EXPLOITING *XYLELLA FASTIDIOSA* PROTEINS FOR PIERCE'S DISEASE CONTROL

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ABSTRACT
The aim of this project is to construct and express in grapevine, a protein or protein chimera capable of inactivating or otherwise interfering with the infectivity of, or disease-induction by, *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) of grapevine. A single-chain, monoclonal (scFv) antibody was selected for its ability to bind to *Xf* cells recovered by centrifugation of *Xf* liquid culture. Immunoblot analysis of total protein extracts from *Xf* cells revealed a single band corresponding to a ~47K protein target of this antibody. The identity of the target is unknown but is not likely to be major outer membrane protein MopB or the protein synthesis elongation factor EF-Tu, both of which have gel electrophoretic mobilities similar to the scFv antibody target. 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. We found *Xf* EF-Tu to be associated with an insoluble fraction which remains after treating *Xf* cells with lysozyme. Expression of *Xf* EF-Tu in *Escherichia coli* altered the cell morphology. 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. These results suggest that EF-Tu may be a signal in grapevine of *Xf* infection, in addition to its role in protein synthesis.

INTRODUCTION
Grapevine cultivars resistant to or tolerant of *Xf* presumably present the best approach to long term, effective, economical and sustainable control of PD. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. However, interference with symptom development (i.e., creation of tolerance) is conceivable, and *Xf* virulence factors are potential targets for interfering with *Xf* infection and symptom induction. A strategy is to create transgenic rootstock(s) that will secrete a protein or proteins into the xylem for transport to scion xylem to provide protection against insect vector-delivered *Xf* or interfere with symptom development. *Xf* surface proteins are candidate targets in this strategy. Examples of *Xf* surface proteins are a major outer membrane protein MopB, 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 (described below), and possibly a form of the protein synthesis elongation factor "temperature-unstable" (EF-Tu). We report here on the single-chain, antibody and then, more extensively, on the properties of *Xf* EF-Tu.

We reported last year 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 ("microbe-associated molecular patterns" = MAMPs) that have been discovered to induce defense responses in a variety of 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 be 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 accumulated defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu and *Xf* EF-Tu gene sequences show 77% identical and 88% similar in amino acid sequence. The regions of identity between the *E. coli* and *Xf* EF-Tu gene sequences also revealed >90% identity with >100 eubacterial EF-Tu sequences (Kunze et al. 2004). Some bacteria have evolved an EF-Tu protein with an 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).

OBJECTIVES

1. Discover or develop peptides and proteins with high affinity for the *Xylella fastidiosa* (*Xf*) cell exterior.
2. Test surface-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity or for interference with disease initiation following inoculation of grapevine or model plant with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins against *Xf* cells in culture and in plants.
4. In collaboration with the Dandekar laboratory, prepare transgenic tobacco and grapevine expressing and xylem-targeting the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*.

RESULTS AND DISCUSSION

A single chain antibody selected for affinity to Xf cell suspension

We obtained a library of single chain (scFv) antibodies, expressed on bacteriophage M13 particles, from the University of Cambridge, UK. Cultures of the rapidly growing and minimally aggregating *Xf* strain H*Xf*A-, which has a transposon insertion in the H*Xf*A gene (Guilhabert and Kirkpatrick 2005), were centrifuged to collect the cells. This cell preparation was used to "pan" the M13-scFv library through three cycles. Ten of the 24 selected M13-scFv clones were sequenced, and all had the same deduced amino acid sequence. Therefore, all subsequent analyses were applied to just one selected scFv antibody, designated A2scFv. To identify the epitope bound by the A2scFv, *Xf* proteins were resolved by electrophoresis and were subjected to immunoblot detection using the A2scFv-p3 fusion protein-carrying bacteriophage particle as the "primary antibody" and anti-M13 major coat protein as the target of the secondary antibody conjugate. A single band was observed with mobility corresponding to slightly less than an apparent molecular weight of approximately 47K (results not shown). This is comparable in electrophoretic mobility to two other *Xf* proteins which we have investigated: *Xf* EF-Tu (formula weight 42.9K) and *Xf* mopB (mobility corresponding to molecular weight 45K). To test whether the A2scFv is recognizing either of these *Xf* proteins, we attempted to interfere with A2scFv binding by pre-incubating two immunoblots with peptide polyclonal antibody against *Xf* EF-Tu or polyclonal antibody against *Xf* MopB. No apparent interference with the binding of the A2scFv-carrying bacteriophage M13 particles was observed (data not shown), but the anti-EF-Tu peptide antibody might not block scFv binding to other parts of EF-Tu. However, results from experiments involving digestion of *Xf* cells with lysozyme, as described below, are not consistent with recognition of either MopB or EF-Tu as possessing the epitope of A2scFv (data not shown).

