OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

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ABSTRACT
In response to the strategy recommended by the Advisory Board, to express plant genes for particularly effective polygalacturonase inhibiting proteins (PGIPs) or other inhibitors of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) in transgenic grape rootstocks, this approach was adopted to enhance grapevine Pierce's disease (PD) resistance. This proposal describes integrated studies aimed at the eventual deployment of that strategy. To ease the path to commercialization, PIPRA investigators will examine relevant Intellectual Property and regulatory issues associated with the use of this strategy. A reliable source of recombinant *Xf* PG will be developed and the PG will be used to screen diverse PGIPs for their ability to effectively inhibit the *Xf* PG enzyme. Grape rootstock lines will be transformed with the most effective PGIPs and signal and target sequences that maximize PGIP expression in the rootstock and its export to the non-transgenic scions. At the conclusion of the project, the capacity of the non-transgenic vines grafted on the transgenic rootstock to resist PD and produce high quality grapes will be tested.

INTRODUCTION
Xylella fastidiosa (*Xf*), the causative agent of Pierce's disease (PD) in grapevines, has been observed in infected portions of vines. Several lines of evidence support the hypothesis that *Xf* uses cell wall-degrading enzymes (CWDEs) to digest the polysaccharides of plant pit membranes separating the elements of the water-conducting vessel system of plants (Thorne et al., 2006). *Xf* CWDEs breakdown and thereby increase the porosity of these these primary cell wall barriers, allowing the systemic expansion of the pathogen. The genome of *Xf* contains genes putatively encoding a polygalacturonase (*Xf*PG) and several ß-1,4-endo-glucanases (EGase), CWDEs that digest cell wall pectin and xyloglucan polymers, respectively. These CWDEs are good candidates as factors that facilitate *Xf* systemic movement and PD development. To demonstrate this, Roper et al. (2007) developed a PG-deficient strain of *Xf* and showed that the mutant bacterial strain was unable to cause PD symptoms, thus identifying the pathogen's PG as a PD virulence factor. Labavitch et al. (2006) reported that introduction of PG and EGase into explanted stems of uninfected grapevines caused breakage of the cell wall of the PM and, subsequently (Labavitch, 2007), demonstrated that substrates for these enzymes, pectins and xyloglucans, are present in grapevine PMs.

PG-inhibiting proteins (PGIPs) produced by plants limit damage caused by fungal pathogens (*B. cinerea*, the gray mold pathogen) as well as by insects (*Lygus hesperus*, the western tarnished plant bug) (Powell et al., 2000; Shackel et al., 2005). PGIPs have been shown to be selective inhibitors of PGs produced by some fungal pathogens and insects, but were reported to be ineffective in inhibiting bacterial PGs (Cervone et al., 1990). However, Agüero et al. (2005) by introducing a pear fruit PGIP gene (Stotz et al., 1993; Powell et al., 2000) into transformed grapevines demonstrated that transgenic vines expressing the pear PGIP exhibit decreased susceptibility to both fungal (*B. cinerea*) and bacterial (*Xf*) pathogens. This result implied that the pear PGIP provided protection against PD by inhibiting the *Xf* PG virulence factor, and in vitro assays using purified, recombinant *Xf*PG expressed in *E. coli*, Roper (2006) demonstrated that *Xf*PG was inhibited by the pear PGIP (Labavitch, 2006). In addition, Agüero et al. (2005) demonstrated that transgenic pear PGIP could be transported across a graft junction of genetically engineered grapevines into the aerial portions of wild-type scions.

The overall goal of the project is to develop transgenic grape rootstock lines that optimally express PGIPs that most effectively inhibit *Xf*PG. The project is designed to identify PGIPs that most effectively inhibit *Xf*PG and to optimally express that PGIP in grape. The optimization of expression includes the use of transformation components with defined Intellectual Property (IP) and regulatory characteristics, as well as sequences that result in the maximal expression of the PGIPs in rootstocks and the efficient transport of PGIP proteins through the graft junctions to inhibit *Xf* PG produced by the pathogen in scions.

