GRAPE RECOGNITION OF *XYLELLA* SURFACE PROTEINS AND THEIR RELATIONSHIP TO PIERCE'S DISEASE SYMPTOM DEVELOPMENT

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Objectives and Activities
Previous results revealed that partially purified fractions containing the protein synthesis elongation factor-temperature unstable (EF-Tu) of *Xylella fastidiosa* (Xf) cause a chlorosis reaction when pressure-infiltrated into *Chenopodium quinoa* leaves. EF-Tu is generally recognized as a microbial signal which allows some plants to detect infection. Results from the laboratories of D.R. Cook and D.G. Gilchrist identified grape promoters, including the 9353 promoter, which is activated by infiltration of Xf cells. Purified *E. coli* EF-Tu protein alone is capable of stimulating this transcription (our previous results). This transcriptional stimulation may be part of the natural pathway of Pierce's disease (PD) symptom development and so may be an appropriate target for disrupting symptom development. Scorch symptoms seen in PD may be due to the innate immune response of the plant. An examination of this system, as proposed in the following specific objectives, will allow this notion to be tested.

Objectives

1. Test Xf EF-Tu for its ability to induce scorching in grape
2. Identify grape receptor for Xf EF-Tu
3. Interfere with Xf EF-Tu-induction of scorching using RNAi or by expression of alternative receptor
4. Characterization of Xf EF-Tu and its immobilization and localization

Production of Xf EF-Tu in vitro

A straightforward test under Obj. 1 would be to purify Xf EF-Tu and infiltrate it into grapevine and other plant leaves. We have observed that the bulk of the EF-Tu protein from Xf cells is present in an insoluble form, as described in previous progress reports, disallowing the use of conventional protein purification methods. One of our approaches to overcoming purification

problems is to synthesize Xf EF-Tu in a commercial *in vitro* protein production system (Thermo Scientific , Mikani et al. 2006). This system uses *in vitro* transcription catalyzed by T7 RNA polymerase followed by *in vitro* translation in an extract from human cell culture. We have cloned the Xf EF-Tu into the appropriate expression vector which contains an internal ribosome entry site for high level translation of uncapped mRNAs. The protein is C-terminally fused to a hexahistidine sequence for easy detection and purification. Initial expression experiments have shown production of the appropriately sized protein (Fig. 1A), based on previous Xf EF-Tu immunoblots of the authentic protein. The amount of protein produced was small, but as seen in Fig. 2, panel B, little full length mRNA was produced compared with the green fluorescent protein (GFP) control mRNA (compare lanes 1 and 3 in panels A and B, Fig. 2). It appears that the amount of plasmid DNA that we provided to the transcription reaction was insufficient (compare topmost band in lanes 1 and 3 of panel B). We aim to produce Xf EF-Tu in amounts comparable to what we have seen for GFP production, by increasing both the amount and purity of the plasmid DNA to be supplied to the transcription system. This should allow valid tests of the symptom-inducing capabilities of Xf EF-Tu in C. *quinoa* and grapevine, as well as grapevine bearing a construction in which the 9353 promoter drives GFP synthesis.