The A2scFv was purified under non-denaturing conditions from solution using its encoded hexahistidine sequence and was labeled with Alexa-488 (fluorescein). The Alexa-488 labeled A2 scFv was incubated with *Xf* cell preparations in phosphate-buffered saline-Triton X100 (PBS-T) (**Figure 1**), and the cell preparations were washed with PBS-T before observing fluorescence. The fluorescence was observed not in association with the cells, as might be expected, but with somewhat larger, amorphous structures that apparently had been collected with the cells during centrifugation or formed during centrifugation. Similar results were observed with the Temecula *Xf* strains bearing mutations in hemaglutinin-like protein genes, H*xf*A- and H*xf*B- (Guilhabert and Kirkpatrick 2005). These experiments do not eliminate the possibility of weak binding of the scFv to intact *Xf* cells, since weak binding would not have been detected after the washing procedures followed here. At this point, both the protein target and the larger-than-cells target of the A2 scFv monoclonal antibody remain unknown.

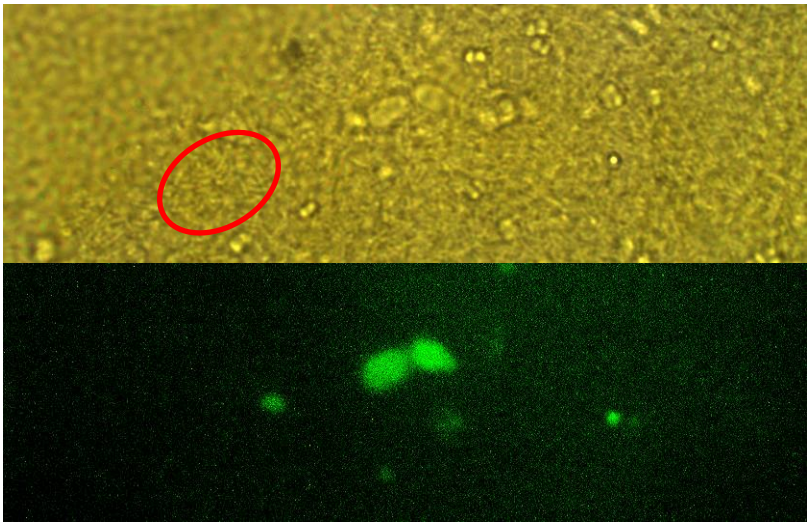

Figure 1. A Temecula strain *Xf* cell suspension, prepared by centrifugation, was observed under white light (upper panel) and 488nm light using an epifluorescence microscope. Alexa-488 (fluorescein) labeled A2scFv monoclonal antibody was added to the cell suspension, which subsequently was washed 3 times with phosphate-buffered saline containing 0.1% Triton X-100 (PBS-T). The red oval identifies an in-focus field of *Xf* cells.