OBJECTIVES

1. Define a path for commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
2. Identify plant PGIPs that maximally inhibit *Xf* PG.
3. Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.
4. Create PGIP-expressing rootstocks and evaluate their PD resistance.

RESULTS AND DISCUSSION

Research Objectives for Year 1-

A. *Use existing pear PGIP-expressing grapes, test PD susceptibility of normal scions grafted to PGIP-expressing and -exporting roots.*

Agüero et al. (2005) described the use of transgenic 'Thompson Seedless (TS)' and 'Chardonnay (Ch)' grapevines expressing the pear fruit PGIP in experiments that showed that (1) high level PGIP expression in grape tissues slows the development of PD symptoms in needle-inoculated vines and (2) PGIP expressed by a transgenic rootstock is transported via the xylem through the graft junction and into the stems of untransformed TS and Ch scions. The inoculation tests were performed on non-grafted transgenic vines, thus both root and shoot tissues would have been expressing the pear PGIP-encoding transgene (PcPGIP). However, to date, we have not shown that PGIP expressed in and translocated from roots into non-transgenic shoots can provide PD protection. Initial tests of this idea will use grafted portions of the transgenic TS and Ch vines that were generated by Dr. Agüero.

We (Greve and Labavitch) have maintained several of these transgenic grape lines and have increased the number of plants available vegetatively. In addition, we have confirmed that they still transport PGIP in the xylem sap. Zac Chestnut, a graduate student funded through this project, also has analyzed the plants by PCR to confirm PcPGIP expression. We are now planning our grafting strategy in consultation with Dr. Andy Walker. Over the Fall and Winter, we will generate plants with pear PGIP-expressing lines as rootstocks and non-transgenic lines as scions. These will be managed in the greenhouse over the Winter and the expression and transport of pear PGIP will be confirmed in early Spring. In Spring, we will do inoculations of these plants and comparable plants with non-transgenic roots and scions and follow the development of disease symptoms and, near the end of the incubation of these inoculated plants, use destructive sampling to determine the extent to which the *Xf* population has spread in the vine. We will use both the virulent "Fetzer" *Xf* strain and the "Fetzer" strain whose single PG-encoding gene was knocked out (Roper et al., 2007). Dr. Roper used the PG knock-out line to demonstrate that the pathogen's PG is a PD virulence factor.

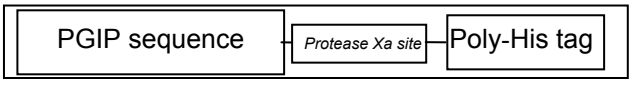

Figure 1. Schematic diagram of constructs for PGIPs and PGIP-like proteins linked to the cleavable poly-His tag and expressed in Arabidopsis.

B. *Express PGIPs in* Arabidopsis *and test for optimal inhibition of* Xf *PG.*

Our strategy is to identify plant PGIPs that are maximally effective in inhibition of the *Xf* PG and identifying that optimal PGIP is another objective of the first year's work. *Arabidopsis* lines have been transformed through the floral dip technique using *Agrobacterium*-mediated transformation vectors (Clough and Bent, 1998). Transformation vectors were assembled using pCAMBIA vectors (http://www.cambia.org.au/daisy/cambia/585.html) that are free from intellectual property restrictions. We have successfully expressed sequences encoding five fruit PGIPs (one from pear and four from tomato) using the CAMBIA vector p1301, linking the PGIPs to the poly-His tag with an intervening protease Xa cleavage site (**Figure 1**). To prepare the transformation vectors, we removed the GUS coding region from p1301 and replaced that sequence with the PGIP full-length coding sequences including the signal peptide sequences for extracellular targeting. We have self-crossed these lines and obtained homozygous progeny identified by resistance to the selectable marker and by PCR for the transgenic sequences. We are evaluating whether the PGIPs we have expressed in *Arabidopsis* using the modified p1301 and the CaMV 35S promoter are active and have appropriate inhibition specificities, and we will continue to include the intervening protease Xa cleavage site so that, if it is necessary for obtaining active inhibiting protein, the poly-His tag used for affinity purification of the expressed PGIPs can be removed by protease Xa after purification. We also have identified three *M. truncatula* PGIP-like sequences and are in the midst of preparing *Arabidopsis* transformation vectors for their expression. For this proposal, we would like to add to the collection 5-6 other PGIP-like sequences that we have identified based on phylogenetic comparisions and charge comparisons of PGIPs (**Tables 1 & 2; Figure 2**).

