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, especially the elongation factor "temperature unstable" (EF-Tu) in recognition of and disease induction by *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) of grapevine, with the goal of interfering with this recognition and, if possible, 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, specific bacterial species have evolved to use EF-Tu for other applications, including binding the bacterium to host cells. Expression of a *Xf* EF-Tu fusion protein in *Escherichia coli* altered the cell morphology and seems sickening to the cell if targeted to the extracellular space. The transformed *E. coli* , when introduced into the petioles of grapevine transformed with reporter constructions driven by a *Xf*-infection-specific promoter, activated synthesis of the reporter. Further analysis showed that purified intact *E. coli* EF-Tu and an N-terminal 18 amino acid peptide of *E. coli* EF-Tu were also capable of inducing reporter gene expression. These results suggest that, in addition to its role in protein synthesis, EF-Tu may be a signal in grapevine of *Xf* infection and that its recognition may be mediated by a known receptor, EFR.

LAYPERSON SUMMARY
The elongation factor "temperature unstable," EF-Tu, is a protein found on the outside of *Xylella fastidiosa* (*Xf*) bacteria. It has been shown that the presence of *Xf* in grape turns on certain grape genes. Purified *E. coli* EF-Tu protein, which is a very close match to the *Xf* EF-Tu protein, is capable of turning on at least one of these same genes. This suggests that the EF-Tu found on the outside of the *Xf* bacteria may be recognized by the grape plant. Release of the *Xf* EF-Tu from the bacteria may explain the distance between the bacteria replication sites in the xylem and the Pierce's disease sites at the leaf margins. Interference with grape recognition of the released EF-Tu may lead to a reduction in the disease 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. That is, at least some EF-Tu proteins act as elicitors. 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. EF-Tu, when introduced at 1 μM by pressure-infiltration into Arabidopsis leaves, 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 grant 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

Grape 9353 promoter activation by purified E. coli EF-Tu

The insoluble character of *Xf* EF-Tu, although interesting as an indicator of possible non-protein-synthesis functions of this protein, has prevented us from purifying *Xf* EF-Tu and therefore from having it available for direct injection into grapevine petiole. As is indicated below, all attempts at expressing intact *Xf* EF-Tu on its own directly in *E. coli* also were not successful. However, *E. coli* transformed to express *Xf* EF-Tu as part of a fusion protein was successful and resulted in possibly biologically active material, as indicated below.

In the previous reporting period, we described the effect of expression of a fusion protein with *Xf* EF-Tu as its amino end and the bacteriophage fd adhesin protein P3 as its carboxyl end. During bacteriophage fd infections or when the P3 gene is expressed in *E. coli*, P3 is targeted to the outside of *E. coli* cells, so we expect the fusion protein to be similarly localized. The fusion protein construction was placed under the control of the *lac* promoter and has the usual P3 signal peptide for extracellular targeting replaced by another signal peptide, dsbA, that should enhance folding for the *Xf* EF-Tu-P3 fusion protein (Steiner et al. 2006).

Work by the laboratory of Prof. Douglas R. Cook resulted in the discovery of a few genes whose expression is associated with *Xf* infection but not with, for example, abiotic stress. The laboratory of Prof. David Gilchrist prepared transgenic Thompson's Seedless grapevine lines bearing a green fluorescent protein (GFP)-encoding sequence under the control of two of the *Xf*-infection specific promoters, one of them being designated here as 9353 (Cook et al., 2005, da Silva et al., 2005, Gilchrist et al., 2007, 2008). The specificity of the 9353 promoter to *Xf* infection was demonstrated by observations of GFP expression after inoculation of *Xf* but not after inoculation of the xylem-invading bacterium *Xanthomonas campestris* (Gilchrist et al., 2008). Last year we reported that a set of four *E. coli* cell suspensions were petiole-injected into 9353:GFP grapevine lines. One suspension was of *E. coli* cells bearing the gene for the *Xf*-EF-Tu-P3 fusion protein and stimulated by exposure to the gratuitous inducer of the *lac* promoter, IPTG. This cell suspension induced accumulation of GFP, whereas the same *E. coli* strain not exposed to IPTG and a control *E. coli* strain bearing a P3 construction (no EF-Tu), and exposed or not to IPTG, failed to mediate GFP accumulation (Bruening et al., 2008). These results suggest that *Xf* EF-Tu alone may be sufficient to trigger transcription under control of the *Xf*-infection-specific 9353 promoter, a conclusion consistent with *Xf* EF-Tu having MAMP activity in grapevine. It is possible that the *Xf* EF-Tu protein represents a signal that is released in some way by the *Xf* bacteria and then accumulates in the leaf margins, leading to PD symptoms at a distance from known sites of bacterial accumulation (Gambetta et al., 2007).

