Objectives and Activities

The objectives of this project, as stated in the proposal, are:

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 or symptom development 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

It is likely that the development of grapevine cultivars resistant to *Xylella fastidiosa* (Xf) presents the best approach to long term, effective, economical and sustainable control of Pierce's disease. The strategy forwarded in our proposal is to create transgenic rootstock(s) that will secrete a protein or proteins into the xylem for transport to scion xylem, where it is expected to provide protection against insect vector-delivered Xf. In the course of characterization of Xf proteins, an outer membrane protein preparation highly enriched in the outer membrane protein MopB was observed to induce a rapid chlorosis when pressure infiltrated into *Chenopodium quinoa* leaves. The chlorosis-inducing activity was traced to a minor component of the membrane protein preparation: Xf protein translation elongation factor-temperature unstable (EF-Tu). This report concerns biological activities of Xf EF-Tu in grapevine.

That Xf EF-Tu should be recognized by *C. quinoa* (and by a specific grapevine promoter, see below) is not surprising. *E. coli* EF-Tu is known to be an example of a bacterial protein that acts as a MAMP, a microbe-associated molecular pattern. MAMPs are considered to be microbe macromolecules that are so common and so essential to microbe function that they serve as a signal in the plant for activation of basal defense against pathogens (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). Arabidopsis (and other brassica species) recognizes *E. coli* EF-Tu and activates a defense response that is effective even against other microbes. A EF-Tu receptor, EFR, has been cloned from Arabidopsis and was shown to confer on transgenic *Nicotiana benthamiana* the ability to recognize, and to mount a defense response against, *E. coli* EF-Tu (Zipfel et al., 2006).

As a participant in protein synthesis, bacterial EF-Tu generally is regarded as a soluble protein residing in the bacterial cell cytoplasm. *E. coli* EF-Tu is not only soluble but is the most abundant soluble protein of the rapidly growing cell (Nilsson and Nissen, 2005). The EF-Tu proteins of some bacteria have been demonstrated to have non-cytoplasmic locations and non-protein-synthesis functions (Granato et al., 2004; Malki et al., 2002; Porcella et al., 1996), presumably in addition to its function in protein synthesis. Xf EF-

Tu and *E. coli* EF-Tu behave differently. Lysozyme treatment of *E. coli* cells resulted in only a very small residual of insoluble material, and the great bulk of cell volume becoming solubilized. In contrast, the same treatment of Xf cells resulted in a fluffy white insoluble material that accounted for a significant fraction of the original packed cell volume. The Xf-derived insoluble material includes most of the Xf cell's EF-Tu, whereas *E. coli* EF-Tu is solubilized by lysozyme treatment (Bruening et al., 2008).

In this reporting period, we present results on stimulation by *E. coli* EF-Tu of expression by a grapevine promoter associated with Xf-infection. We also present data suggestive of the same promoter activation by the amino-end 18 amino acids of Xf EF-Tu. Activity by such a peptide would be consistent with a role for a grape homolog of the Arabidopsis EFR protein, which is required for recognition of the EF-Tu proteins from a variety of bacteria (Zipfel et al., 2006). The possibility that a homolog of the Arabidopsis EFR protein could be involved in Xf-sensing by the grape mediated by the Xf EF-Tu protein could explain certain aspects of Pierce's disease biology, most importantly how a xylem-limited epiphyte could cause disease at the leaf margin when little if any bacteria can be detected at this site. If this notion is correct, disruption of this interaction could be exploited for control of Pierce's disease symptoms, which might represent a reaction similar to a plant's version of anaphylactic shock.

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

We have attempted in numerous experiments to express intact Xf EF-Tu intracellularly in *E. coli*. No clones have been obtained, suggesting that intracellular EF-Tu may be sickening to *E. coli*. As is indicated below, we have successfully expressed Xf EF-Tu as part of a fusion protein targeted to the *E. coli* outer membrane. The fusion protein construction was modified by deletion of non-EF-Tu sequences, except for retention of the targeting signal peptide. This should cause the Xf EF-Tu protein to be produced and exported for escape from the apparently EF-Tu-sensitive cytoplasm and easy purification. We have attempted to make 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 below has substantial adverse effects, perhaps it is not surprising that we have not been able to generate Xf EF-Tu either in or as an export from *E. coli*.

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 this protein and therefore from having it available for direct injection into grapevine petiole. As is indicated above, attempts at expressing intact Xf EF-Tu 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 has been 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). 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 Pierce's disease symptoms at a distance from known sites of bacterial accumulation (Gambetta et al., 2007).