The total charge on proteins is determined by pH and the sequences of each protein. The total charge of the proteins may serve as a general guide towards predicting whether PGs and PGIPs interact and therefore whether a specific PGIP is likely to

inhibit specific PGs. Certainly, the specific local chemistry is most important, but the total charge may serve as a deal breaker, so to speak, of the possibility that a PG interacts with a PGIP. Dan King (Taylor Univ.) is beginning to examine the *Xf*PG protein, as it is quite unusual. The charge of the *Xf*PG is unusually positive (+22). The only PGs King has come across with such positive charges are putative plant PGs, such as a grape PG (AAK81876). Interestingly, the grape PGIP is particularly positive as well (+19). Regardless of local chemistries, it would be hard to imagine the *Xf*PG and the grape PGIP proteins having a strong interaction for each other. From another point of view, the pear PGIP has shown some ability to inhibit the *Xf*PG, and the pear PGIP has a particularly small charge (+9). **Table 1** shows some examples of the total charges of PGs and PGIPs at six pH values.

Table 1. Total Protein Charge vs. pH of selected PGs and PGIPs

	PG									PGIP								
pH	*Xf* PG	*F. moniforme* PG	*A. niger* PGC	*A. niger* PGB	*A. niger* PGA	*A. niger* PG2	*A. niger* PG1	Tomato PG	Grape PG	Grape	Pear	Bean2	Bean1	Tomato	Arabidopsis 1	Arabidopsis 2	Apple	Kiwi
3.50	40.99	17.90	4.79	27.25	3.88	21.94	10.46	30.88	32.19	31.51	23.07	22.74	24.74	30.82	28.52	36.37	24.22	25.21
4.00	31.30	11.25	-9.53	18.45	-11.40	12.86	-2.13	25.51	27.26	24.98	16.07	17.62	19.62	24.43	21.86	29.49	17.42	18.85
4.50	22.24	5.44	-23.56	9.92	-26.43	4.08	-14.38	20.03	22.4	18.63	9.27	12.95	14.95	18.01	15.23	22.56	10.88	13.31
5.00	16.39	2.07	-32.08	4.67	-35.56	-1.35	-21.80	16.26	19.23	14.54	5.03	10.17	12.17	13.8	10.96	18.02	6.793	10.18
5.50	11.90	-0.13	-36.17	1.86	-39.77	-4.36	-25.41	13.54	17.15	11.84	2.64	8.55	10.55	11.14	8.405	15.22	4.344	8.333
6.00	6.79	-2.36	-38.46	-0.20	-41.90	-6.69	-27.53	10.68	15.14	9.21	0.73	7.13	9.129	8.733	6.236	12.75	2.216	6.637

This total charge analysis has suggested to us that by examining PGIPs that have been identified in other plants, we can select PGIPs that are likely to be good candidates for inhibiting *Xf*PG and express these PGIPs in *Arabidopsis* for evaluation. To accomplish this, we have identified 52 non-redundant PGIPs in GenBank (**Table 2**) and we have evaluated their sequence similarities (**Figure 2**).

Table 2. List of PGIP sources.