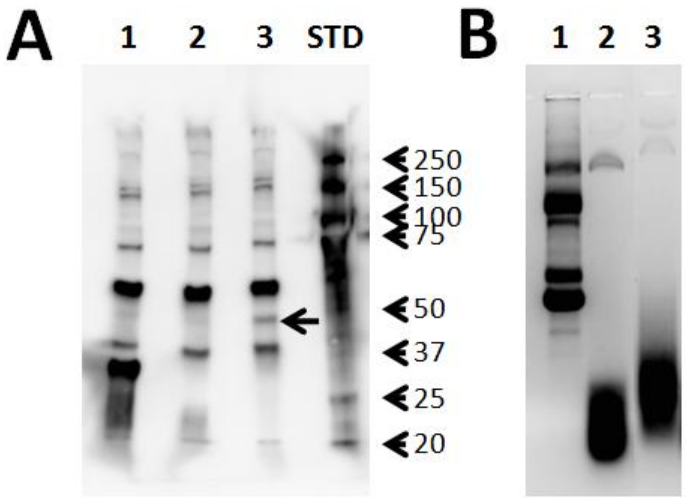

Figure 1. *In vitro* translation of Xf EF-Tu protein and analysis of the mRNA provided to the translation system. A. *In vitro* production of GFP protein (lane 1), cowpea mosaic virus 24KDa protease (lane 2), and Xf EF-Tu (lane 3) from *in vitro* transcribed mRNAs (panel B). The arrow next to lane 3 points out the band corresponding in mobility to the Xf EF-Tu protein. The protein was detected with anti-His_6 antibody. B. *In vitro* transcripts that programmed the *in vitro* translations. The lanes in panel B correspond to the proteins described for panel A.

Construction of Xf EF-Tu expression constructs for in planta expression

An alternative to *in vitro* production of Xf EF-Tu is *in planta* production. Two binary constructs are in progress that should allow us to produce the Xf EF-Tu protein with cytoplasmic or apoplastic targeting by agroinfiltration. Recognition of Xf EF-Tu is expected to be extracellular (apoplastic). The apoplastic targeting sequence from the plant pathogenesis protein PR-1(P14) (Vera et al. 1989), has been shown by Jim Lincoln of the David Gilchrist laboratory to direct green fluorescent protein (GFP) to the apoplast (Fig. 2.)

Two binary vector constructs for *Agrobacterium*-mediated expression are being made with and without the P14 signal peptide to determine if the recognition is taking place extracellularly as expected. Initially, the plan was to insert the P14 sequence at the amino end of Xf EF-Tu;

however, we were concerned that the amino end may not match the authentic amino end found in bacteria. The state of the amino end is likely to be significant because the known receptor for *E. coli* and other EF-Tu's in brassicas, EFR appears to recognize the EF-Tu amino end (Kunze et al. 2004). The construction in progress will have a *Cowpea mosaic virus* 24K protease (CPMV 24KPro, a protease capable of releasing itself from the interior region of a polyprotein) between the P14 signal peptide and the Xf EF-Tu coding sequence to create a defined amino end to the EF-Tu protein.

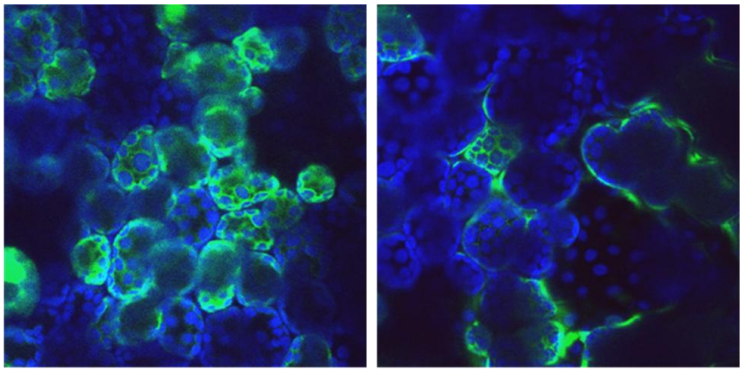

Figure 2. Expression of synthetic GFP protein (SGFP) from Agrobacterium in Xanthi tobacco with or without the P14 signal peptide. Notice the cytoplasmic localization of a SGFP alone (left panel) and the apoplastic localization with the P14 signal peptide (right panel). Image kindly provided by Dr. Jim Lincoln.

CPMV 24KPro is known to cleave specific Q/M or Q/G sites within the virus polyprotein to produce mature viral proteins. The CPMV 24KPro/Xf EF-Tu construct is designed such that the methionine immediately after the cleavage site is the first amino acid normally produced during translation of the Xf EF-Tu protein. Due to the presence of the CPMV 24KPro between the P14 signal peptide and the XF EF-Tu protein, we cannot be sure that the P14 will remain attached to the Xf EF-Tu protein long enough to successfully target the Xf EF-Tu to the apoplast. To test the effectiveness of the apoplast delivery, we are preparing a derivative of the P14-SGFP construct used in the right panel of Figure 2 in which a CPMV 24KPro is being placed between the P14 and the SGFP. By examining whether the SGFP is targeted to the apoplast or the cytoplasm, we will be able to determine whether the 24KPro action is slow enough to allow apoplastic delivery.