Xf protein synthesis elongation factor "temperature unstable" (EF-Tu) in an insoluble fraction of a Xf extract
Since EF-Tu was found in MopB preparations, and the MopB purification procedure (Bruening and Civerolo 2004) includes extraction from insoluble material, it is likely that at least some EF-Tu of *Xf* is in an insoluble form. Results presented in **Figure 2** suggest that EF-Tu is associated with an insoluble fraction from which some other proteins, including MopB, had been released. H*Xf*A- cells were exposed to a proprietary detergent solution, "BugBuster®," or they were exposed to lysozyme, or to both. Prior exposure of H*Xf*A- cells to BugBuster® solution reduced the intensity of, or eliminated, a few protein bands (**Figure 2**, lanes 1 and 2), whereas treatment with lysozyme had no apparent effect on the pattern of protein bands (lane 4 compared to lane 1). Incubating the cells with BugBuster® and lysozyme together resulted in diminution or elimination of several protein bands (lane 3 compared to lane 1), including a prominent band with a mobility corresponding to an apparent molecular weight of about 43K, i.e., to the mobilities of *Xf* EF-Tu and *Xf* MopB.

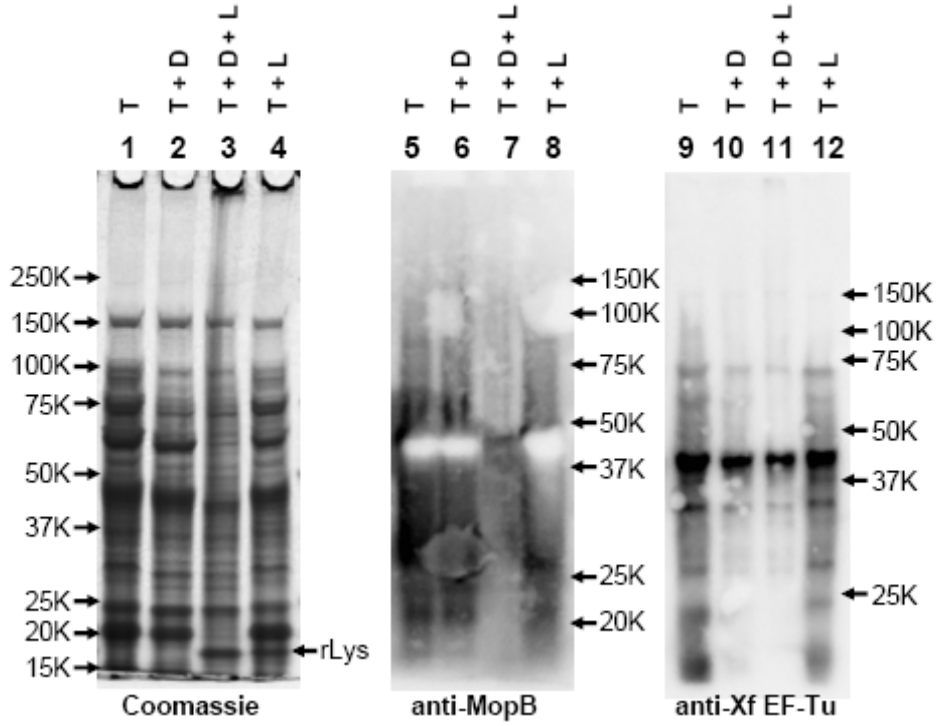

Figure 2. Effects of lysozyme treatment on the recovery of MopB and EF-Tu from an insoluble fraction of *Xf* cells. Each of the three images, lanes 1-4, 5-8 and 9-12, was derived from a different 12% polyacrylamide gel after electrophoresis of extracts of the relatively rapidly growing *Xf* strain H*Xf*A-. Cells were harvested from liquid culture in late logarithmic phase. Cells were washed with water and cell pellets were quick-frozen in liquid nitrogen and stored at -70˚C. Rapidly thawed cells were suspended to approximately 0.3 µg/µL total protein in 20 mM Tris-HCl, pH 8, alone (T, lanes 1, 5 and 9), in Tris-buffered "BugBuster®" (EMD Biosciences, proprietary detergent solution, T+D, lanes 2, 6 and 10), in Tris-buffered BugBuster® containing 45 U/µL recombinant lysozyme (EMD Biosciences, T+D+L, lanes 3, 7 and 11), and in Tris-buffered 45 U/µL recombinant lysozyme (T+L, lanes 4, 8 and 12). Samples were incubated at room temperature for 15 min with mixing, and insoluble material was collected by centrifugation at 14K rpm for 10 min. Precipitates were suspended in one-eighth the original volume of water, were mixed with SDS-mercaptoethanol-dithiothreitol disruption solution and were heated before loading the equivalent of 27 µg (lanes 1-4) or 8.5 µg (lanes 5-12) of starting protein on the gels. Detection was by staining with Coomassie Brilliant Blue (lanes 1-4), by immunoblotting using anti-MopB (lanes 5-6) or anti-EF-Tu peptide polyclonal antibody. Horse radish peroxidase-conjugated goat-anti-rabbit secondary antibody was located using the DuraSignal (Pierce) chemiluminescense system. The locations of bands for molecular markers and a presumed band for recombinant lysozyme (rLys) are indicated by arrows.