Common name	Species	Protein [a]	Accession Number[b]
Common bean, pinto bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP1	AJ864506
Common bean, pinto bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP2	AJ864507
Common bean, pinto bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP3	AJ864508
Common bean, pinto bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP4	AJ864509
Soybean	*Glycine max* cv. Williams 82	GmPGIP1	AJ972660
Soybean	*Glycine max* cv. Williams 82	GmPGIP2	AJ972661
Soybean	*Glycine max* cv. Williams 82	GmPGIP3	AJ972662
Soybean	*Glycine max* cv. Williams 82	GmPGIP4	AJ972663
Ume (Japanaese apricot)	*Prunus mume* cv. Dali	PmuPGIP	DQ364056
Chinese plum, Japanese plum	*Prunus salicina* cv. xiaohuangli	PsaPGIP	DQ364055
Common pepper	*Capsicum annum* cv. arka abhir	CaPGIP	AM181174
Chinese cabbage	*Brassica rapa* subsp. *pekinensis*	BrPGIP	AY964100
Granny Smith apple	*Malus* x *domestica* cv. Granny Smith	MdPGIP	DQ185063
Peach	*Prunus persica*	PpePGIP	AY903218
Wild plum, American plum	*Prunus americana*	PamPGIP	AY883418
Flemish Beauty pear	*Pyrus communis* cv. Flemish Beauty	PcFPGIP	AY333105
Asian pear	*Pyrus pyrifolia* cv. Kinchaku	PpyKuPGIP	AY333103
Potato	*Solanum tuberosum* cv. Istrinskii	StPGIP	AY662681
Wild carrot	*Daucus carota*	DcPGIP	AY081214
False Spiraea	*Sorbaria sorbifolia*	SsPGIP	AF196947
Chinese Firethorn	*Pyracantha fortuneana*	PfPGIP	AF196929
Taiwanese Photinia	*Photinia serratifolia*	PsePGIP	AF196907
Oneseed Hawthorne	*Crataegus monogyna*	CmPGIP	AF196881
Mahaleb cherry	*Prunus mahaleb*	PmaPGIP	AF263465
Bartlett pear	*Pyrus communis* cv. Bartlett	PcBPGIP	L09264
Thale cress	*Arabidopsis thaliana* (Col.)	AtPGIP1	NM_120769
Thale cress	*Arabidopsis thaliana* (Col.)	AtPGIP2	NM_120770
Bowman's root	*Gillenia trifoliata*	GtPGIP	AF196915
Flowering Quince	*Chaenomeles speciosa*	CspPGIP	AF196871
Rape	*Brassica napus* cv. DH12075	BnPGIP1	AF529692
Rape	*Brassica napus* cv. DH12075	BnPGIP2	AF529694
Rape	*Brassica napus* cv. DH12075	BnPGIP3	AF531456
Rape	*Brassica napus* cv. DH12075	BnPGIP4	AF531457
Apricot	*Prunus armeniaca* cv. Marille Bauer	ParPGIP	AF020785
Cherry tomato	*Solanum lycopersicum* cv. VFNT Cherry	LePGIP	L26529
Kiwi	*Actinidia deliciosa* cv. Hayward	AdPGIP	Z49063
Iyokan	*Citrus iyo*	CiPGIP1	AB016205
Iyokan	*Citrus iyo*	CiPGIP2	AB016206
Rough lemon	*Citrus jambhiri*	CjPGIP1	AB013397
Rough lemon	*Citrus jambhiri*	CjPGIP2	AB015198
Sweet orange	*Citrus sinensis* cv. Hamlin	CsiPGIP	Y08618
Mikan	*Citrus unshiu*	CuPGIP	AB016204
Carrot	*Daucus carota* cv. Autumn King	DcAFP	AF055480
Red River Gum	*Eucalyptus camaldulensis*	EcPGIP	AF159168
Rose gum (Flooded gum)	*Eucalyptus grandis*	EgPGIP	AF159167
Shining gum	*Eucalyptus nitens*	EnPGIP	AF159171
Sydney Blue Gum	*Eucalyptus saligna*	EsPGIP	AF159170
Timor White Gum	*Eucalyptus urophylla*	EuPGIP	AF159169
Kumquat	*Fortunella margarita*	FmPGIP	AB020529
Trifoliate orange	*Poncirus trifoliata*	PtPGIP	AB020528
Asian pear	*Pyrus pyrifolia* cv. Kikusui	PpyKiPGIP	AB021791
Red raspberry	*Rubus idaeus* cv. Autumn Bliss	RiPGIP	AJ620336

