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

Principal Investigator:
Abhaya M. Dandekar
Dept. of Plant Sciences
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Cooperators:
David Gilchrist
Dept. of Plant Pathology
University of California
Davis, CA 95616

John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616

Ana Maria Ibanez
Dept. of Plant Sciences
University of California
Davis, CA 95616

Cecilia Aguero
Dept. of Plant Sciences
University of California
Davis, CA 95616

Reporting period: The results reported here are from work conducted September 2005 to September 2006.

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.

We collected xylem exudate 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 5 of them were found in the TIGR Vitis vinifera gene index. The signal sequences of xylem proteins Chi1b and similar to NtPRp27 were fused to the mature pear polygalacturonase inhibiting protein (pPGIP)-encoding gene. The expression of these chimeric genes will be evaluated in transient and permanent transformations in order to evaluate their ability to target pPGIP to the xylem. 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* and *A. rhizogenes*.
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 transiently transformed grapevines.
   b. analysis of the expression and secretion of pPGIP in grapevines bearing roots transformed via *A. rhizogenes*.

RESULTS

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 (Buhrz et al., 2004). cDNA sequences of 5 of them were found in the TIGR *Vitis vinifera* gene index. However, it was possible to predict the signal peptide in 2 contigs only (TC 39929 and TC 45857, annotated as Chi1b and similar to NtPRp27 respectively; see Figure 1). 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) (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).

<p>| Table 1. Construction of vectors for the expression of mature PGIP with various signal peptide sequences. |</p>
<table>
<thead>
<tr>
<th>No</th>
<th>Signal Peptide</th>
<th>Reporter Gene</th>
<th>Promoter</th>
<th>Marker Genes</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>Mature PGIP</td>
<td>CaMV35S</td>
<td>GUS and Kan</td>
<td>pDU05.1002</td>
</tr>
<tr>
<td>2</td>
<td>Rice amylase-Ramy3Dsp</td>
<td>Mature PGIP</td>
<td>CaMV35S</td>
<td>GUS and Kan</td>
<td>pDU05.0401</td>
</tr>
<tr>
<td>3</td>
<td>Xylem sap protein XSP30-XSP30sp</td>
<td>Mature PGIP</td>
<td>CaMV35S</td>
<td>XSP</td>
<td>pDU06.0201</td>
</tr>
<tr>
<td>4</td>
<td>Chi1b signal peptide</td>
<td>Mature PGIP</td>
<td>CaMV35S</td>
<td>GUS and Kan</td>
<td>pDU05.1910</td>
</tr>
<tr>
<td>5</td>
<td>NtPRp27 signal peptide</td>
<td>Mature PGIP</td>
<td>CaMV35S</td>
<td>GUS and Kan</td>
<td>pDU05.1910</td>
</tr>
</tbody>
</table>

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. 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 *Vitis vinifera* ‘Thompson Seedless’ has been initiated for all 5 vectors and this step takes some time. Once we obtain plants, leaf tissue will be examined for PGIP expression and the positive plants will be subjected to the analysis of the expression and secretion of PGIP.

CONCLUSIONS

Through the study of the proteins present in xylem exudates of ‘Chardonnay’, we have found 2 good candidates to investigate the effect of using grape signal sequences on xylem targeting. In addition we have produced 2 other chimeric genes containing the signal peptide of a xylem-specific protein in cucumber and the signal sequence of rice amylase. The results obtained with permanent transformations with these genes will provide, in the short term, valuable information for the
identification of signal peptides that will deliver proteins to grapevine xylem with high efficiency. In the long term, the development of an efficient secretory system will be essential to target therapeutic proteins to the xylem of grapevine.

REFERENCES


FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Research Grant Program.
DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF XYLELLA

Project Leaders:
Abhaya M. Dandekar  Goutam Gupta  Karen McDonald  Meghan Norvell
Department of Pomology  B-1, MS M888, LANL  Chem Engr and Material Sci  B-1, MS M888, LANL
University of California  Los Alamos, NM 87545  UC Davis, Davis, CA 95616  Los Alamos, NM 87545
Davis, CA 95616  gxg@lanl.gov  kamcdonald@ucdavis.edu  mek@lanl.gov
amdandekar@ucdavis.edu

Collaborators:
George Bruening  Edwin L. Civerolo
Dept of Plant Pathology  SJVASC
University of California  Parlier, CA 93468
davis, CA 95616  eciverolo@fresno.ars.usda.gov
gebruening@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT
Xylella fastidiosa (Xf), is a gram-negative xylem-limited bacterium and causative agent of Pierce’s disease (PD) in California grapevines. During very early stages of Xf infection, specific carbohydrates/lipids/proteins on the outer membrane of Xf interact with plant cells and are important for virulence (Pieters, 2001). Design of a protein inhibitor that interrupts this step of the plant-Xf interaction will be useful in anti-microbial therapy and controlling PD. In this UC/LANL project, we have developed a novel protein-based therapy that circumvents the shortcomings of traditional antibiotics. We have designed a chimeric anti-microbial protein with two functional domains (Figure 1). One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill Xf. In this chimera, human neutrophil elastase (HNE; 5-10) is the SRD that recognizes MopB, the major outer membrane protein of Xf (Bruening et al., 2002). The second domain is cecropin B, a lytic peptide that targets and lyases gram-negative bacteria. We have combined HNE and cecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized and cloned into different vectors for insect and plant transformation. Five transformed insect cell lines are being evaluated and production and processing of the protein is being optimized in liter size preps. Plant transformation experiments have been completed and we have obtained plants of Nicotiana tabacum var benthamiana and plants of Vitis vinifera ‘Thompson Seedless’ transformed with this gene that are being generated for the analysis of gene expression and protein production. The proteins obtained from the transgenic insect and plant cell lines will be used to test for antimicrobial activity against Xf.

INTRODUCTION
Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. Xylella fastidiosa (Xf) is a devastating bacterial pathogen that causes Pierce’s disease (PD) in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorched disease in numerous other agriculturally significant plants including almonds in California (http://danr.ucop.edu/news/speeches). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the availability of the whole genome sequences of PD and CVC strains of Xf offer new avenues to directly target and inactivate the pathogen. In this project, we are developing a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of Xf. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (Pieters, 2001; Baquero and Blazquez, 1997). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several immediate and long term impacts.