Immunoblot analysis using chemiluminescense detection is subject to a characteristic bleaching reaction when the target protein is present above a threshold amount. The apparent MopB signal of Fig. 2, lanes 5, 6 and 8, shows bleaching, as expected for the 8.5 µg of *Xf* cell total protein and the known abundance of MopB. In contrast, the anti-MopB antibody provided only a weak signal from the insoluble fraction left after treatment with BugBuster® and lysozyme (lane 7). The anti-MopB antibody was raised against an immunogen MopB preparation that likely contained traces of EF-Tu, so it is possible that the lane 7 signal at about 43K apparent molecular weight reflects EF-Tu rather than MopB. Most of the EF-Tu remained in the insoluble fraction after treatment with BugBuster® and lysozyme (Fig. 2, compare lanes 11 and 9). Anti-*Xf*

H*Xf*A polyclonal antibody (gift from Tanja Voegel and Bruce Kirkpatrick), which cross-reacts with *Xf* H*Xf*B, and the A2 scFv antibody, gave a greatly diminished signal for cells treated with BugBuster® and lysozyme (data not shown). Thus, it appears that the treatment with the two reagents results in solubilizing two outer-membrane proteins, MopB, H*Xf*B, and the scFv target, but not EF-Tu.

The insoluble product of the BugBuster® and lysozyme incubation (**Figure 3A**) was applied to a 50%-to-80% (w/v) sucrose gradient, which was centrifuged under conditions which should result in the various components of the digest moving through the gradient and reaching their own density. The main band of material **(Figure 3B**) was found to retain EF-Tu according to immunoblot results (**Figure 3C**). The centrifugation process, in effect, performs multiple washing steps as the main band material moves from the top of the tube to its isopycnic position, suggesting that the association of EF-Tu with the insoluble material is strong. Presumably this EF-Tu is not participating in protein synthesis but, as has been found for other systems (Dallo et al. 2002, Granato et al. 2004), *Xf* EF-Tu may have more than one function.