Once these constructs are completed, we will test them in *C. quinoa*, Arabidopsis, which is the source of the original EFR receptor, and in SR-1 tobacco, which has been shown in the Civerolo laboratory to mimic Pierce's disease symptom development (Francis et al. 2007). More importantly, we will be using them in transient expression assays in combination with Agrobacterium containing constructs that express authentic Arabidopsis EFR and the grape 9353 promoter-GFP constructs that was used by the Gilchrist laboratory to make their transgenic Thompson seedless grapevines that we have used previously (Gilchrist et al. 2007). We will be doing these assays in *Nicotiana benthamiana*, which does not contain its own EFR receptor. In addition, grapevine EFR homologs will be tested in place of the Arabidopsis EFR constuct. Any grapevine EFR homologs that stimulate 9353 promoter activity as determined by GFP expression levels in the presence of the Xf EF-Tu expression will be used to make RNAi constructs to test if such constructs can reduce Pierce's disease symptoms in the presence of wildtype Xf bacteria.

EF-Tu and mopB localization in Xf cells by electron microscopy and immunogold labeling

We have previously shown that treatment of Xf cells with Bugbuster (EMD BioSciences), a commercial detergent solution, and recombinant egg white lysozyme, followed by low speed centrifugation leads to recover of roughly two thirds of the original cell pellet material (Bruening et al. 2008). In contrast, similar treatment of *E. coli* cells or *Xanthomonas campestris* cells results in complete clearing of the solution with insignificant recovery of material after low speed centrifugation. Centrifugation of the Bugbuster and lysozyme-treated, centrifugation-recovered material through a 50 to 80% sucrose gradient, leads to a substantial band of intermediate density (Bruening et al. 2008) . Immunoblots of the proteins present shows that most of the EF-Tu protein is present in this sucrose buoyant band. Ms. Darlene Hoffmann of USDA-ARS Parlier examined these samples by electron microscopy with and without immunogold labeling and using anti-mopB and EF-Tu antibodies to probe intact HxfA- and HxfB- Xf cells. These Xf strains, containing transposon-inactivated genes for hemagglutinin-like proteins A and B, respectively, were selected because of their rapid growth in culture. Additionally, Bugbuster-treated cells, Bugbuster and lysozyme-treated material from the same cell types, and Bugbuster and lysozyme-treated material purified from the top of a 50% to 80% sucrose gradient also were examined.

Fig. 3, panel A, shows intact HxfA- Xf cells. I estimate that the long axes of the cell sections are approximately 2 microns long and the short axes are approximately 0.5 microns across. Fig. 3, panel B shows the HxfA- Xf cells treated with Bugbuster and recombinant egg white lysozyme. This is a typical field for this treatment. In other fields, a few intact cells are visible, although the majority of the material is these roughly circular sections of particles. The largest have a diameter of ~0.7 microns. Assuming that an intact cell has a surface area roughly equal to a cylinder with a diameter of 0.5 microns and a height of 2 microns, a 0.7 micron diameter spheres should have approximately the same surface area. This suggests that the material that makes up these spheres is either just outside or inside the cell's membrane. In the absence of some presumably internal structure that would confer on the cells a rod-like structure, it would be reasonable to assume that the cells would take on a spherical aspect. These spheres are not seen in electron micrographs of Xf cell treated by Bugbuster alone. Fig. 3, panels C and D, show the distribution of mopB and EF-Tu within intact HxfA- cells using immunogold labeling with antibodies against these two proteins. As expected, mopB, which is presumed to be the major outer membrane protein of Xf, is localized to the periphery of the cells. As EF-Tu is a component of the cellular translational machinery, it would be expected that it would be localized to the interior of the cells; however, the majority of this protein is also localized to the cellular periphery consistent with a possible role in a cytoskeleton-like structure.