As described previously, changes were noted in *E. coli* cells expressing the *Xf* EF-Tu-P3 fusion protein, compared to control cells. Growth was greatly slowed and cells became elongated and more fragile. An alternative hypothesis, to the hypothesis that the *Xf* EF-Tu-P3 fusion protein acts as a MAMP, is that the *Xf* EF-Tu-P3-expressing cells ruptured after injection into the petiole, thereby releasing *E. coli* EF-Tu. That is, the stimulator of 9353 promoter-controlled GFP expression could be *E. coli* EF-Tu rather than the *Xf* EF-Tu-P3 fusion protein, serving as a surrogate for *Xf* EF-Tu. It also is possible that both the *E. coli* and the *Xf* EF-Tu proteins are MAMPs in grapevine.

To test *E. coli* EF-Tu for MAMP activity in grapevine, we injected *E. coli* EF-Tu protein, purified by covalent chromatography (Caldas et al., 1998), into the petioles of 9353:GFP grapevine and examined them for GFP expression after various times post injection. As **Figure 1** reveals, GFP accumulation in pith cells, which is the characteristic response of the 9353:GFP grapevine petioles to infection by *Xf* cells, was observed in petioles that had been injected days earlier with *E. coli* EF-Tu (right hand three panels).

Controls not injected (leftmost panels) or injected with bovine serum albumin (BSA, middle panels) did not show fluorescent pith cells. This result shows that expression from the 9353 promoter of grapevine is stimulated by the presence of purified *E. coli* EF-Tu. The EF-Tu amino acid sequences of *Xf* and *E. coli* are 77% identical and 88% similar. Therefore, it is entirely

possible that *Xf* EF-Tu, could it be obtained in pure and injectable form, also would induce GFP accumulation in 9353:GFP grapevine.

Full length EF-Tu protein expression extracellularly appears to be toxic to expressing E. coli cells

As mentioned above, expression of *Xf* EF-Tu extracellularly from *E. coli* cells seems to have a toxic effect on the cells. Our initial construction produced the *Xf* EF-Tu protein either as untethered *Xf* EF-Tu with a small addition of amino acids at the C-terminus, which should accumulate in the extracellular space, or as EF-Tu fused to the N-terminus of the bacteriophage fd p3 protein, which should anchor the entire fusion protein to the outside of the *E. coli* . These two forms of the *Xf* EF-Tu are produced from the same gene construction by the action of a suppressible stop codon that separates the *Xf* EF-Tu from the bacteriophage fd P3. Previous experiments showed a marked decrease in growth of *E. coli* expressing this initial construct containing the suppressible stop codon
within two hours of induction.

