Project Report - March, 2012

Project Title: Genomic sequencing of biocontrol strain EB92-1 and identification of elicitor(s) of effective defense in *Vitis vinifera* against Pierce's Disease.

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Reporting Period: The results reported here are from work conducted from 11/13/09- 2/28/12

List of Objectives and Activities Accomplished:

Objective 1: obtain nearly the complete EB92-1 genome DNA sequence (1 year).

This work was completed, reported and made available online in 2010 and published last year (Zhang et al., 2011). Basically, the draft genome of 2,478,730 bps (~194X coverage) of *Xylella fastidiosa* (Xf) biocontrol strain EB92-1 was obtained using Roche 454 GS (FLX titanium) pyrosequencing. Based on the size of the PD strain Temecula1 genome (2,519,802 nt), this draft EB92-1 genome is ~98% complete The genome was assembled into 168 contigs with an average contig length of 12,977 bps using Newbler v. 2.3 (454 Life Sciences). There were 2,343 predicted protein-encoding genes. By far the majority of the primary BLAST (1) hits were to Temecula1; 92% of predicted EB92-1 proteins had more than 99% identity with Temecula1 proteins. Comparisons using MAUVE 2.3.1 revealed a high level of synteny with Temecula1. Plasmid sequence similar to the Temecula plasmid (pXFPD1.3) was also found in EB92-1. No unique or additional genes were found in EB92-1 that were not previously identified in Temecula1. However, eleven genes found in Temecula1 were not found in EB92-1; ten of these encoded predicted secreted pathogenicity effectors.

Objective 2: compare EB92-1 with Temecula and identify all unique ORFs and differences, ranking the top 40 candidate ORFs for evaluation as elicitors (1 month).

Essentially, the draft genome of *Xylella fastidiosa* (Xf) biocontrol strain EB92-1 revealed: 1) that it was nearly identical in gene order and sequence to Pierce's Disease (PD) strain Temecula; 2) no unique or additional genes were found in EB92-1 that were not previously identified in Temecula, and 3) EB92-1 appeared to be missing genes encoding 10 potential pathogenicity effectors found in Temecula (Zhang et al 2011). The latter included a type II

secreted lipase (LipA; PD1703), two identical genes from a duplicated prophage region encoding proteins similar to zonula occludens toxin (Zot; PD0915 and PD0928) and all six predicted hemagglutinin-like proteins (PD0986, PD1792, PD2108, PD2110, PD2116 and PD2118). PCR analyses and subsequent sequencing of the PCR products confirmed that all 10 genes were missing, at least from their expected locations, in EB92-1.

No additional hemolysins or colicins were found to date in the draft EB92.1 genome that were not found in Temecula. More importantly, no additional hemolysins or colicins were present in Temecula that were not found in EB92.1. The fact that the complete repertoir of known Temecula Type I effectors were found, with 100% identity, in EB92.1 (which does not cause PD), means that PD symptoms per se are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated (Flores-Cruz et al. 2009).

Objectives 3: evaluate two defense response assays designed to test the hypothesis that EB92-1 produces an elicitor that Temecula does not.

Objective 4: perform "avirulence" assay screens using up to 40 Temecula transconjugants carrying up to 40 candidate ORFs identified from the EB92-1 genome in Objective 2 (1 year)..

Objective 5: perform defense response assay screens to confirm any suspected elicitors identified in Objective 4 and identify any elicitors that may be polygenic in nature (ie., LPS or cell fractions) using the best of 3 assays determined by Objective 3 (1 year).

Ojectives 3, 4 and 5 were not pursued when it became apparent from the results of sequence comparisons in Objective 2 that Temecula produced pathogenicity effectors that EB92-1 did not. No genes were identified in EB92-1 that were not already present in Temecula. EB92-1 appeared to be a more highly evolved PD strain than Temecula, having lost specific pathogenicity factors---some redundant---that cause disease. In order to prove that this was indeed the case four of these missing genes were functionally tested to determine if they actually contributed to disease or not, and all four were found to enhance PD symptoms.

