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

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ABSTRACT
The aim of this project is to understand the role played by surface proteins of *Xylella fastidiosa* (*Xf*), especially the translation elongation factor "temperature unstable" (EF-Tu), in disease induction by this bacterium, the causative agent of Pierce's disease (PD) of grapevine, and to use this information to interfere with disease induction. Previously we demonstrated that *Xf* EF-Tu induces chlorosis when pressure infiltrated into leaves of *Chenopodium quinoa*, suggesting that EF-Tu may be a protein recognized by plants as a signal of *Xf* infection. Although the primary function of EF-Tu in eubacteria is in protein synthesis, certain bacterial species have evolved to use EF-Tu for other applications, including binding the bacterium to host cells. EF-Tu is associated an insoluble fraction of *Xf* cells and hence cannot be purified by conventional methods. The use of *Agrobacterium* to program plant cells to synthesize and target this protein to the plant apoplast has allowed the testing of EF-Tu's ability to cause PD-like symptoms in both *Nicotiana tabacum* cv. SR-1 and Thompson seedless grapes without purifying the EF-Tu protein. For both plant species, infiltration of *Agrobacterium* carrying an engineered binary vector allowed in planta expression and apoplast secretion of EF-Tu, resulting in PD-like symptoms in the absence of *Xf* cells or other *Xf* proteins. To introduce Agrobacterium into the difficult-to-infiltrate grapevine leaf, we developed a new infiltration procedure. To locate EF-Tu within the *Xf* cell, we treated *Xf* cells with detergent and lysozyme and examined the resulting spheroplast-like structures, which were found to retain EF-Tu. The EF-Tu that is present in these spheroplast-like structures, compared to EF-Tu in intact cells, is far more accessible to staining by fluorescent anti-EF-Tu antibody. Intact *Xf* cells were examined by immunogold electron microscopy of thin sections from centrifuged *Xf* cell pellets. EF-Tu and MopB, a major outer membrane protein of *Xf*, were very similarly distributed, primarily near the cell surface. These results support our earlier assertion that a fraction of EF-Tu is associated with the outer or inner membrane of the *Xf* cell surface.

LAYPERSON SUMMARY
The elongation factor "temperature unstable," EF-Tu, is an abundant soluble protein in most bacteria but in *Xylella fastidiosa* (*Xf*) is a protein found mainly at or just under the bacterial cell surface and in an insoluble fraction when the cells are disrupted. Insolubility prevents purification of EF-Tu by conventional means and makes determining the functions of *Xf* EF-Tu difficult. *Agrobacterium tumefaciens* is a bacterium capable of delivering genes to plant cells. We engineered *A. tumefaciens* to induce, when infiltrated into leaves, plant cells to generate *Xf* EF-Tu and to secrete the protein into intercellular spaces, bypassing the need to purify EF-Tu. Infiltrated leaves of both tobacco and grapevine developed symptoms characteristic of Pierce's disease (PD). That *Xf* EF-Tu, in the absence of *Xf* cells or any other *Xf* protein, appears to induce PD-like symptoms suggests that host plant reaction to EF-Tu released from *Xf* cells may be important in PD and may explain the observation of leaf-margin scorching in natural *Xf* infections even though *Xf* bacteria may be present only more centrally in the leaf or petiole. Understanding how this protein is recognized and how recognition leads to symptoms may lead to new ways of ameliorating PD symptoms.

INTRODUCTION
Long term, economical and sustainable control of Pierce's disease (PD) is likely to be achieved most effectively by deploying grapevine cultivars resistant to or tolerant of *Xylella fastidiosa (Xf)*. Interference with symptom development (i.e., creation of tolerance) is conceivable by preventing the full functioning of *Xf* virulence factors. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. *Xf* surface proteins are candidates for symptom-inducing factors. Examples of *Xf* surface proteins are a major outer membrane protein MopB (Bruening and Civerolo 2004), the hemagglutinin-like minor outer membrane proteins H*Xf*A and H*Xf*B (Guilhabert and Kirkpatrick 2005), a protein that is recognized by a single chain, monoclonal antibody (Bruening et al. 2008), and possibly a form of the protein synthesis elongation factor "temperature-unstable" (EF-Tu) (Bruening et al. 2008). We reported earlier that EF-Tu was the major component of a minor trailing band observed after electrophoresis of partially purified MopB through sodium dodecyl sulfate- (SDS-) permeated polyacrylamide gel. *Xf* EF-Tu was recovered by elution from excised gel pieces from the trailing band and was shown to induce chlorosis in *Chenopodium quinoa* (Bruening et al. 2007), whereas *Xf* MopB produced in transformed *E. coli* failed to induce chlorosis in *C. quinoa*. These observations suggest that the chlorosis-inducing factor in our MopB preparations may be *Xf* EF-Tu and not MopB, formerly the candidate chlorosis-inducing factor.