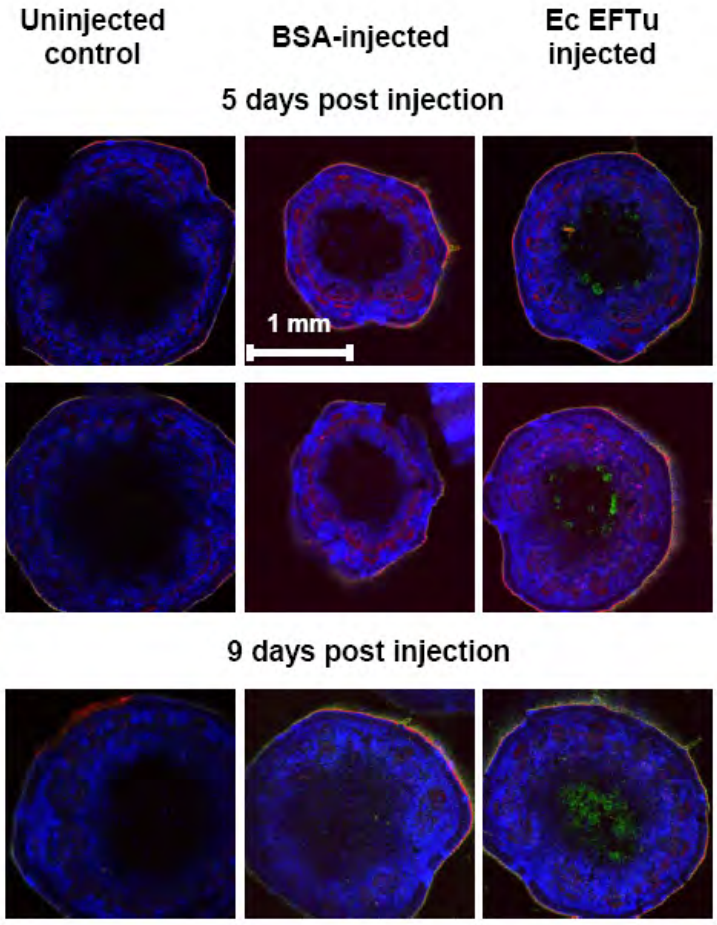

Figure 1. Either chromatographically purified *E. coli* EF-Tu or bovine serum albumin (BSA) (each at about ~0.9 µM in water) was injected into petioles of transgenic grapevine containing a green fluorescent protein (GFP) open reading frame under control of the grapevine *Xf*-infection-specific 9353 promoter. Each petiole was injected twice, ~0.5 cm apart and near the stem. At five and nine days after injection, leaves were removed and cross-sections were prepared at a site ~1 cm towards the lamina from the injection site. *E. coli* EF-Tu (Ec EFTu) was far more effective than BSA at inducing GFP accumulation We have attempted in numerous experiments to express intact *Xf* EF-Tu in a pure extracellular form from *E. coli* . No clones have been obtained, suggesting that extracellular EF-Tu may be very sickening to *E. coli* . The fusion protein construction was modified by insertion of a frameshift downstream of the *Xf* EF-Tu coding region. This should cause the *Xf* EF-Tu protein produced and exported to be free of the cell for easy purification. We have attempted to produce this construct several times, being careful to ensure that all of the steps are working appropriately, but have been unsuccessful each time. We have recovered only the starting material or constructions that have been substantially deleted in the *Xf* EF-Tu region. Given that even the fusion protein described above has substantial adverse effects, perhaps it is not surprising that we have not been able to generate *Xf* EF-Tu as an export from *E. coli* .

Our interest in the idea that *Xf* EF-Tu has a detrimental effect on the cells relates to results from the Gilchrist laboratory that suggests that high density cultures of *Xf* bacteria contain substantial numbers of non-viable cells. To investigate the

possibility that there is a role for the free form of the *Xf* EF-Tu in the deaths of the *E. coli* cells that were expressing it, we made constructs that deleted portions of the p3 region including the suppressible stop but maintained the reading frame so the C-terminal anchor sequence would hold the *Xf* EF-Tu to the cells themselves. If the free form of *Xf* EF-Tu is required to generate this phenotype, removing the suppressible stop should relieve this phenotype. An experiment with *E. coli* bearing this deleted construct without the suppressible stop codon showed a decrease in growth with similar kinetics compared to that seen with induced cells containing the initial construction suggesting that this aspect of the phenotype does not depend on the presence of a free form of the *Xf* EF-Tu. However, we cannot be sure that the presence of the free form of *Xf* EF-Tu would not generate a more extreme phenotype. We are currently extending this experiment by adding back the deleted region without the suppressible stop codon to more closely match the original construct.