IV. Summary of major research accomplishments

A predicted type II secreted esterase, LipA, (PD1703), was entirely missing from EB92-1, evidently as a result of a deletion. This conclusion was confirmed by PCR analysis and subsequent sequencing. PD1703 is an apparent *lipA* ortholog of *Xanthomonas oryzae*; *lipA* is known to directly contribute to pathogenic symptoms of *X. oryzae* by degradation of host cell walls, eliciting programmed cell death.

The predicted type II secreted esterase, LipA (PD1703) exhibited strong esterase activity in *Xanthomonas* citri, *E.coli* and EB92-1 on agar plates. PD1703 was cloned with its native promoter (690bp) and predicted secretion leader peptide in pBBR1MCS-5 (downstream from the *lacZ* promoter), creating pSZ26. An *in vitro* lipase assay was conducted using Tween 20 as the substrate and 0.01% Victoria Blue B as indicator. Agar plates containing the substrate and indicator were poured and wells created by removal of agar with a cork borer. Culture supernatants from centrifuged cells grown to late mid-log phase (ca. OD = 0.7) were added to the

wells. The supernatant from the EB92-1 exconjugant carrying cloned PD1703 (pSZ26), demonstrated relatively strong amounts of secreted lipase in these culture supernatants (the crude supernatants were not concentrated or purified). These levels of lipase activity were not present in the supernatants of wild type Temecula or EB92-1, nor in *X. citri* B21.2, *E. coli* Mach1-T1, *X. citri* B21.2 and *E. coli* Mach1-T1 transconjugant with another lipase (PD1702, with its native promoter) cloned from Temecula1, or these same strains carrying the empty vector pBBR1MCS-5.

Crude protein extracts of the predicted type II secreted esterase, LipA (PD1703), overexpressed in BL21 (DE3)/pET-27b, induced hypersensitive cell collapse in tobacco and citrus. Crude protein was extracted from *E. coli* BL2 (DE3) carrying pET27b and expressing PD1703 from *lacZ* . An amount of crude protein suspension that measured 15-18 mg/ml elicited a rapid cell collapse that was visible starting 14 hrs post inoculation in tobacco. The reaction became stronger by 2 days. By comparison, 4-5 ug/ml of purified *X. oryzae* LipA were required to elicit browning of rice in infiltrated zones by degradation of cell walls.

Temecula PD1703 lipase (in pSZ26), PD0928 Zot (in pSZ41), and PD0986 (in pPC3.1) hemagglutinin all contribute to the pathogenic symptoms elicited by *Xylella fastidiosa* on grapes. The following results were reported in detail by Gabriel et al. (2011) at the PD annual meeting. PD1703 (lipase), PD0928 (ZOT) and PD0986 (hemagglutinin) were all cloned with their native promoters (690bp) in pBBR1MCS-5 (downstream from the *lacZ* promoter). For pathogenicity assays, 4-6 week old *V. vinifera* cv. Carignane were inoculated with 10 ul cultures of each strain. Bacterial cultures were diluted in SCP buffer and were inoculated by stem puncture.

Pathogenic symptoms elicited by Temecula began to appear by the 5th week after inoculation, and continued to develop up to 12 weeks. Most of the inoculated plants were killed after 12 weeks. Plants inoculated with EB92-1/pSZ26 (Lipase) and EB92-1/pSZ41 (ZOT) showed slightly delayed pathogenesis with visible symptoms becoming evident by the end of 6 weeks. The infection progressed slowly and remained restricted to 9-10 internodes in case of strains pSZ26 and pSZ41. At the end of 3 months, plants inoculated with EB92/pSZ26 reached a total of 30% infection and EB92/pSZ41 reached a total of 22 %. EB92-1/ pBBRMCS5 (empty vector) never reached higher than 12% during the period). Plants inoculated with EB92-1/pPC3.1 (hemagglutinin) exhibited symptoms almost as rapidly as the wild type (by the 5th week after inoculation) but with reduced severity (40% infection for EB92-1/pPC3.1 vs. 62% for Temecula by 48 days post inoculation).

The infection of EB92-1/pPC3.1 (hemagglutinin; PD0986) was surprisingly fast by comparison with the other two transconjugants, with visible symptoms elicited by the transconjugants becoming evident by the beginning of the 5th week in two independent experiments (15 grape plants inoculated with transconjugants total). All plants inoculated with these transconjugants showed prominent necrotic areas very similar to those observed with the wild type.