EF-Tu is one of a small number of highly conserved eubacterial macromolecules that have been categorized as "microbe-associated molecular patterns" (MAMPs) because of their ability to induce defense responses in specific plants (Jones and

Dangl 2006). Flagellin, chitin, certain lipopolysaccharides, and a few other molecules are other MAMPs. EF-Tu is the most abundant soluble protein of rapidly growing *E. coli* cells, so it is reasonable for it to serve as a signal for the presence of bacteria. The MAMP activity of *E. coli* EF-Tu is illustrated by alkalization of the medium of cultured *Arabidopsis thaliana* cells on exposure to subnanomolar concentrations of EF-Tu. When introduced at 1 µM concentration by pressure-infiltration into Arabidopsis leaves, *E. coli* EF-Tu induced resistance to *Pseudomonas syringae* and caused Arabidopsis to accumulate defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu and *Xf* EF-Tu gene sequences show 77% identity and 88% similarity in amino acid sequence, and both proteins induce chlorosis when pressure infiltrated into C. chenopodium leaves (Bruening et al. 2007). Those regions that show identity between the *E. coli* and *Xf* EF-Tu gene sequences also showed >90% identity with >100 eubacterial EF-Tu sequences (Kunze et al. 2004). Some bacteria have evolved an EF-Tu protein with at least one additional function, beyond participating in polypeptide chain elongation or acting as an elicitor. *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu as an adhesin that is responsible for the binding of these bacteria to human cells, and, in the case of *M. pneumoniae*, antibody to EF-Tu was demonstrated to interfere with attachment to human cells (Dallo et al. 2002, Granato et al. 2004). Therefore, it will not be surprising if *Xf* EF-Tu is found to be capable of inducing reactions in grapevine, including reactions that lead to symptom development. This work is an extension of our previous project entitled "Exploiting *Xylella fastidiosa* Proteins For Pierce's Disease Control"**.** The objectives for the current project are given below.

OBJECTIVES

1. Test *Xf* EF-Tu for its ability to induce scorching in grapevine
2. Identify a grapevine 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

RESULTS AND DISCUSSION

Xf EF-Tu expression from Agrobacterium induces PD-like symptoms in SR-1 Nicotiana tabacum

Results reported here advance Objective 1. The *Xf* cell surface location and known MAMP activity of EF-Tu suggest that accumulation of this protein in the intercellular spaces of leaves could induce scorching symptoms at locations not reached by *Xf* cells. The occurrence of *Xf* EF-Tu in an insoluble fraction after disruption of *Xf* cells prevents EF-Tu purification by conventional liquid phase separation methods. Therefore, we took the alternative approach of inducing plant cells to synthesize *Xf* EF-Tu and to secrete the protein into the (extracellular) apoplast. The apoplastic targeting sequence P14 from the plant pathogenesis-related protein PR-1 (Vera et al. 1989) has been shown by Jim Lincoln of the David Gilchrist laboratory to direct green fluorescent protein (SGFP) to intercellular spaces (data not shown).

The state of the expressed EF-Tu amino end could be significant to its biological activity because the known receptor for *E. coli* and other EF-Tu's in brassicas, designated elongation factor receptor (EFR), appears to recognize the EF-Tu amino end (Kunze et al. 2004). Therefore, the *Cowpea mosaic virus* 24K protease (CPMV 24KPro, a protease that includes cleavage of Q/M bonds in its range of specificity and capable of releasing itself from the interior region of a polyprotein) was interposed between P14 and *Xf* EF-Tu (**Figure 1**, construction E1) to create the native methionine amino end of the *Xf* EF-Tu protein. The *Xf* EF-Tu construct, P14-CPMV24KPro-*Xf* Ef-Tu, that replaces the SGFP with *Xf* EF-Tu, construct was difficult to make. Creation of construction E1, but not G1, required strong selection accomplished by use of a chloroamphenicol resistance gene cassette, suggesting a sickening effect of *Xf* EF-Tu on E. coli even without intentional expression of the protein.