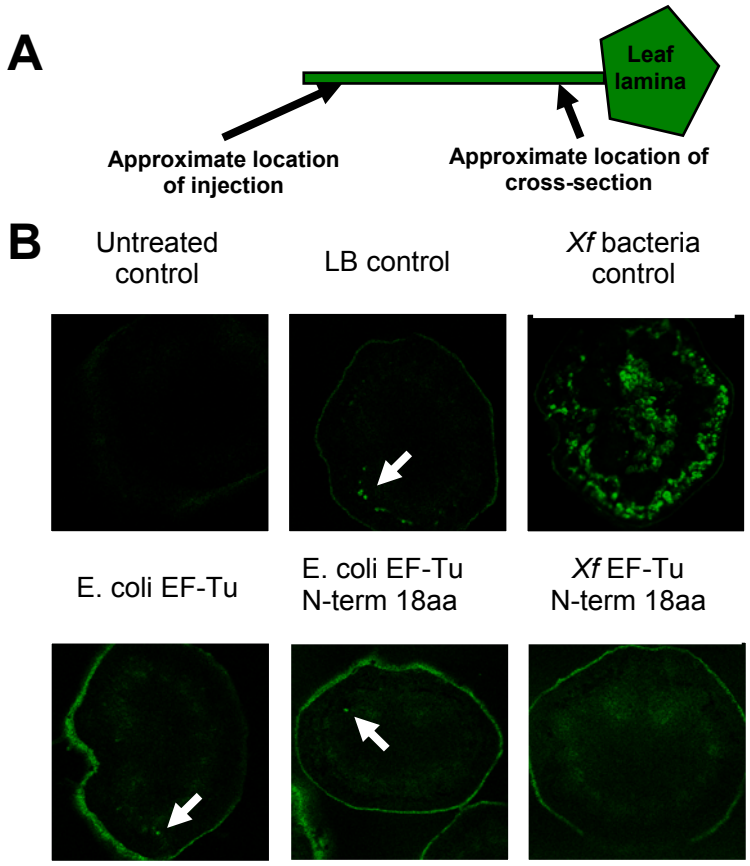

Figure 2. Full length *E. coli* EF-Tu protein solution was prepared at 0.9 µM and injected as in **Figure 1**. Peptides corresponding to the amino-end 18 amino acid residues of *Xf*-EF-Tu and *E. coli* EF-Tu, both amino-end acetylated, were prepared at 200 µM in water. **A**. Injection sites and cross-section sites in the petiole are shown relative to the leaf lamina. The petiole injections were as described in figure 1. **B**. At 17 days post-injection, leaves with petioles were collected and cross-sections both near the injection sites and near the leaf lamina were examined. No GFP expression was seen near the injection site. The three upper panels show results for an uninjected control, a control petiole exposed to bacteria-free LB microbiological media [known to activate GFP expression from the 9353 promoter (Lincoln, unpublished observation)], and a control petiole exposed to *Xf* bacteria (image provided by Lincoln from previous experiments). The lower three panels show three experimental injections: full length *E. coli* EF-Tu protein and amino-end peptides of *E. coli* and *Xf* EF-Tu proteins, respectively. Images were captured and provided by James Lincoln, Gilchrist laboratory

Grape 9353 promoter stimulation by E. coli and a Xf EF-Tu-amino-end-sequence-derived peptide

We obtained a commercially synthesized peptide having a sequence corresponding to the 18 amino acid sequence of the amino end of *E. coli* EF-Tu, followed by tyrosine and cysteine residues for convenient labeling or immobilization. A second peptide of similar design corresponds to the *Xf* EF-Tu amino end. The two 20-mer peptides are amino-end acetylated, as is *E. coli* EF-Tu. Kunze et al. (2004) demonstrated that an *E. coli* EF-Tu peptide of this design was recognized by, and induced defense responses in, Arabidopsis.

Neither of our synthetic peptides induced development of chlorosis when solutions were pressure-infiltrated in to leaves of *C. quinoa*, suggesting that recognition of EF-Tu in *C. quinoa* operates under a different mechanism from EF-Tu recognition by

brassica species. To test for the possible activation of the 9353:GFP gene of grapevine by the peptides, peptide solutions were petiole-injected.