Xf EF-Tu accessiblity changes upon Bugbuster and lysozyme treatment of Xf cells

We had previously tested Alexa-488-labeled anti-EF-Tu antibody with intact cells and seen little if any reaction. Given the appearance of the Bugbuster and lysozyme-treated HxfA- or B- Xf cell material as empty shells, we wanted to see if there was any change in the accessibility of the Xf EF-Tu, which we know by immunoblot analysis is still associated with this fraction. To do this, we exposed intact HxfB- Xf cells and HxfB- Xf cells that had been treated with Bugbuster

and lysozyme then recovered either by centrifugation, or by centrifugation through a 50% to 80% sucrose gradient to Alexa-488-labeled anti-EF-Tu antibody for 1 hour. The cells

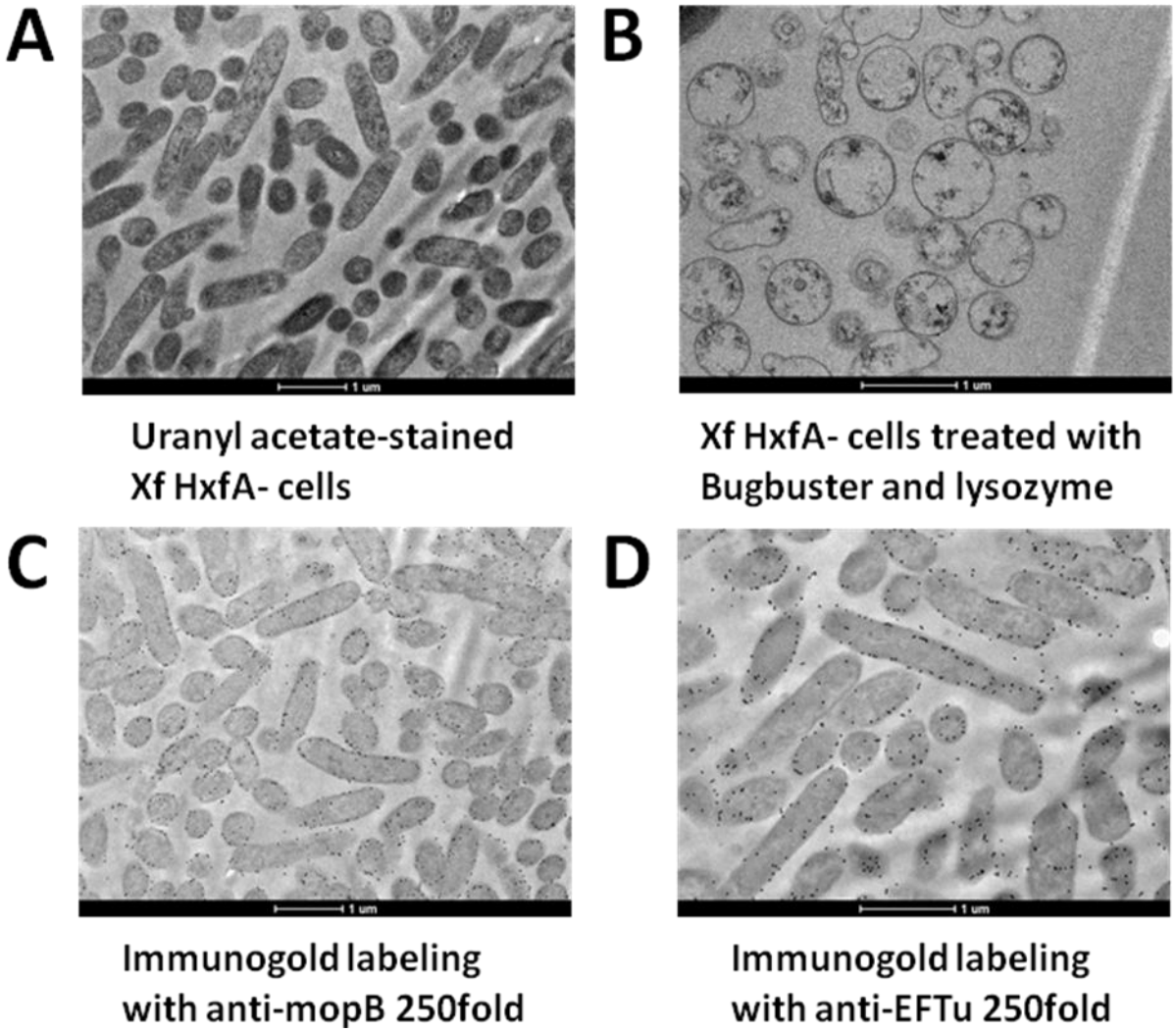

Figure 3. Thin section transmission electron micrographs of Xf cells with or without immunogold labeling with anti-mopB or EF-Tu antibodies. Panel A. Intact HxfA- Xf cells. Panel B. HxfA- Xf cells treated with Bugbuster detergent and recombinant egg white lysozyme. Panel C and D. Immunogold labeling of intact HxfA- Xf cells with 250 fold dilution of anti-MopB and anti-EF-Tu antibody respectively. We thank Darlene Hoffmann for producing these electron micrographs.

or cell material were washed 3 times and then examined in the bottoms of Eppendorf tubes using the laser confocal microscope.

As seen previously, the intact cells did not substantially bind the Alexa-488-labeled anti-EF-Tu antibody; however, after treatment with the Bugbuster and lysozyme, much more of the antibody bound to both the pellet material after the centrifugation only treatment and the sucrose buoyant