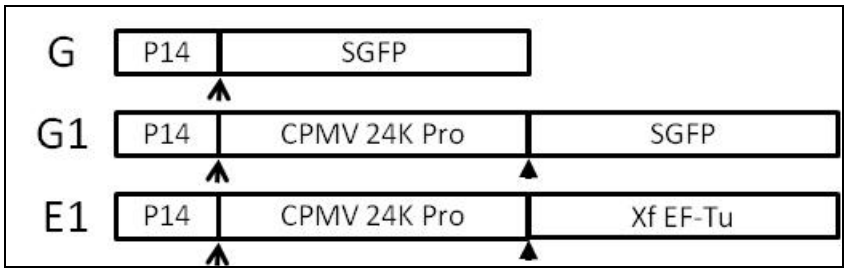

Figure 1. Coding portions of constructs used to export SGFP or *Xf* EF-Tu to the apoplast after Agrobacterium infiltration. P14 and SGFP are defined above. CPMV 24K Pro is the protease of *Cowpea mosaic virus*. The open arrowheads locate the cleavage site of the P14 apoplastic targeting sequence removing protease and the closed arrowheads locate the cleavage site of the CPMV 24K Pro, intended to release *Xf* EF-Tu with the authentic amino end methionine.

The presence of the CPMV 24KPro in construction E1 could interfere with apoplast targeting of *Xf* EF-Tu. Therefore, agroinfiltration into *N. benthamiana* of constructions G (gift from the Gilchrist laboratory) and G1 were compared (**Figure 2**). As expected, construction G targeted GFP to the intercellular spaces. Construction G1 produce little or no GFP signal but apparently induced a spongiform appearance suggesting loss of cells in the stomatal cavities. Similar results were observed for agroinfiltration of *N. tabacum* line SR-1 (not shown). Although these results, and our observation of only limited accumulation of 24KPro (not shown), both suggest that construction E1-mediated accumulation of *Xf* EF-Tu will be limited, the loss of plant cells could result in greater release of EF-Tu into intercellular spaces.

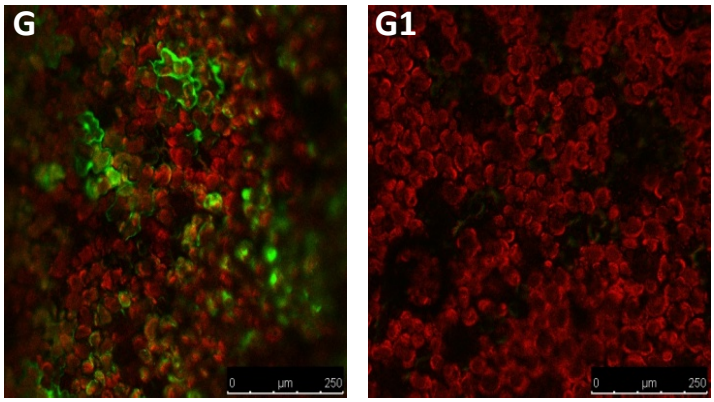

Figure 2. Laser confocal microscope images of *N. benthamiana* infiltrated with Agrobacterium strain GV2260 containing the binary vector pCB4NN with inserts G or G1 (**Figure 1**).

Agroinfiltration of constructs G (image not shown), G1 and E1 resulted in development of chlorosis within two days. However, the leaf areas infiltrated with construct E1 (P14-CPMV24KPro-EF-Tu) SGFP (**Figure 3**, panel A, right leaf panel) showed an incipient necrosis at two days post infiltration and, at five days, substantial necrosis (**Figure 3**). Areas infiltrated with G or G1 constructions remained chlorotic at five days post infiltration. These necrotic symptoms are similar to those seen for SR-1 tobacco inoculated with *Xf* (Francis et al. 2008).