[a]Protein names given to match abbreviations used in **Figure 2.**
[b]GenBank nucleotide accession numbers.

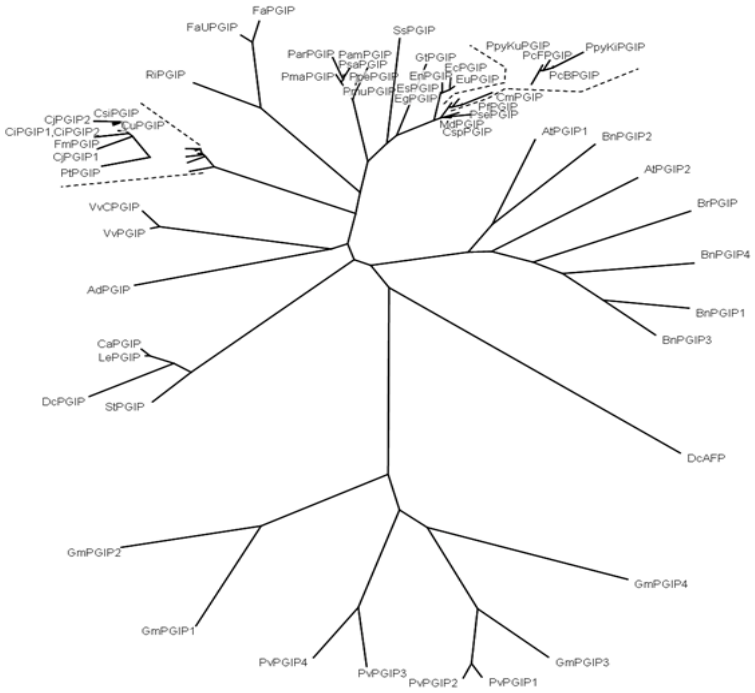

Figure 2. Unrooted phylogenetic tree of PGIPs.

C. *Optimally express* Xf *PG, using recombinant protein expression systems*

Since previous *E. coli* PG over-expression studies resulted in significant amounts of protein but little activity (Roper, 2006), we (with co-PI Booth at Texas State U. - San Marcos) will utilize a Drosophila expression system. For expression in Drosophila, we probably want two versions of the *Xf*PG, if possible; one with the antibody epitope and one without (in case the epitope causes trouble). The *Xf*PG gene will be ligated into pMTBiP/V5-HisA (**Figure 3**) and transformed into Drosophila Schneider 2 cells using protocols provided by Invitrogen and modified by Prof. Booth. Existing cloned *Xf*PG will be reamplified by PCR to create an NcoI site at the N terminus and probably an XhoI site at the C terminus. The sequence of the *Xf*PG will be checked and the C terminus of the inserted *Xf*PG will be modified by PCR to produce an in-frame fusion to C terminal additions (V5 (the epitope for detection) + 6xHis (for affinity purification with Ni)) without an intervening stop codon. A second expression construct will also be prepared that includes the *Xf*PG stop codon before the C terminal additions in case they interfere with protein folding. More detailed assays of active preparations (Gross, 1982; Roper et al., 2007) will be done at UC Davis to characterize the expressed recombinant *Xf* PG. Once enzyme activity is confirmed, expression will be optimized by varying the concentration and time of exposure to the inducer followed by purification with nickel affinity chromatography.