material recovered from the sucrose gradient. As our electron micrographs show that the pellet material is substantially made up of what appear to be spherical cellular shells, the fluorescently labeled antibody binding suggests that the sucrose buoyant material is also these spherical cellular shells.

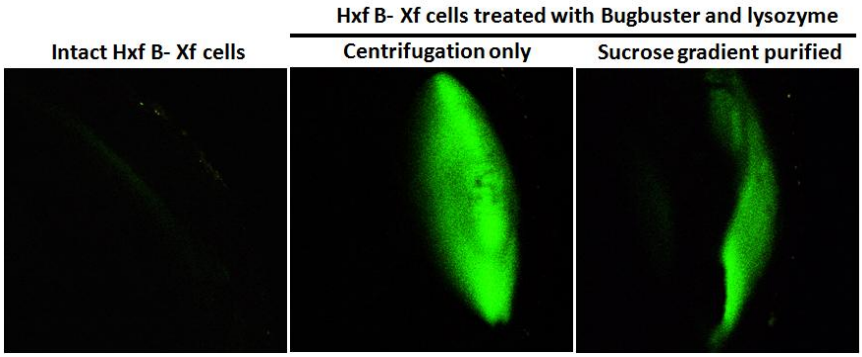

Figure 4. Binding of Alexa-488-labeled anti-EF-Tu antibody to intact HxfB- Xf cells and HxfB- Xf cells treated with Bugbuster and lysozyme and then centrifuged directly or through a sucrose gradient. The image shown cells collected in the bottom of Eppendorf tubes by centrifugation.

C. quinoa infiltration assays with Bugbuster and lysozyme treated Xf cells

Once we realized that the Xf EF-Tu was more accessible in the Bugbuster and lysozyme-treated material, we tested these same samples for chlorosis when pressure infiltrated into *Chenopodium quinoa* (the CqC assay). Water and various dilutions of intact HxfB- Xf cells, HxfB- Xf cells that had been treated with Bugbuster and lysozyme then recovered by centrifugation, and HxfB- Xf cells that had been treated with Bugbuster and lysozyme then recovered from a 50% to 80% sucrose gradient were pressure infiltrated into *Chenopodium* plants. After 3 days, the plants were examined for the chlorosis that is indicative of the reaction to partially purified EF-Tu from Xf or *E. coli*.

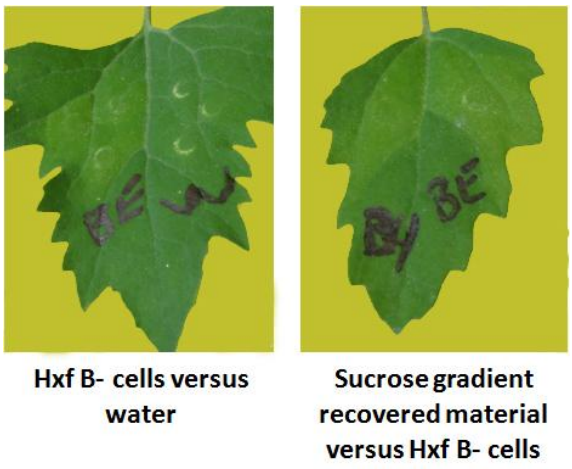

Figure 5. CqC assays comparing chlorosis induced by water, intact HxfB- Xf cells and HxfB- Xf cells treated with Bugbuster and lysozyme and then either centrifuged only to recover the infiltrated material or centrifuged through a sucrose gradient then recovered before infiltration.