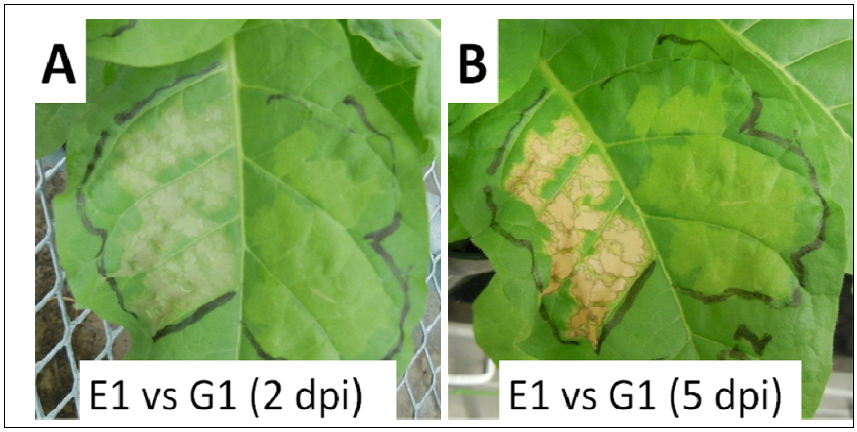

Figure 3. Opposite half-leaf agroinfiltrations of SR-1 tobacco with constructs E1 and G1 (**Figure 1**) for expression of P14-CPMV24KPro-SGFP and P14-CPMV24KPro-*Xf* EF-Tu, respectively. Photographs were taken at two and five days post infiltration.

The obvious interpretation of the **Figure 3** result is that EF-Tu is recognized by SR-1 tobacco, resulting in the development of symptoms characteristic of *Xf* infection of SR-1 tobacco but without an actual *Xf* infection or the presence of *Xf* cells or even the presence of any other *Xf* protein. There is, however, a reservation about this interpretation. Although similar binary vectors were used for the E1 and G1 constructions, the 3' and 5' untranslated regions flanking the fusion protein open reading frames (ORFs) of **Figure 1** are different. The E1 construct has CPMV RNA2 5' and 3' untranslated regions to increase translation efficiency (pEAQ-HT vector from George Lomonosoff , Sainsbury Laboratory, Norwich, UK) (Sainsbury and Lomonossoff 2008). The G and G1 constructs use the TMV omega 5' untranslated region (Gallie et al.

1987). Therefore, it is conceivable that the untranslated regions contribute to the differential seen in **Figure 3**. Constructs with the same 3' and 5' regions are complete and will be tested shortly. EF-Tu and GFP constructs without CPMV 24KPro also are being prepared. Another complicating factor is the possibly sickening effects of the constructs on *E. coli* and *A. tumefaciens* itself. We will be preparing constructs with introns inserted into either the CPMV24KPro, or *Xf* EF-Tu coding regions, or both, to allow the bacteria to escape the putative toxic effects of these constructions.

Xf EF-Tu expression from Agrobacterium induces PD-like symptoms in Thompson seedless grapevine

Results reported in this section advance Objective 1. The leaves of tobacco plants are readily pressure infiltrated with *Agrobacterium* cell suspensions by simply pressing a syringe (no needle) against the lower leaf epidermis, supporting the leaf at the opposite upper epidermis, and gradually pressing the syringe piston. The infiltrated leaf area is demarcated by the appearance of water logging. Grapevine leaves resist pressure infiltration so effectively that only a region corresponding to the bore of the syringe end shows water logging or, subsequently, reporter gene (typically GFP) activity. For most infiltration sites, no reporter activity is observed under the microscope (J. E. Lincoln, personal communication).

To test the constructions for symptom induction by agroinfiltration into grapevine, the infiltration process must be improved. In the new method developed here, grapevine leaves, detached or still on the excised cane or the intact potted plant, were very lightly abraded on the lower epidermis by dusting with 600 grit carborundum, wetting with water, and then rubbing very lightly with a gloved finger for 15 -20 seconds. The leaf was rinsed with distilled water and placed, lower epidermis up, on a glass frit filter support connected to house vacuum. The weak vacuum holds the leaf in place. 200ul of Agrobacterium suspension in dilute wetting agent was applied to the lower epidermis. Substantial entry of the liquid was evidenced by apparent water soaking in a number of small areas spread over carborundum-rubbed area of the leaf. At one day post infiltration (dpi), there was no evidence of macroscopic damage or any negative effects on the leaf from the infiltration process.

GFP fluorescence was observed in areas infiltrated with either the G (**Figure 4**.) or G1 (data not shown) constructions. A distinction between the results for the *Nicotiana* species and grapevine is that the 24KPro of the G1 construction did not seem to cause cell death in grapevine.