D. *Evaluate IP and licensing status of the plant expression construct components for the PGIP-based rootstock strategy.*

For this project, we proposed to develop a PGIP plant transformation construct that optimally confers tolerance to PD AND that has maximum legal freedom-to-operate (FTO) with respect to the underlying intellectual property, thus providing the maximum potential to gain regulatory approval. The results of a preliminary IP audit of the original PGIP expression construct (see Agüero et al. 2005) revealed the DNA module contained a number of proprietary components including: the CaMV35S promoter and the nptII (kanamycin) selection marker patented by Monsanto Company, the GUS marker gene patented by CAMBIA, and the pear PGIP gene patented by the University of California. Licensing these proprietary technologies, particularly from the private sector, may prove to be a hurdle for commercial deployment. Furthermore, this analysis also noted the presence of the GUS-marker gene and His tags, which although necessary during the proof-of-concept phase, may compromise downstream regulatory approval of commercial transgenic plants. To advance this project beyond

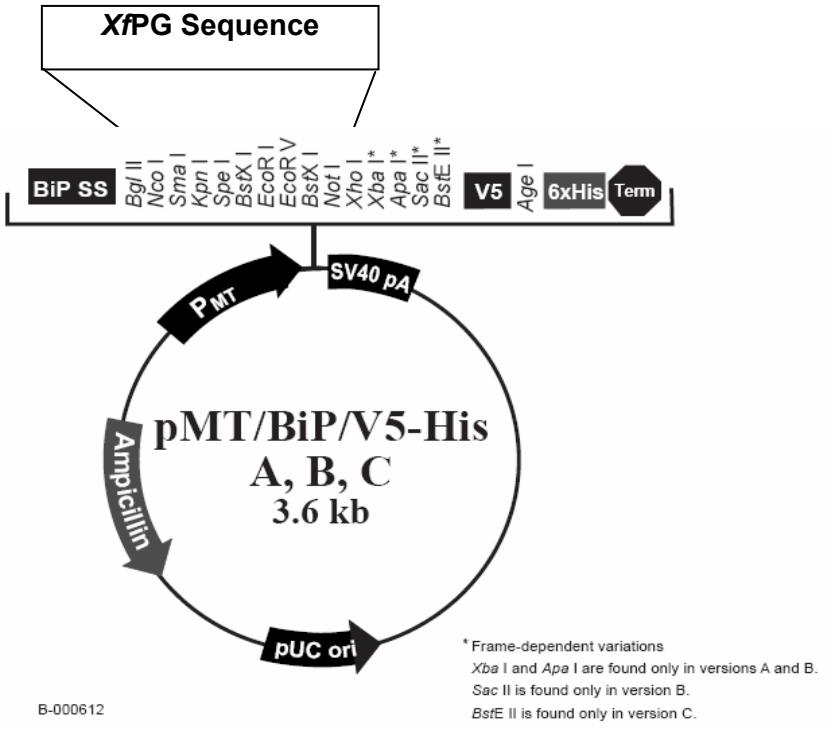

Figure 3. Schmatic diagram of the pMTBiP vector used to transform Drosophila cells for *Xf*PG expression

the "proof of concept" stage, the Public Intellectual Property Resource for Agriculture (PIPRA) is conducting an in-depth analysis of all component technologies that will be integrated into the PGIP gene construct as well as the enabling technologies required to transfer the PGIP construct into a grape rootstock. This analysis also will assess the likelihood that components of the PGIP gene construct will be able to gain regulatory approval for commercialization.

CONCLUSIONS

1. Because of the limited time that the funds have been available for this project (2 months), this report documents only the activities accomplished in that time.
2. *Xf*PG protein has an unusually high positive charge at the pH expected in plant tissue.
3. The sequences for currently available PGIPs have been collected and compared so selections of additional PGIPs to be expressed in *Arabidopsis* represent the diversity of PGIP sequences.
4. The total charge of the PGIP proteins can be used as an indicator of the likelihood of interaction with and inhibition of the *Xf*PG.

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