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

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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.

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 endoplasmatic recticulum (Nielsen et al., 1997). Generally, signal peptides are interchangeable and secretion of non-secreted proteins becomes possible by the fusion of a signal peptide at the N-terminus of the mature protein; however, changing the signal sequence of recombinant proteins can affect the degree of protein production (Yoshida et al., 2004).

In previous research, we fused the sequence coding for the signal peptide of XSP30, a xylem-specific protein from cucumber (Masuda et al., 1999), to the green fluorescent protein (GFP) reporter gene. Contrary to what we expected, fluorescence was only detected inside the cells. Our results suggested that either the XSP30 signal peptide is not recognized by the grape secretory machinery or GFP is not secretion competent. If the first hypothesis is correct, signal sequences obtained from proteins present in grape xylem sap would constitute better candidates for delivery of transgene products to the xylem.

Interestingly, we have also found that the product of the pPGIP encoding gene from pear fruit, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al., 2005). These results show that pPGIP is secretion competent in grapes and constitutes a good alternative to GFP. We intend to use the sequence encoding the mature pPGIP fused to the signal peptides for analysis.

We have collected xylem exudate from plants of *Vitis vinifera* 'Chardonnay' and analyzed its protein composition by two-dimensional gel electrophoresis. The purpose of this project is to fuse the signal sequences of these grape xylem sap proteins to the mature pPGIP-encoding gene in order to evaluate their ability to target pPGIP 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 (Buhz 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).

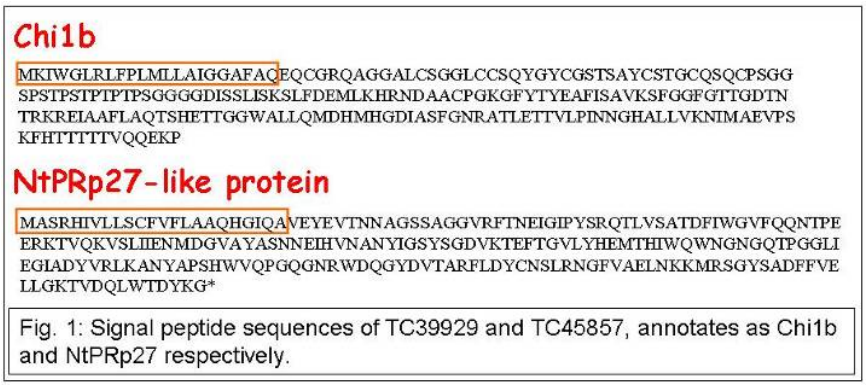

Fig. 1: Signal peptide sequences of TC39929 and TC45857, annotates as Chi1b and NtPRp27 respectively.

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. 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 need 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.

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DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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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 lyses 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*.

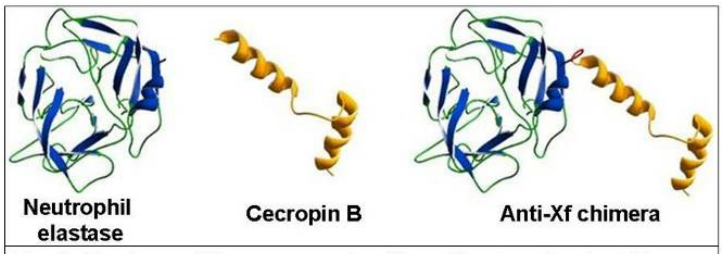

Fig. 1: Structures of the components of the chimeric anti-microbial proteins for the rapid clearance of *Xylella*.

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 scorch 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, 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.