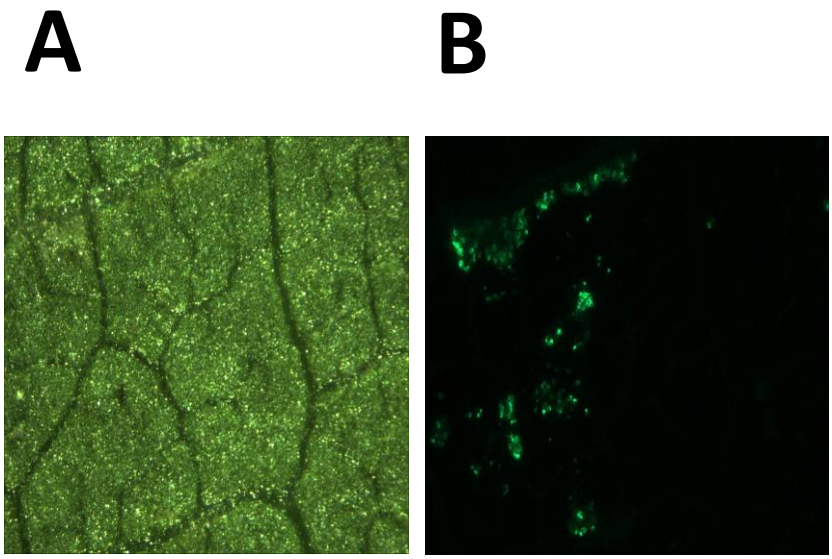

Figure 4. Images of grape leaf infiltrated with *Agrobacterium* containing the G (P14-GFP) construct. A. White light image. B. 36 second exposure using 488nm light and GFP expression filters. Note the SGFP expression is directly below the gouge seen in the upper left of panel A.

Figure 5 shows results from an experiment in which the E1 construction, designed to express EF-Tu, was agroinfiltrated into leaves on a detached cane of Thompson Seedless grapevine. A necrosis was visible at one dpi, and the necrotic area expanded with time. These symptoms were similar to those seen for E1-infiltrated SR-1 tobacco (**Figure 3**). Infiltration with the GFP-expressing G1 construction did not induce necrosis (image not shown).

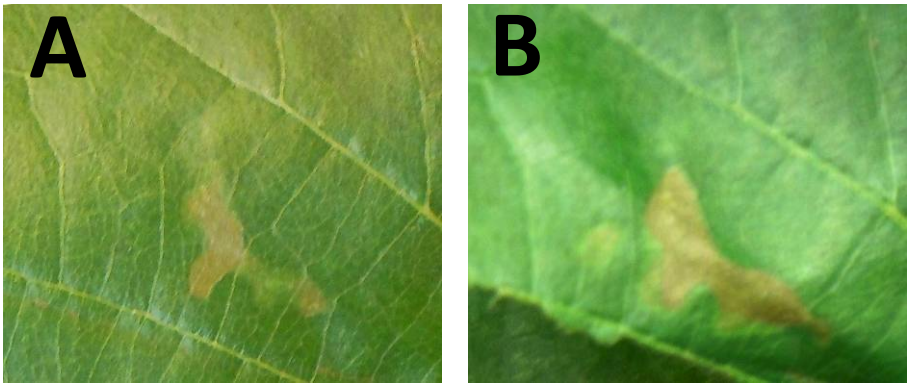

Figure 5. Symptoms induced on Thompson seedless grapevine leaves after infiltration with *Agrobacterium* containing the E1 construct (**Figure 1**). A. Symptoms at one dpi. B. Symptoms at two dpi. The yellowing that appears below the brown area in panel B is a photographic artifact resulting from removal of an infiltrated panel for microscopy, which allowed a leaf below to be observed through the opening.

