**Progress report for CDFA Project # 08-0171**

## I. Project title

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

## II. Principal investigators and cooperators


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## III. List of objectives and description of activities conducted to accomplish each objective

(This report covers research progress from July 1, 2010 through February 28, 2011)

***Objective 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.

- **A.** Evaluate IP and licensing status of the plant expression construct components for the PGIP-based rootstock strategy (Year 1)
- **B.** Assemble grape transformation vectors utilizing PIPRA vectors with defined IP characteristics (Year 2)

***Objective 2*:** Identify plant PGIPs that maximally inhibit *X. fastidiosa* PG.

- **A.** Use existing pear PGIP-expressing grapes, test PD susceptibility of normal scions grafted to PGIP-expressing and -exporting roots (Years 1 and 2)
- **B.** Identify plant PGIPs that are efficient inhibitors of *Xf*PG (Year 1)
- **C.** Express PGIPs in *Arabidopsis thaliana* and test for optimal inhibition of *X. fastidiosa* PG (Years 1 and 2)
- **D.** Optimally express *X. fastidiosa* PG, using recombinant protein expression systems (Year 1)
- **E.** Model PGIP and *X. fastidiosa* PG interactions to identify optimal PGIPs for PD defense (Years 1 and 2)

***Objective 3*:** Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.

- **A.** Make transformed grape lines using the best PGIP candidates, promoters etc. (Years 2 and 3)

***Objective 4*:** Create PGIP-expressing rootstocks and evaluate their PD resistance.

- **A.** Molecular analysis of putative marker free transgenic grape plants (Year 3)

**B.** Evaluate transgenic grape lines for optimal expression and export to scions of selected PGIPs (Year 3)

**C.** Evaluate transgenic lines for susceptibility to *X. fastidiosa* (Year 3)

## IV. Summary of major research accomplishments and results for each objective

*Objective 1: A path to commercialization of transgenic rootstocks*

**A.** PIPRA Intellectual property (IP) analyst, Gabriel Paulino, has served as the main liaison for issues associated with the potential commercialization of transgenic grapevine rootstocks for several CDFA PD/GWSS Board funded projects. He has obtained the necessary APHIS-USDA authorizations to test PGIP-based PD control strategies in vineyards in Solano and Riverside Counties. 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pear fruit PGIP (pPGIP) gene were planted in a jointly operated field trial in Solano County during July, 2010. More details can be found in the report "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell).

IP analysis was done on ten PGIP sequences (**Table 1**).

**IP summary table**

| PGIP Sequence | | No. Disclosures | No. Patents |
|---|---|---|---|
| AJ864507<br>PvPGIP (Broadbean) | NUCLEOTIDE | 9 | 4 |
| | PROTEIN | 9 | 2 |
| AM180652<br>OsPGIP1 (Rice) | NUCLEOTIDE | 10 | 8 |
| | PROTEIN | 4 | 2 |
| EU711352<br>GsPGIP (Cotton) | NUCLEOTIDE | 11 | 3 |
| | PROTEIN | 0 | 0 |
| NM120770<br>AtPGIP2 (Arabidopsis) | NUCLEOTIDE | 9 | 5 |
| | PROTEIN | 6 | 0 |
| LO9264<br>PvPGIP (Pear) | NUCLEOTIDE | 6 | 0 |
| | PROTEIN | 1 | 0 |
| AF499451<br>VvPGIP (Grape) | NUCLEOTIDE | 1 | 0 |
| | PROTEIN | 0 | 0 |
| L26529<br>LePGIP (Tomato) | NUCLEOTIDE | 0 | 0 |
| | PROTEIN | 1 | 0 |
| AY903218<br>PpePGIP (Peach) | NUCLEOTIDE | 1 | 0 |
| | PROTEIN | 1 | 0 |
| Mehill 2004 | NUCLEOTIDE | 0 | 0 |
| | PROTEIN | 0 | 0 |
| Y08618<br>CsiPGIP (Citrus) | NUCLEOTIDE | 0 | 0 |
| | PROTEIN | 0 | 0 |

**Table 1**, Summarized Intellectual Property analysis of plant PGIPs.

Of the sequences examined, none are associated with active patents. Applications were made about 2 years ago on three families of PGIP proteins and these applications will be reexamined to see if they are still active. None of the PGIPs that will be pursued in detail (see below) have active patents.