V. Publications or reports resulting from the project:

Gabriel, D. W. and D.L. Hopkins. 2009. Role of Type I Secretion in Pierce's Disease. Symposium Proceedings of the 2009 Annual Pierce's Disease Meeting, December 9-11, 2009, pp 86-91.

Flores-Cruz, Z, Reddy, S, Hopkins, D.L., and D. W. Gabriel. 2009. Potential offensive role of the Type I Secretion System in *Xylella fastidiosa. Poster* PS2-50, presented at the 2009 Annual Pierce's Disease Meeting, December 9-11, 2009.

Gabriel, D.W. and S. Zhang. 2010. Genomic sequencing of biocontrol strain EB92-1 and identification of elicitor(s) of effective defense in Vitis vinifera against Pierce's Disease. Symposium Proceedings of the 2010 Annual Pierce's Disease Meeting, Dec. 15-17, 2010, pp 84-86.

Zhang, S., Flores-Cruz, Z., Kumar, D., Chakrabarty, P., Hopkins, D.L. and D. W. Gabriel. 2011. The genome of *Xylella fastidiosa* biocontrol strain EB92-1 is very similar to Pierce's Disease strains. J Bacteriol 193: 5576–5577.

Gabriel, D.W., Zhang, S., and P. Chakrabarty. 2011. Three new pathogenicity effectors of Pierce's Disease not found in biocontrol strain EB92-1. Symposium Proceedings of the 2011 Annual Pierce's Disease Meeting, Dec. 13-15, 2011, pp 59-64.

VI. Presentations on research

Flores-Cruz, Z, Reddy, S, Hopkins, D.L., and D. W. Gabriel. 2009. Potential offensive role of the Type I Secretion System in *Xylella fastidiosa*. Poster presentation at the IS-MPMI meetings, July 19-23, Quebec, Canada.

Gabriel, D. W. and D.L. Hopkins. 2009. Role of Type I Secretion in Pierce's Disease. Symposium Proceedings of the 2009 Annual Pierce's Disease Meeting, December 9-11, 2009, pp 86-91.

Flores-Cruz, Z, Reddy, S, Hopkins, D.L., and D. W. Gabriel. 2009. Potential offensive role of the Type I Secretion System in *Xylella fastidiosa. Poster* PS2-50, presented at the 2009 Annual Pierce's Disease Meeting, December 9-11, 2009.

VII. Research relevance statement

Rarely has one genomic sequence provided so much specific guidance for identification of pathogenicity factors relevant to pathogenicity—in this case, PD caused by Xf. Of the 10 genes found missing in biocontrol strain EB92-1 but found in PD strain Temecula, nine appear to be involved in pathogenicity, based on functional analyses (one lipase, one zonular occludens toxin member (two are missing), and one hemagglutinin (six are missing). These genes were demonstrated to be at least partially responsible for generating PD symptoms. The one hemagglutin examined, in particular, exhibited strong symptoms when replaced in EB92-1. Therefore methods developed to interfere with the mechanism of action or the secretion of these

genes, in particular the hemagglutinins, would likely be able to control PD.

VIII. Lay summary of current year's results

Xylella fastidiosa (Xf) strain EB92-1 is infectious to grapevines but causes no symptoms and has been used for biological control of Pierce's Disease (PD). We determined the genomic DNA sequence of EB92-1 to 98% completion, allowing comparisons of this strain to strain Temecula, which causes PD. Most of the EB92-1 genes were nearly identical in gene order and protein sequence with those found in Temecula. No unique or additional genes were found in EB92-1 that were not previously identified in Temecula. However, 11 genes found in Temecula were not found in EB92-1; 10 of these encoded predicted secreted pathogenicity effectors that had not previously been associated with PD. Four of these missing genes were functionally tested to determine if they actually contributed to disease or not, and all four were found to enhance PD symptoms. This data identifies new molecular targets with potential to suppress disease symptoms.

IX. Status of funds

Approximately 80% of the funding provided has been spent, and the remainder will be spent on completing functional work for two additional publications anticipated from this award. 100% of the funds are expected to be spent by 8/31/12.

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

None generated to date.