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 Fig. 1 reveals, GFP accumulation in pith cells, which is the characteristic response of the 9353:GFP grapevine petioles to infection by Xf, was observed in petioles that had been injected days earlier with *E. coli* EF-Tu (right hand three panels).

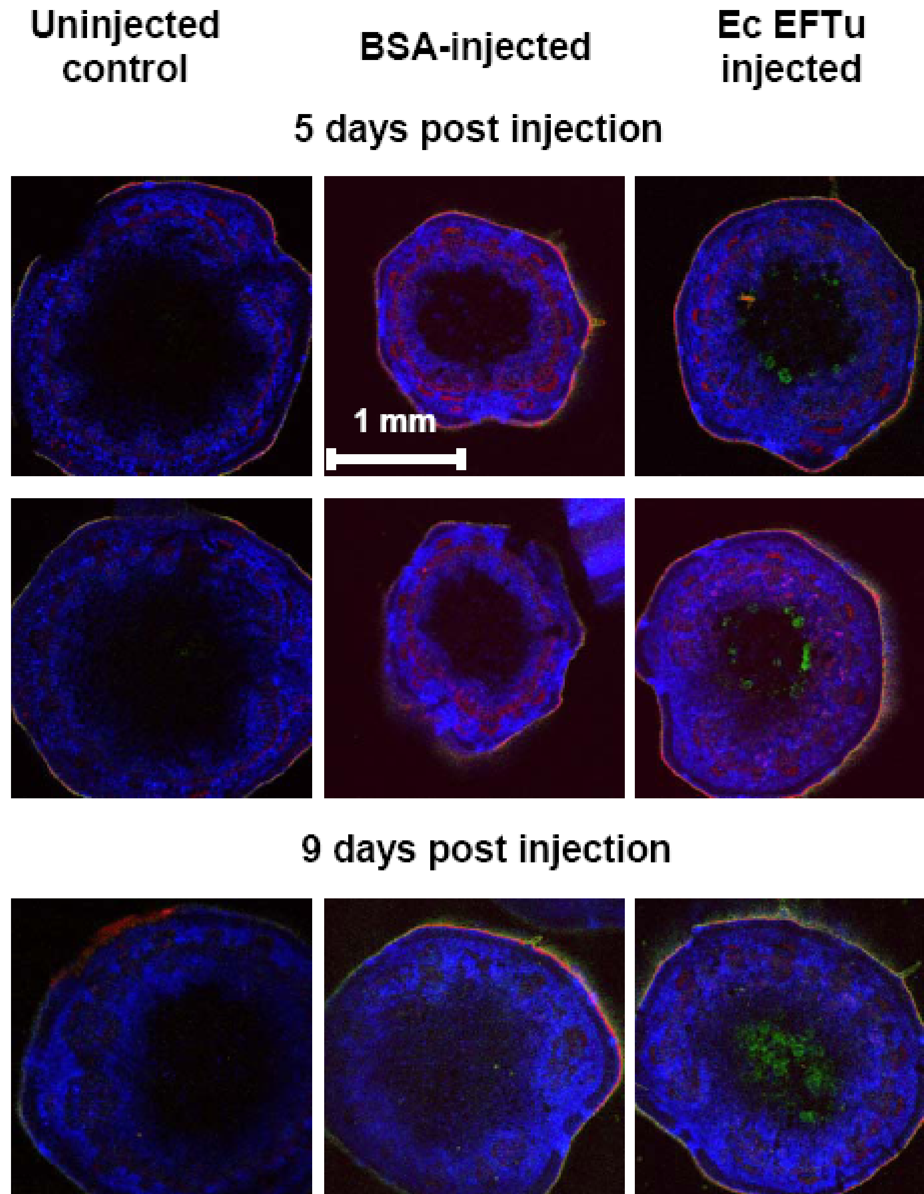

Figure 1. Either chromatographically purified *E. coli* EF-Tu or bovine serum albumin (BSA) (each at about ~0.9 µM in water) was injected into petioles of transgenic grapevine containing a green fluorescent protein (GFP) open reading frame under control of the grapevine Xf-infection-specific 9353 promoter. Each petiole was injected twice, ~0.5 cm apart and near the stem. At 5 and 9 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.

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 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.