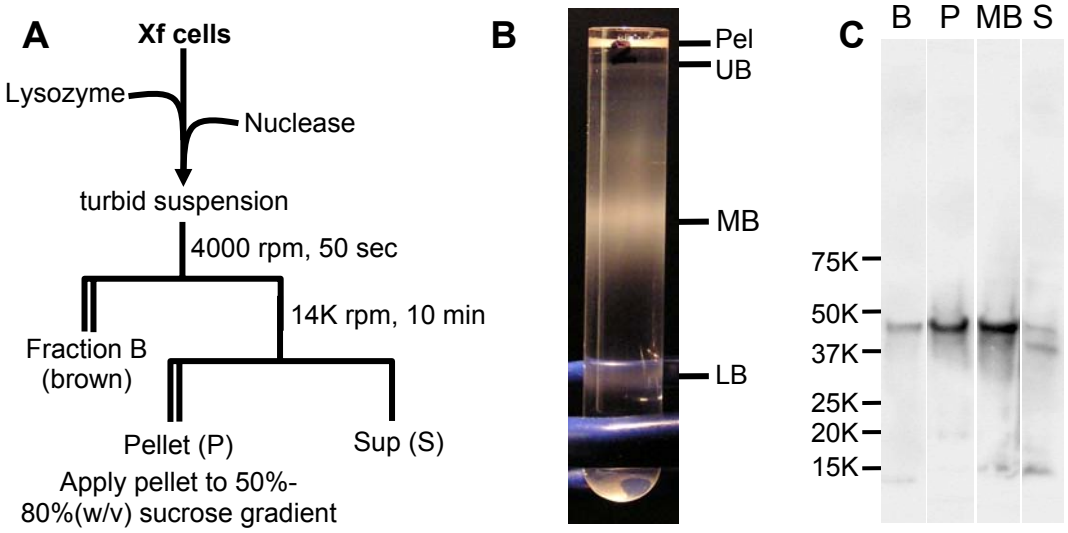

Figure 3. *Xf* EF-Tu is tightly associated with an insoluble fraction released from *Xf* cells by treatment with lysozyme **A.** *Xf* cells were suspended in a mild detergent solution ("BugBuster®," EMD Biosciences), and the suspension was incubated at room temperature with lysozyme and then with a general nuclease (Benzonase, EMD Biosciences), which greatly reduced the viscosity of the suspension. Differential centrifugation produced a small brown precipitate (B), a white, fluffy precipitate (P), and a supernatant fraction (S). **B.** The P fraction was applied to a 50%-to-80%(w/v) sucrose gradient in 20 mM Tris-HCl, pH 8. Centrifugation was for 4 hr at 35,000 rpm in a Beckman SW-41Ti rotor at 4˚C. The tube was mounted in a blue clamp, visible near the bottom of the image. In addition to the main band (MB) of white, turbid material, material also accumulated at the bottom of the tube, in a pellicle (Pel) floating on the gradient, and in two other small bands (UB and LB). **C.** Fractions from centrifugation and sucrose gradient centrifugation were heated in SDS-urea solution and were analyzed by SDS-PAGE on an 8-16% gradient gel. The bulk of the material reacting with peptide antibody to *Xf* EF-Tu in an immunoblot was associated with the MB fraction, which had a buoyant density of about 1.24 g/mL.

Phenotype of Xf EF-Tu expression in E. coli cells

An *E. coli* strain was designed and constructed to express a fusion of the M13-like single-stranded DNA bacteriophage fd outer membrane protein P3 with *Xf* EF-Tu, *Xf* EF-Tu forming the amino end of the fusion. P3 is an adhesin protein responsible for initiating attachment of the bacteriophage M13 particle to the bacterial F-pilus. Prior to extrusion of the bacteriophage particle from the infected cell, or when P3 is expressed in transformed but uninfected cells, P3 resides in the cell outer membrane. Therefore, our expectation is that the fusion protein EF-Tu-P3 will be targeted to the outer membrane. When cells from the EF-Tu-P3-expressing and control P3-expressing strains were collected and treated with lysozyme (**Figure 4**), an insoluble residue remained. The insoluble material was subject to analysis by sucrose gradient centrifugation and electrophoresis in a fashion similar to the analysis of *Xf* cells presented in **Figure 3. Figure 4A** reveals a difference between the results obtained for P3-generating and EF-Tu-generating E. coli, in both amount and buoyant density of the product. Wildtype *E. coli* cells, incubated under the conditions reported in the **Figure 2 or Figure 3** legends, in BugBuster® and lysozyme solution, were completely liquefied, leaving no insoluble residue (data not shown). These results suggest that targeting a protein to the outer membrane may induce the accumulation of a new, lysozyme-resistant substance in the *E. coli* cell. Presumably proteases of *E. coli* prevent the accumulation of more than a trace of intact EF-Tu-P3 (Fig. 4C).