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

This section is concerned with Objective 4. *Xf* strains HxfA- and HxfB- were used in these experiments. These *Xf* strains bear transposon-inactivated genes for hemagglutinin-like proteins A and B (Guilhabert and Kirkpatrick, 2005) and were selected because of their rapid growth in culture. We have previously shown that treatment of *Xf* cells with Bugbuster (EMD BioSciences), a proprietary detergent solution, and recombinant egg white lysozyme, followed by low speed centrifugation leads to recovery of roughly two thirds, by volume, of the original cell pellet material (Bruening et al. 2008). In contrast, similar treatment of *E. coli* cells or *Xanthomonas campestris* cells resulted in complete clearing of the solution with insignificant recovery of material after low speed centrifugation. The *Xf*-derived material that survived Bugbuster and lysozyme treatment was layered on a 50 to 80% sucrose gradient and centrifuged, resulting in a substantial band of intermediate density (Bruening et al. 2008). Immunoblots showed 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, comparing untreated and Bugbuster- and lysozyme-treated *Xf* cells. A rough estimate of the surface area of intact HxfA- *Xf* cells cells (**Figure 6A**), which are approximately 2 µ long and 0.5 µ diameter, is comparable to an estimate for the surface area of the apparently spherical entities, of average diameter 0.7 µ, that survived treatment of HxfA- *Xf* cells with Bugbuster and lysozyme (**Figure 6B**). The spherical particles were not seen in electron micrographs of *Xf* cell treated with Bugbuster alone.

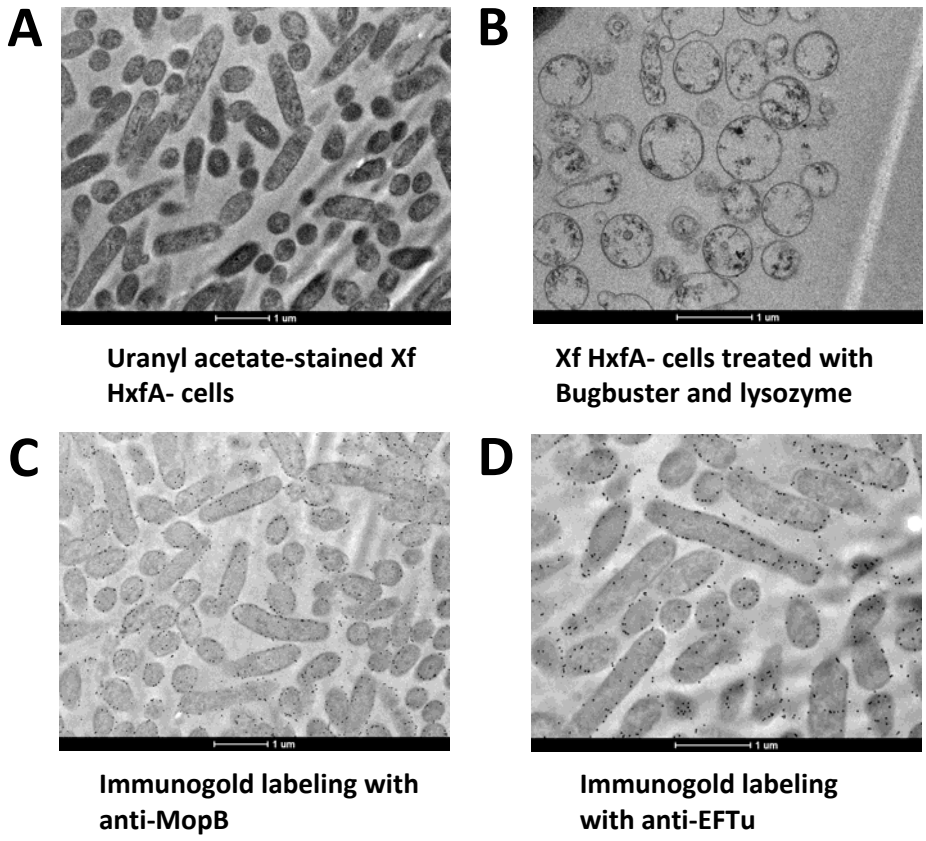

Figure 6. Thin section transmission electron micrographs of *Xf* cells and material released from *Xf* cells by treatment with detergent and lysozyme. **A**. intact HxfA- *Xf* cells. **B**. HxfA- *Xf* cells treated with Bugbuster detergent and recombinant egg white lysozyme. Material was centrifuged, and the resulting pellet was sectioned. Sections were stained with uranyl acetate to prepare the sample for microscopy. For C and D, sections were not stained but were exposed to rabbit polyclonal antibody raised against (**C**) partially purified *Xf* MopB (abundant outer membrane protein) or against (**D**) a synthetic peptide from the *Xf* EF-Tu amino acid sequence coupled to keyhole limpet hemocyanin, each antibody at a dilution of 1:250. The secondary was immunogold-labeled goat anti-rabbit antibody.