**B.** PIPRA has assembled a transformation vector to optimize eventual freedom to operate (FTO) issues for commercialization. This vector (pPIPRA, Figure 1) will be used to transform rootstock grape lines for PGIP expression.

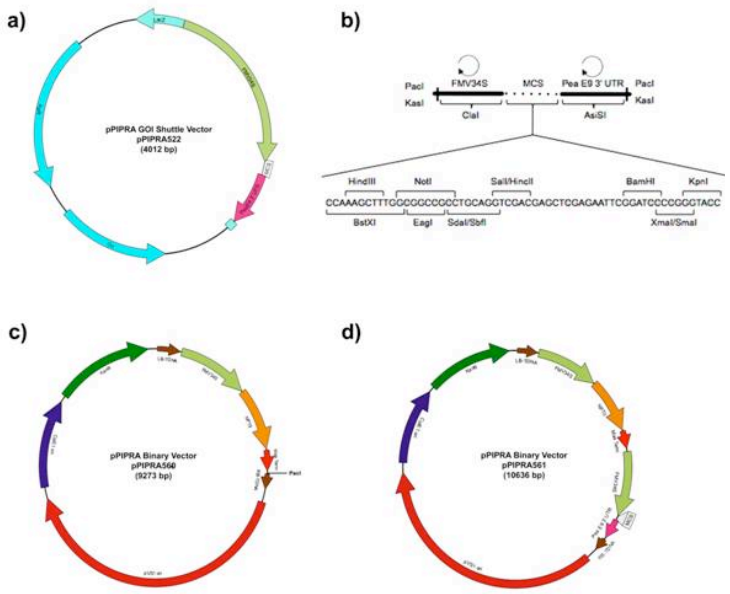


**Figure 1**. (**a**) pPIPRA Gene of Interest (GOI) Shuttle Vector Map. is a pUC19 vector with a modified multi-cloning site (MCS) that allows two-step cloning of GOI into the plant transformation vector. (**b**) GOI cassette with MCS, with unique restriction sites. The promoter is FMV34S and the 3'UTR is pea Rubisco E9. The GOI cassette can be inserted into the pPIPRA binary T-DNA vector at the PacI (direct cloning), and KasI (indirect cloning, involving blunt treatment of both GOI cassette and binary vector) sites. (**c**) pPIPRA binary T-DNA vector has maximum FTO and includes, ColE1 for *E. coli* and pVS1 for maintenance in *Agrobacterium*/*Rhizobium* and kanamycin for bacterial resistance. This binary vector has the FMV34S promoter, NPTII and Mas terminator as the selectable marker cassette. (**d**) Complete pPIPRA binary T-DNA vector

*Objective 2: Identify plant PGIPs that maximally inhibit X. fastidiosa PG*

**A.** Propagation, grafting and susceptibility testing of grape lines expressing and exporting pPGIP

The transgenic 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pPGIP described in Aguero *et al.* (2005) have been maintained in the UC Davis Core Greenhouse Complex. More individual plants of each cultivar expressing pPGIP and control plants not expressing pPGIP have been rooted during this reporting period with the help of an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA). Non-lignified stem segments, three nodes in length, were transferred to individual sites within the cloner. Roots began forming on dark-grown, constantly misted basal regions in 1-2 weeks. The application of 1000 ppm IBA to basal regions immediately after cutting did not result in increased rooting time or yield.