Comparison of **Figure 3B and Figure 4A** reveals that accumulation of EF-Tu-P3 in *E. coli* cells and EF-Tu in *Xf* cells do not generate lysozyme-resistant, insoluble materials of similar densities.
E. coli transformed and induced, by IPTG, for P3 expression increased in culture at a rate that was comparable to that for the corresponding untransformed strain. However, the *E. coli* strain bearing the EF-Tu-P3 construction grew slowly and very slowly after induction by IPTG. Cells from the two cultures, as viewed by light microscopy, had very different appearances. The presumed EF-Tu-P3-expressing cells were larger, in both length and diameter, than the P3-expressing or wildtype cells (data not shown). These results are consistent with incorporation of *Xf* EF-Tu, but not *E. coli* EF-Tu, into the *E. coli* cell wall.

Does EF-Tu of Xf act as a MAMP or contribute to the symptoms seen on Xf-infected grapevines?
The above results suggest that *Xf* EF-Tu is present in *Xf* in an immobilized or insoluble form unlikely to be active in protein synthesis. What function might this altered form of *Xf* EF-Tu have? The chlorotic response of *C. quinoa* leaves to pressure-infiltrated *Xf* EF-Tu (Bruening et al. 2007), although no defense response has been documented, suggests that *Xf* EF-Tu may be a MAMP in some plants. It is well established that local concentrations of *Xf* cells and symptom intensities on leaves do not correlate (Gambetta et al. 2007). Therefore, symptom development could be the result of factor(s) secreted or otherwise released by *Xf* cells. Such factors could accumulate at the leaf margin, for example, where scorch symptoms are first observed. Whether a *Xf* factor such as EF-Tu is involved in symptom induction or in defense response in grapevine, the relevant events must include recognition of the factor by grapevine as may be indicated, for example, by altered transcription.

Previous work from the laboratory of Prof. Douglas Cook identified grapevine promoters whose transcription was increased specifically in *Xf*-infected plants (Cook et al. 2005, da Silva et al. 2005). Transgenic Thompson Seedless grapes containing constructs with these promoters coupled to a green fluorescent protein (GFP) sequence were prepared in the laboratory of Prof. David Gilchrist (Gilchrist et al. 2007). Plants of the transformed line have been demonstrated to accumulate GFP in the pith region of petioles after inoculation of *Xf* but not after inoculation of another xylem-invading bacterium, *Xanthomonas campestris* (Gilchrist et al. 2008).

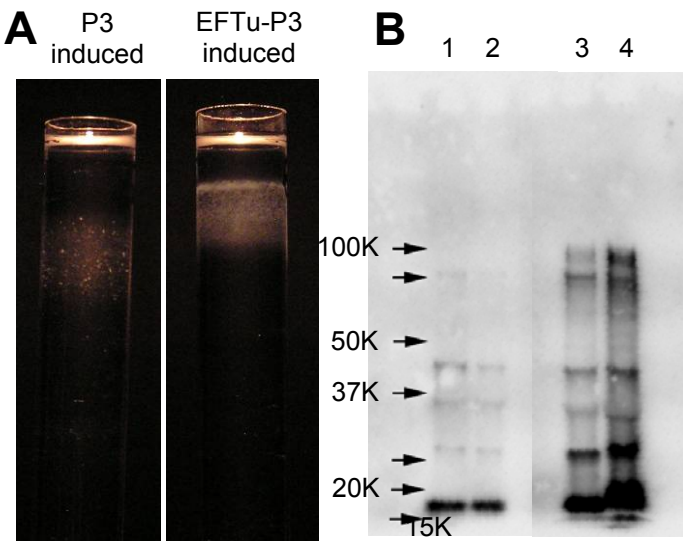

Figure 4. Expression of *Xf* EF-Tu in *E. coli* alters the insoluble residual found after digestion with lysozyme **A.** A 40 mg/mL (wet weight) suspension of *E. coli* cells that had been induced with IPTG was exposed to 30 U/µL recombinant lysozyme in BugBuster® detergent for 15 min and then to 0.05U/µL nuclease (EMD benzonase) for 10 min at room temperature. The resulting white precipitate was applied to the top of preformed 45%w/v – 75%w/v sucrose gradients. Gradients were centrifuged at 35,000 rpm for 4 hr at 4˚C. **B.** An immunoblot was prepared using anti-*Xf* EF-Tu antibody. Lanes 1 and 3 received the flocculent lysozyme digestion product whereas lanes 2 and 4 were loaded with material recovered from the sucrose gradients. The uppermost band in lanes 3 and 4 has a mobility expected for a *Xf* EF-Tu-P3 fusion protein. Arrows indicate the locations of reference proteins by their molecular weight.