Grape 9353 promoter stimulation by E. coli and Xf EF-Tu amino end 18 amino acid sequence

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 Fig. 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 (Fig. 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 Fig. 1 and Fig. 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 Fig. 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).

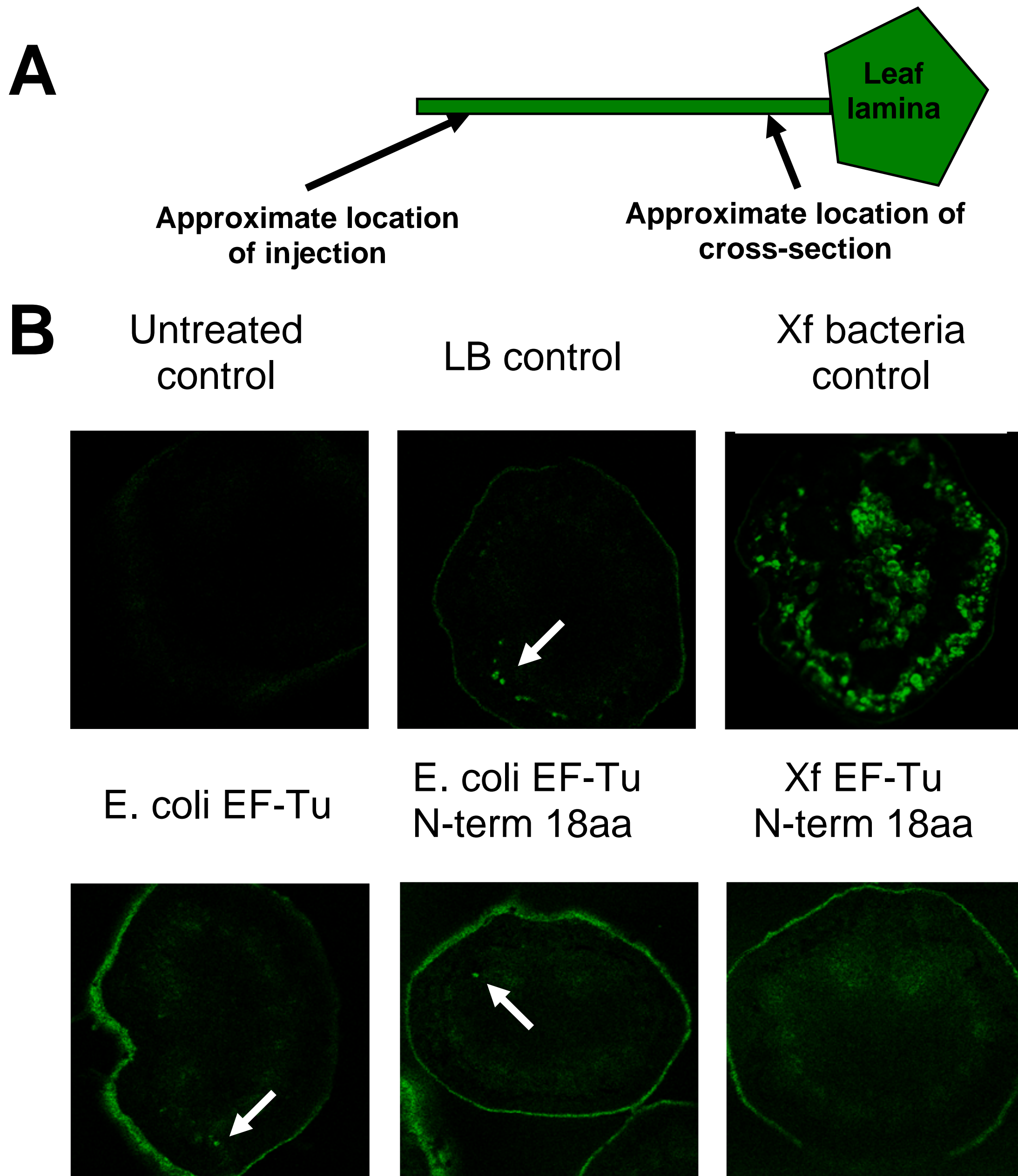

Figure 2. Full length E. coli EF-Tu protein solution was prepared at 0.9 µM and injected as in Fig. 1. Peptides corresponding to the amino-end 18 amino acid residues of Xf-EF-Tu and E. coli EF-Tu, both amino-end acetylated, were prepared at 200 µM in water. **A**. Injection sites and cross-section sites 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 LB 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

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