Even though the GFP expression seen after injection of either full length *E. coli* EF-Tu protein or an amino-end 18 amino acid EF-Tu peptides, the peptide signal was weak compared to that seen for *Xf* bacteria (compare **Figure 2**, lower left and middle with upper right), it is in the same region of grapevine petiole seen when the cross-sections from near the leaf lamina of positive samples are examined. The amino end peptide has been shown to invoke response from Arabidopsis that is dependent on the EFR receptor (Kunze et al., 2004). Hence, grapevine may have an EFR-like receptor that could be responsible for signaling involving the 9353 promoter.

The response to injection of the *Xf* EF-Tu amino-end 18-mer peptide, although also weak, was more generalized spatially (**Figure 2**, lower right panel). It is known that the strength of the response by the Arabidopsis EFR receptor to an amino-end 18-mer peptide from *Xf* EF-Tu is weaker than that seen with the *E. coli* EF-Tu peptide (Kunze et al., 2004). It is also possible that the overall weak responses seen when compared to previous experiments (compare **Figure 1** and **Figure 2**) may be due to the reaction of the plant itself at the time of this assay. It has been suggested that the overall reaction of the plant to the *Xf* bacteria might be reduced during the winter months (Gilchrist and Lincoln, personal communication). To determine which is the case, we will repeat this experiment when the weather improves. (The green margins seen in the lower images of **Figure 2** represent overflow of the red autofluorescence seen by the confocal microscope into the green photomultiplier tube. The white arrows indicate sites of GFP expression which did not correspond to sites with strong red autofluorescence) follow up experiment was performed using the purified intact *E. coli* EF-Tu and a peptide corresponding to the amino-end 18 amino acid sequence of *E. coli* EF-Tu at the same concentrations that were used above. Similar levels and locales for GFP expression were seen using the intact EF-Tu protein and the N-terminal peptide (compare **Figure 3** left and right panels).

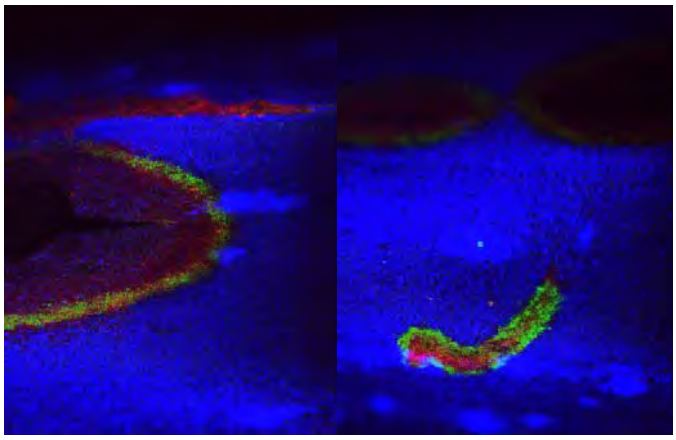

Figure 3. Confocal micrograph of 9353 promoter-GFP transgenic Thompson Seedless grapes infiltrated with intact *E. coli* EF-Tu (left panel) and *E. coli* EF-Tu N-terminal 18 amino acid peptide (right panel) in water. Images are of intact petioles with their long axis aligned left to right. Confocal microscopy kindly performed by Dr. Jim Lincoln.Both samples show a region of GFP expression near the site of infiltration, although the GFP response is higher in the peptide injected petiole. This could be due to the higher molar concentration of the peptide used. To confirm this result and to allow assays without consideration for the visibility of the GFP signal, we are currently checking these results by quantitative reverse transcription PCR using the actin mRNA as a control and both the GFP mRNA and the native 9353 mRNA as a more sensitive assay.

CONCLUSIONS

Xf EF-Tu, when expressed extracellularly on *E. coli* cells, leads to induction of a known *Xf*-responsive promoter as well as conferring a deleterious phenotype on the *E. coli* cells. Injection of either purified *E. coli* EF-Tu or its N-terminal 18 amino acids leads to qualitatively similar responses from this same promoter. The known EF-Tu receptor from Arabidopsis, EFR, recognizes both EF-Tu and the amino end peptide, suggesting that the grapevine response may be due to the presence of a grapevine EFR homologue. This recognition could be involved in disease symptom development and may explain the apparent separation of *Xf* bacterial accumulation site and PD symptoms sites. If so, it may be possible to interfere with this recognition and block Pierce's disease symptoms.

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