Grafted plants are being generated to verify the transport of pPGIP protein from transgenic rootstocks, across the graft junction, into scion tissue not expressing any foreign PGIP. Grafts of six 'Thompson Seedless' plants and one 'Chardonnay' plant have been formed by a modified wedge grafting technique whereby scion sections of 1 to 2 nodes were stripped of foliage and cut with perpendicular apical ends and wedge basal ends. These sections were fitted into notched rootstock stems of equal maturity. The grafts were secured with Parafilm M, a clothespin, and a translucent bag to prevent desiccation. Other green grafting techniques, such as chip budding, have been attempted with limited success.

UC Davis Biochemistry and Molecular Biology Ph.D. candidate, Victor Haroldsen, has shown that pPGIP protein produced in transgenic rootstocks crosses the graft junction and can be identified in scions of grafted grape and tomato plants. The pPGIP protein has been identified in wild-type tomato scion leaf tissue (Figure 2). For these experiments, Haroldsen used existing stocks of polyclonal pPGIP antibodies after concentrating leaf extract samples 30-fold. Once the monoclonal antibody we are preparing (PI Powell) is available, its increased specificity will be used for quantification of pPGIP crossing the graft junction into wild-type tissues.

To date, insufficient numbers of grafted plants have been generated to allow testing of the PD susceptibility of the scion portions of plants with pPGIP expressing rootstocks.

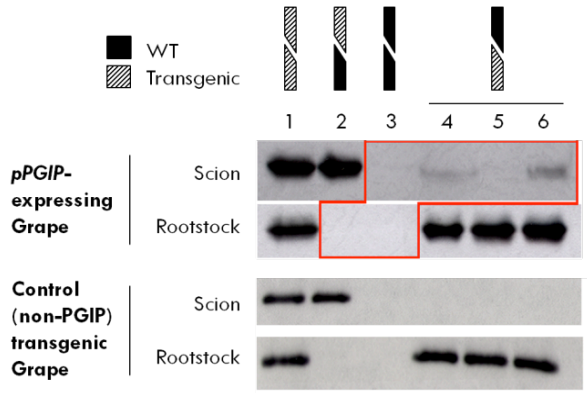


**Figure 2.** Western blot of leaf extracts taken from rootstock and scion portions of grafted 'Thompson Seedless' grapevines. Transgenic vines are expressing either pPGIP or NPTII (control). pPGIP is visualized crossing from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4-6). This movement is not seen in the reciprocal graft (lane 2).

**B.** Selection of PGIPs as PD defense candidates and PGIP-XfPG modeling

Fourteen candidate PGIPs were previously selected for *in vitro* and *in vivo* XfPG inhibition assays based on predicted protein charge and phylogenetic analyses. The homology models created for XfPG, the polygalacturonic acid (PGA) substrate for PG, and each of the candidate PGIPs have provided unique predictive tools to interpret the inhibition mechanisms and physical interactions between XfPG and the PGIPs (Labavitch, 2009). Dynamic *in silico* reaction simulations predicted that two clusters of amino acids, #63-74 and #223-226, must be unblocked for XfPG to cleave PGA. The long columns of electronegative residues on the concave faces of the PGIP's leucine rich repeat structure bind to these critical regions (Fig. 3). This information coupled with surface chemistry mapping predicts that pPGIP, CsiPGIP (citrus), and OsPGIP1 (rice) will be the best inhibitors of XfPG.