As before, the intact HxfB- cells (Figure 5, left panel, left half leaf) give a strong chlorotic reaction upon pressure infiltration when compared to water (right half leaf). Material recovered from the sucrose gradient showed a comparable chlorotic reaction (Figure 5, right panel, left half

leaf) to that seen with infact HxfB- cells (right half leaf), even though the protein concentration was approximately 4 times lower in the sucrose gradient material.

Summary of major research accomplishments

For Objective 1, we are making progress in the production of Xf EF-Tu using an *in vitro* protein production system. We have produced appropriately sized protein and are prepared to attempt to produce increased amounts to test in the CqC assay and in grapevine for its effect on Xf-responsive promoters. We are also making progress on constructs for *in vivo* production of Xf EF-Tu using Agrobacterium-mediated transient expression with intra- and extracellular targeting. For Objective 2 and 3, we are waiting for results from Xf EF-Tu production before addressing these further. For Objective 4, we have used electron microscopy to preliminarily localized both mopB and EF-Tu to the periphery of the cell. We have also found that treatment of Xf cells with detergent and lysozyme removes most of the cell material but leaves a substantial structure that can be recovered by centrifugation. These residual structures bind strongly to fluorescent anti-EF-Tu antibody while intact cells do not. They also give a strong chlorotic reaction when pressure infiltrated into *Chenopodium quinoa* at an apparently lower total protein concentration.

We have identified a Xf cellular substructure that is resistant to lysozyme and detergent treatment and that contains a substantial amount of accessible Xf EF-Tu as evidenced by binding of anti-EF-Tu antibody, and that can react with plant tissue, as evidenced by the chlorotic reaction seen when this material is infiltrated into *Chenopodium quinoa*. Comparisons with *E. coli* and *Xanthomonas campestris* suggests that the EF-Tu of Xf takes a form that is very different from the EF-Tu in most bacteria. Possibly EF-Tu has a structural role in Xf that allows the spherical particles to survive lysozyme and detergent treatment. This material might be responsible for initiating Pierce's disease symptoms. Further experiments to determine if this material will stimulate 9353 promoter activity and scorch symptoms in grapevines will begin shortly.

Reports from the project

Paul Feldstein, Grape Recognition of *Xylella* Surface Proteins and Their Relationship to Pierce's Disease Symptom Development**,** Proceedings, 2009 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Research relevance

Identification of an essential bacterial recognition pathway that could lead to Pierce's disease symptom development might allow control of these symptoms through interference with this pathway at an early step using RNA interference. This could give a robust, simple and more acceptable form of symptom control.

Lay summary of current year's research

We have previously identified a bacterial protein, designated EF-Tu that may be involved in the development of Pierce's disease symptoms. It has been difficult to work with this protein

directly from the Xf bacteria. To help us understand the relationship between symptoms and recognition of this protein, we are producing EF-Tu in different ways so that we can more easily study it. We have made progress in making it completely outside the cell and hope to make enough to test its effect on various plants. We are also preparing ways to make the protein directly in plants to tests its ability to induce symptoms.

We have also found that the Xf bacteria has a structure either on or just below its surface that seems to be quite stable. This is unlike other bacteria, like *Xanthomonas*, which is a bacteria that lives in a similar environment to Xf. This structure is rich in EF-Tu, which we believe is involved in recognition and Pierce's disease symptom development. Perhaps this structure plays a role in stimulating symptom development too, possibly by presenting the plant with a concentrated form of the stimuating protein.

Status of funds

Currently, 75% of the first year award funds have either been spent or encumbered. 88% of the awarded salary money has been expended or encumbered and 19% of budgeted supply money has been spent.

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