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As was observed previously, intact *Xf* cells bind antibody to *Xf* MopB but did not bind Alexa-488-labeled anti-EF-Tu antibody sufficiently to show localized fluorescence. However, the material that survived treatment with Bugbuster detergent and lysozyme was found to bind the fluorescent anti-EF-Tu antibody tightly enough to survive centrifugal washing. As is shown by **Figure 7**, detergent- and lysozyme-treated *Xf* cell material that was partially purified by sucrose gradient centrifugation bound the fluorescent antibody. However, much more of the fluorescent antibody bound to the pellet material

recovered after conventional centrifugation, suggesting that the EF-Tu, though immobilized in the treated *Xf* cell material, can be released by the multiple washing steps that occur as the treated *Xf* cell material passed from the top of the gradient to its buoyant position.

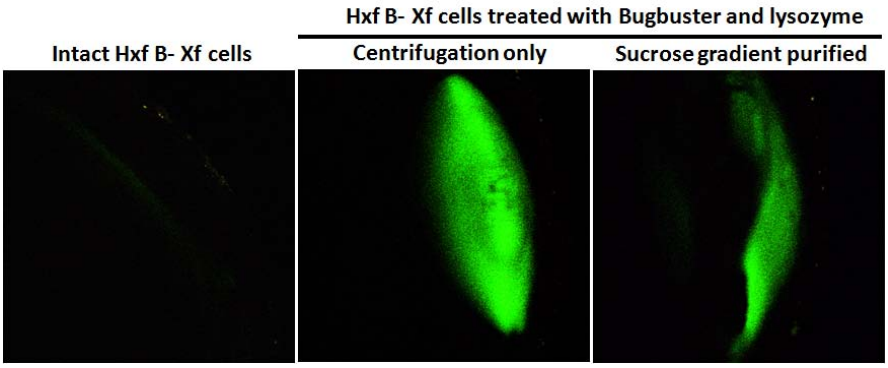

Figure 7. Binding of Alexa-488-labeled anti-EF-Tu antibody to HxfB- *Xf* cells that had been treated with Bugbuster and lysozyme. Intact HxfB- *Xf* cells and HxfB- *Xf* cells incubated with detergent and lysozyme were compared. The latter were treated in two ways, either recovered after incubation by conventional centrifugation or by conventional centrifugation followed by centrifugation through a 50% to 80% sucrose gradient and then recovery from sucrose solution by another centrifugation step. Preparations were exposed to Alexa-488-labeled anti-EF-Tu antibody for one hour, and the materials were washed three times by centrifugation. After the final centrifugation, the bottoms of the Eppendorf tubes were examined under 488 nm illumination using a stereo microscope.

Figure 6, panels C and D, provide evidence about 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*, appears to localized primarily at 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 *Xf* EF-Tu appeared to be distributed similarly to the distribution for MopB, also apparently localized to the cellular periphery, consistent with a possible role for *Xf* EF-Tu in a cytoskeleton-like structure. That exposure of XxfA- cells to detergent and lysozyme makes EF-Tu available for binding fluorescent antibody, whereas MopB antibody can react directly of HxfA- cells suggests that EF-Tu may not be as close to the cell surface as MopB is. A preliminary analysis of the spatial relationship between the immunogold particles bound by antibody to MopB and the immunogold particles bound by antibody to EF-Tu suggests that they are essentially coincident, but more sophisticated analyses are in progress.

CONCLUSIONS

Xf EF-Tu has been demonstrated to be present in a peripheral location of the *Xf* cell, similar to that of the outer membrane protein MopB, using fluorescent antibody and immunogold labeling. A peripheral location could allow for the release and recognition by the plant of EF-Tu, consistent with our earlier observation that *E. coli* EF-Tu protein, introduced as the purified protein into leaf intercellular spaces, is capable of activating a *Xf*-responsive grapevine promoter. Here we used transient expression and constructions designed to cause EF-Tu to be synthesized in leaf cells and be secreted into leaf intercellular spaces. This construct induced PD-like symptoms in tobacco and grapevine, without the presence of *Xf* cells or other *Xf* proteins. Very likely, grapevine has a receptor, possibly similar to the EFR receptor of *Arabidopsis*, that can detect *Xf* EF-Tu. The system developed here should allow us to identify regions of the *Xf* EF-Tu protein molecule that are recognized by the grapevine receptor. With this information in hand, we can attempt to reduce PD symptom development by interfering with this recognition.

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