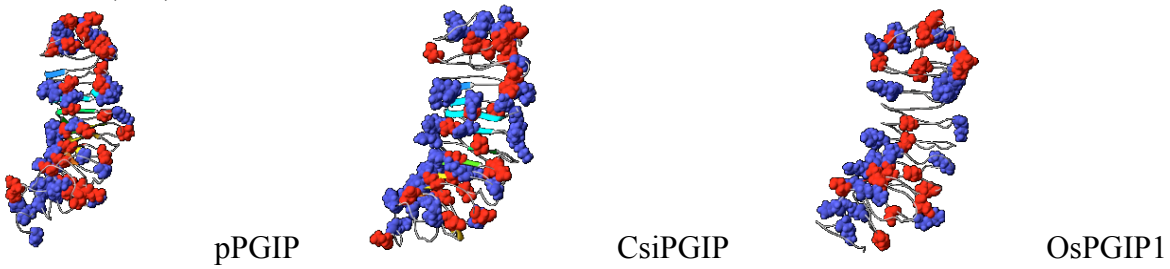


**Figure 3**. Homology models of three PGIPs predicted to be good candidates to inhibit XfPG. The column of electronegative residues (red) on the concave face of each protein may align with critical residues on XfPG important for inhibition.

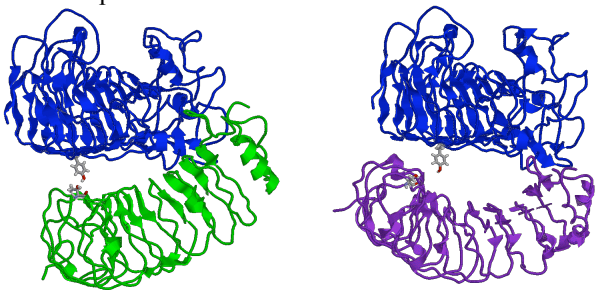

**Figure 4.** PG-PGIP complexes. Tyr303 of XfPG (blue) binds strongly with a region of pPGIP (green); this is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

A closer look at the dynamic reaction simulations highlighted other residues that may also influence PG-PGIP binding. Strong hydrogen bonding occurs between residues on pPGIP and Tyr303 of XfPG, bringing them together in a potentially inhibitory manner (Fig. 4). Electrostatic repulsions between VvPGIP residues and XfPG Tyr303 prevent a

similar alignment and may predict a failure to inhibit XfPG. Combining modeling predictions and future inhibition data will allow us to evaluate the predicted interactions and infer other potentially useful interactions between the candidate PGIPs and other PGs.

Adding to the information gained from the 14 candidate PGIP homology models, other unpublished PGIP sequences from non-vinifera *Vitis* varieties will be modeled in the future. These sequences are being pursued as part of a collaboration, currently in negotiation, with a research group at Stellenbosch University, South Africa. The sequences are the property of an industry board associated with the Institute for Wine Biotechnology at Stellenbosch University. We will be making models of these non-vinifera PGIPs to compare to the modeled structure of VvPGIP from *Vitis vinifera* cv. 'Pinotage'.

**C.** XfPG expression and purification

The XfPG expression system utilizing Drosophila S2 cells was developed to yield sufficient amounts of active, stable XfPG protein for *in vitro* inhibition assays. The cloning strategy fused the coding sequence of XfPG to a C-terminal histidine tag for purification and an N-terminal targeting sequence for protein secretion (Labavitch, 2009). The medium from transiently transfected cells induced to express XfPG had a small amount of PG activity, as shown by radial diffusion assay (Taylor and Secor, 1988); this activity decreased over time. Current work is focused on generating stably transfected recombinant cell lines to provide more consistent stocks of XfPG.

