buhtz, A., Kolasa, A., Arlt, K., Walz, C., and Kehr, J. 2004. Xylem sap protein composition is conserved among different plant species. Planta 219:610-618.
8. Horton, R.M., Cai, Z.L., Ho, S.N., and Pease, L.R. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques 8:528-35.
9. Trexler, M.M., McDonald, K.A., and Jackman, A.P. (2002). Bioreactor production of human α_1-antitrypsin using metabolically regulated plant cell cultures, Biotechnol Prog 18: 501-508.
10. 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).
11. Tylor R.J. and Secor G.A. 1988. An important diffusion assay for qualifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopathology 78:1101-1103.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.