Significant accumulation of GFP was observed by confocal microscopy in the transgenic grapevine petioles after inoculation of the petiole with *E. coli* cells of the strain transformed for expression (**Figure 4B, lanes 3 and 4)** of the EF-Tu-P3 fusion protein, provided the cells were induced by exposure to IPTG (**lower right panel, Figure 5**). This image does not show GFP accumulation in pith cells, which is unlike the pith-cell accumulation seen when *Xf* cells were inoculated (Gilchrist et al. 2008). Significant GFP signals were not observed for the *E. coli* cells not exposed to IPTG or to cells transformed for P3 protein expression (**Figure 5**). In the **Figure 5** experiment, *E. coli* cells had been stored at 4°C overnight before infiltration. Cold storage may lead to substantial bacterial death (data not shown). Therefore, based on these results, we are not able to connect the observed induction of GFP synthesis to live cells actively accumulating EF-Tu-P3 or even to intact, EF-Tu-P3-containing cells. In a subsequent experiment, the transformed *E. coli* cells were held at room temperature, and GFP accumulation in pith cells was observed (not shown). The results presented here suggest that *Xf* EF-Tu protein may act as an elicitor that is recognized by grapevine. Whether this recognition has a role in symptom development or defense against *Xf* infection remains to be determined.

CONCLUSIONS

A single-chain monoclonal antibody was isolated that reacts with a *Xf* protein that is most readily accessible in unusual structures found in liquid cultures of *Xf* cells but is also present in *Xf* cells. This protein is a potential target for interfering with *Xf* growth or colonization of grapevine. The protein synthesis elongation factor of *Xf* was demonstrated to have a bound form that may be recognized by grapevine in symptom development or defense reactions.

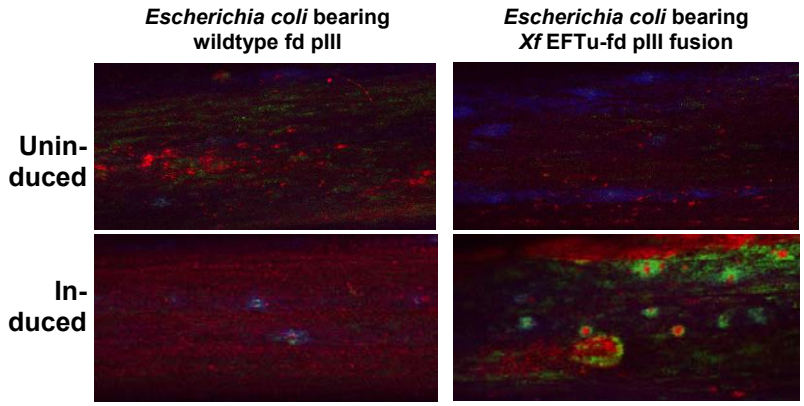

Figure 5. A *Xf*-specific grapevine promoter is activated by *E. coli* expressing EF-Tu-P3. The transgenic Thompson seedless grape plants bear constructions for green fluorescent protein (GFP) expression under the control of the *Xf*-infection inducible 9353 promoter (da Silva et al. 2005, Gilchrist et al. 2007). Transgenic grape petioles were inoculated with *E. coli* cells bearing either P3 (left panels) or EFTu-P3 (right panels), all at 2 x 10^8 cells per ml. An aliquot of cells was supplied with the gratuitous inducer IPTG (lower two panels) for two hr before storing the cells overnight at 4^{o}C and inoculation. At 13 days post inoculation, transverse sections of petiole taken a few mm from the inoculation site.

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