**D.** Expression of PGIPs in Arabidopsis and tobacco for XfPG assays

| **Protein (Organism)** | **Cloning Progress Checkpoints** | | | | |
|---|---|---|---|---|---|
| | Source tissue acquired | PGIP cDNA isolated | Transformed into *E. coli* | Transformed into *Agrobacterium* | Plant transformation |
| AtPGIP1 (Arabidopsis) | ✓ | ✓ | ✓ | O | - |
| AtPGIP2 (Arabidopsis) | ✓ | ✓ | ✓ | O | - |
| BnPGIP1 (Rapeseed) | ✓ | ✓ | O | - | - |
| CaPGIP (Pepper) | ✓ | O | - | - | - |
| CsiPGIP (Orange) | ✓ | O | - | - | - |
| FaPGIP (Strawberry) | ✓ | ✓ | O | - | - |
| OsPGIP1 (Rice) | ✓ | ✓ | O | - | - |
| OsPGIP2 (Rice) | ✓ | ✓ | O | - | - |
| PvPGIP2 (Bean) | ✓ | ✓ | O | - | - |
| PpePGIP (Peach) | O | - | - | - | - |
| PfPGIP (Firethorn) | ✓ | O | - | - | - |
| pPGIP (Pear) | ✓ | ✓ | ✓ | ✓ | ✓ |
| LePGIP (Tomato) | ✓ | ✓ | ✓ | ✓ | O |
| VvPGIP (Grape) | O | - | - | - | - |
| XfPG (Xylella) | ✓ | ✓ | ✓ | ✓ | ✓ |
| pPGIP::XfPG | ✓ | ✓ | ✓ | O | - |

**Table 2.** Cloning progress chart. Checkmarks indicate completed checkpoints while circles indicate work in progress.

The strategy for cloning each of the 14 candidate PGIPs for transformation into *Agrobacterium tumefaciens* (EHA105 pCH32) continues (Table 2; Labavitch, 2009). The full-length XfPG construct was successfully cloned into the transformation vector which was then transformed into *Agrobacterium*. This construct and the pPGIP::XfPG fusion construct (in progress) provide a potential diagnostic tool to test the efficacy of each PGIP *in planta* using an infiltration system (described below). The advantage of this assay is that it should be quicker than testing the lines in Arabidopsis or grape stably transformed to express PGIPs. It has been reported that the infiltration assay will work on grape and tomato leaves and as this approach provides advantages in terms of time and cost, we will continue to develop and use this echnique for testing the inhibition of PGs by different test PGIPs.

Co-infiltration of *Agrobacterium* cultures harboring XfPG and either pPGIP or LePGIP (tomato PGIP) in pCAMBIA-1301 was carried out as described by Joubert *et al.* (2007). Fully formed leaves of *Nicotiana benthamiana* and *N. tabacum* were infiltrated with constant manual pressure using a needle-less syringe, thus forcing bacterial cells into the abaxial leaf tissue. In most cases, initial infiltration zones were marked on the adaxial surface and had measured areas of approximately 35 $mm^2$. Visual symptom development was observed at 24 and 72 hours post infiltration (hpi, Figure 5). Infiltration with cultures harboring the XfPG construct resulted in marked wilting, localized water soaking, and chlorotic lesions developing in the infiltration zone. Leaves co-infiltrated with XfPG and PGIP cultures displayed attenuated symptoms while leaves infiltrated with just PGIP or empty vector cultures showed no symptom development. LePGIP was less effective than pPGIP at inhibiting wilting and lesion development when co-infiltrated with XfPG. Further work to quantify the results will provide a measure of the extent of XfPG inhibition by each cloned PGIP. We anticipate that the fusion construct pPGIP::XfPG will yield more easily scored results than the native XfPG construct because the pPGIP signal sequence has been shown to target the translated XfPG protein to the cell apoplastic space where it can either degrade the pectin-rich middle lamellae and cell walls or be inhibited by any co-infiltrated PGIP. PGIP is naturally targeted to the apoplast. Furthermore, the assay will be done using our existing lines that express the pPGIP (grape and tomato lines) or the LePGIP (tomato lines). We also anticipate doing this assay with the grafted lines which translocate the pPGIP from the rootstock into the scion (described above).

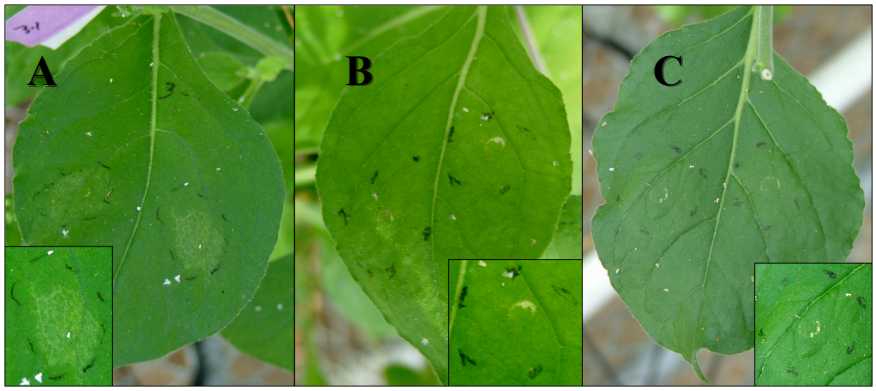


**Figure 5.** Transient expression of XfPG, pPGIP, and LePGIP in tobacco *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with XfPG (A). Symptoms are reduced when XfPG is co-infiltrated with *Agrobacterium* expressing the pPGIP (B) or LePGIP (C). Inserts show details of infiltration sites. Black marks indicate the borders of the initial leaf zone infiltrated.

**E.** Modeling of PGIP:XfPG interactions is covered under **B** above.

*Objective 3: Maximize PGIP expression in and transport from roots*

The transformation vector to be used in grape transformation has been reevaluated for its effectiveness. Information pertaining to potential signal sequences targeting PGIPs to xylem tissues for transport to and across graft junctions into wild-type scions has been reported by the project "*In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*" (PI: A. Dandekar).

*Objective 4: Create PGIP-expressing rootstocks and evaluate their PD resistance*

Multiple rootstock genotypes are being considered for transformation with the optimized vectors containing the candidate PGIP constructs. Different rootstock varieties are adapted to diverse climates, soil conditions, and disease pressures. St. George (Rupestris du Lot), 101-14 (Millardet et de Grasset), and Freedom rootstocks are transformable by the UC Davis Plant Transformation Facility, providing time for production of embryogenic calli. St. George and 101-14 are both adapted for moist, clay soils and have moderate to high phylloxera resistance; 101-14 is less resistant to high salinity and drought-prone, deep soils, but provides greater nematode resistance. Freedom provides a high level of nematode resistance, but is very susceptible to phylloxera and imparts higher than average scion vigor so it should be used in sandier soils. We are appraising the predicted usefulness of each of these varieties for our field evaluations in Solano and Riverside counties.

**Conclusions**

The ability of one of the candidate PGIPs discussed here, pPGIP, to provide PD resistance to wild-type scions will be determined by the field trials. This evaluation will be a key step in advancing the use of transgenic rootstocks for PD control in commercial applications becausethe pPGIP, thus far, is the only PGIP that has been demonstrated to inhibit XfPG Homology models of all 14 candidate PGIPs have been constructed and critical residues for XfPG-PGIP interaction were discovered. Recombinant XfPG, produced from transiently transfected Drosophila cells, was purified and shown to have a low level of PG activity. Further work to clone and express the candidate PGIPs continues. A more efficient assay, a co-infiltration assay on tobacco leaves, has been developed to assess PGIP inhibition of XfPG. Grape leaves will also be tested for their suitability for this assay. *In planta* co-infiltration assays have shown that both pPGIP and LePGIP are able to inhibit the chlorotic lesion development in tobacco leaves that is caused by XfPG-harboring *Agrobacterium*.

**References cited:**

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Stotz HU, Powell ALT, Damon SE, Greve LC, Bennett AB, Labavitch JM. 1993. Molecular Characterization of a Polygalacturonase Inhibitor from *Pyrus communis* L. cv Bartlett. Plant Physiol. 102: 133-138.

Taylor RJ, Secor GA. 1988. An Improved Diffusion Assay for Quantifying the Polygalacturonase Content of *Erwinia* Culture Filtrates. Phytopathol. 78: 1101-1103.

## V. Publications or reports resulting from the project: None

## VI. Presentations on research:

Chestnut, Zachary, Panel Discussion participant, Pathogen and Disease Management, 2010 Pierce's Disease Research Symposium, San Diego, CA, 15-17 December, 2010

Labavitch, J.M., Powell, A.L.T. Powell, Bennett, A., King, D., and Booth, R. 2010. Optimizing grape rootstock production and export of inhibitors of *Xylella fastidiosa* polygalacturonase activity. Proceedings of the 2010 Pierce's Disease Research Symposium, San Diego, CA, 15-17 December, 2010 (poster).

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Powell, A.L.T., Dandekar, A., Labavitch, J.M. Tools to identify polygalacturonase-inhibiting proteins transmitted across grapevine grafts. Proceedings of the 2010 Pierce's Disease Research Symposium, San Diego, CA, 15-17 December, 2010 (poster).

## VII. Research relevance statement

The CDFA Pierce's Disease (PD) and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery from grafted rootstocks of PD control factor candidates, including polygalacturonase-inhibiting proteins (PGIPs). Optimal PGIPs for inhibition of *Xylella fastidiosa* (*Xf*)

polygalacturonase (PG) are being selected from several plant sources. Fourteen candidate PGIPs have been chosen and homology models were generated to predict interactions with and potential inhibition of XfPG. PGIPs from pear, rice, and orange were determined to be the most likely inhibitory proteins for XfPG. Recombinant protein expression systems have been developed for XfPG and each candidate PGIP. Initial inhibition assays have shown that the pear fruit PGIP is a more effective inhibitor of XfPG than the PGIP from tomato, however both PGIPs limit XfPG symptom development in tobacco leaf infiltration assays. Expression of additional PGIPs to test is underway and other non-vinifera *Vitis* PGIPs are being pursued.

The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *X. fastidiosa*. The project is designed to identify specific PGIPs that optimally inhibit the virulence factor, XfPG, and to express these PGIPs in grape rootstocks to provide PD protection in scions. The expression of PGIPs will utilize transformation components with defined intellectual property (IP) and regulatory characteristics, as well as expression regulating sequences that result in the maximal production of PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of vines so that Xf movement is limited in infected scion tissues

## VIII. Lay summary of current year's results

*Xylella fastidiosa* (*Xf*) uses a key enzyme, polygalacturonase (PG), to spread from the initial point of inoculation throughout the grapevine; this spread leads to PD symptom development. Plant proteins called PG-inhibiting proteins (PGIPs) are produced by many plants and selectively inhibit PGs from bacteria, fungi, and insects. Pear fruit PGIP is known to inhibit XfPG and to limit PD development in inoculated grapevines which have been transformed to express the pear protein. PGIPs are graft transmissible so we are interested to determine which PGIP best inhibits XfPG and how effectively this PGIP prevents PD development in *Xf* inoculated wild-type scions when it is expressed in transgenic rootstocks. We have modeled 14 candidate PGIPs to predict how they physically interact with XfPG and to combine this knowledge with *in vitro* and *in planta* assay results measuring the ability of each candidate PGIP to inhibit XfPG. For these inhibition assays we are developing separate systems to generate high levels of active XfPG and PGIPs. The best XfPG inhibiting PGIPs will be expressed in test grape rootstock germplasm and, after grafting, their ability to limit PD development in non-transgenic scions will be determined.

## IX. Status of funds

We currently (as of 10 March 2011) have 47.3% of the original total budget of $520,428 remaining in the project account. This breaks down to 46% of the originally budgeted Personnel costs (including benefits), 48% of the originally budgeted Supplies and Other Expenses (including the costs of our sub-contracts with Taylor Univ. and Texas State Univ.) and 70% of the originally budgeted Travel expenses (only $4,430). We will be submitting a request for a no-cost extension, that will include some shifts of funds in the main budget categories.

## X. Summary and status of intellectual property produced during this research project:

No patents have been filed but the intellectual property status of PGIPs under consideration is described in Objective 1.