ASSESSMENT OF THE PROCESS OF MOVEMENT OF *XYLELLA FASTIDIOSA* WITHIN SUSCEPTIBLE AND RESISTANT GRAPE VARIETIES

Project Leader:
Steve Lindow
Plant and Molecular Biology
University of California
Berkeley, CA
icelab@socrates.berkeley.edu

Cooperators:
Clelia Baccari
Plant and Molecular Biology
University of California
Berkeley, CA
cbaccari@nature.berkeley.edu

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ABSTRACT
We are studying the process of movement of *Xylella fastidiosa* (*Xf)* cells between xylem vessels and through plants by analyzing the changing proportion of genetically distinct strains, initially introduced into the plants by distance and time from point of inoculation. We are also determining whether bottlenecks in movement of *Xf* cells in plants are more extreme in resistant plants than in susceptible plants, and whether this phenomenon can be exploited as a tool to screen germplasm for resistance to *Xf.* We expect that the process of movement of *Xf* involves a progressive and sequential colonization of a large number of xylem vessels that is limited by anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem). The resulting in bottlenecks practically limit the number of *Xf* cells that can move from one vessel to another, and thus constitute a major factor that confers resistance in plants.

INTRODUCTION
Xylella fastidiosa (Xf) has a rather unique means of colonizing plants and causing symptoms, which make strategies of disease control that are useful in other bacterial diseases ineffective. Many agriculturally important plants besides grapevines, including citrus, almond, alfalfa, and coffee, are susceptible to diseases caused by *Xf* (Hopkins 1989). The bacterium is transmitted to new host plants during xylem sap feeding by sharpshooter vectors and then multiplies and spreads from the site of inoculation to colonize the xylem; a water transport network of vessels composed of dead, lignified cells. Vessels are interconnected by channels, called bordered pits, that allow the passage of xylem sap but block passage of larger objects due to the presence of a pit membrane (Choat et al. 2003, Esau 1977). Bacterial cells attach to the vessel wall and multiply, forming biofilm-like colonies that can, when sufficiently large, occlude xylem vessels, blocking water transport (Alves et al. 2004, Fry and Milholland 1990a, Newman et al. 2003). In susceptible plants, leaf scorching, fruit shriveling and other symptoms result, likely due to the increased stress of xylem blockage as colonization ensues. However, within the majority of host plants, *Xf* behaves as a harmless endophyte (Freitag 1951). The population size of *Xf* in grapevines resistant and susceptible to Pierce's disease (PD) is highly correlated with symptom expression (Alves et al. 2004, Fry and Milholland 1990a, Fry and Milholland 1990b, Hopkins 1981, Newman et al. 2003, Krivanek and Walker 2004). A much higher proportion of vessels are colonized by *Xf* in symptomatic tissues that in non-symptomatic tissues (Newman et al. 2003). However we still lack an understanding of the process of colonization and what specifically about high populations of *Xf* leads to symptom expression. The pathways by which water moves through plants via the xylem are spatially complex. It is simplistic to consider axial water movement in stems via xylem vessels as simple vertical "pipes". Indeed, xylem vessels themselves often follow complicated paths through a tissue with respect to each other (Figure 1). More importantly, the water in a given vessel is in contact with that of different vessels as well as with those in adjacent tracheids via the many pits in the cell walls (Figure 1). Pits are adjacent to one another on either side of the cell wall and thus come in pairs. The pit membrane is composed of the primary cell wall and middle lamella of adjacent cell walls of the pit pairs (Esau 1977). In a bordered pit the secondary cell wall forms a border over the pit membrane leaving a small opening called a pore. While secondary cell walls can be thickened via the intrusion of lignin and other polymers into the cellulosic matrix of the primary cell wall, pits represent local "thinning" of the primary wall with only a minimal amount of cellulose and pectin, which allows relatively free diffusion of water and solutes from one cell to another. Thus instead of a limited number of vertical "pipes' that conduct water through a stem, there are thousands of alternative pathways that water might travel in a tissue. The interconnectivity of the xylem cells is presumably one means by which the plant overcomes injuries or other insults that would disrupt the movement of water via a given xylem element by shunting it to adjacent cells.

In the context of PD it thus becomes obvious that in order for water movement in a stem to be so restricted that disease develops, a large percentage of the xylem pathways must be blocked for disease to occur. Yet, while over 40% of the xylem vessels in a single section of an infected grape stem may be infested with *Xf* (Newman et al. 2003) this alone is unlikely to explain water stress. Serial sections of grape tissue however, demonstrated that different xylem vessels are blocked in different cross sections; the percentage of occluded vessels in one of several sections along 5 mm of petiole was five times that of a single cross section. Given that inoculation of grape with *Xf* must occur in a relatively few sites on a vine, it is clear that the pathogen has the ability to move both axially and radially in xylem tissues. Such extensive movement must take some time, explaining why the disease is "progressive" and appears only several weeks after inoculation.

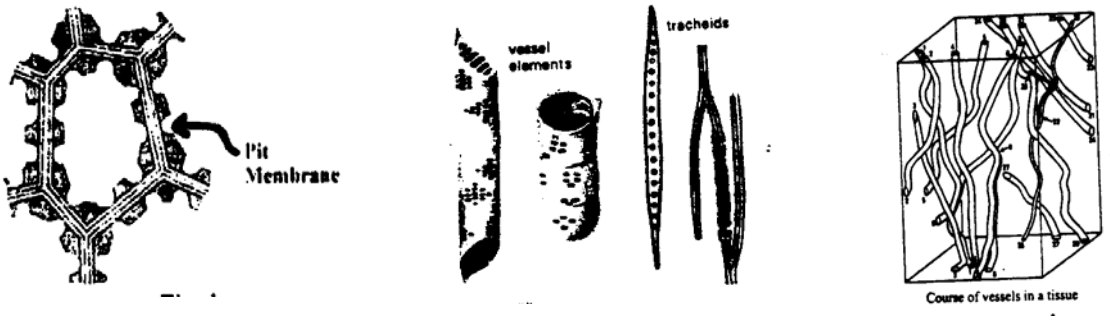

Figure 1. Structure and arrangement of xylem vessels.

Dispersion of *Xf* through the xylem network likely involves following the natural course of the xylem stream through the plant, but would be expected to require a mechanism for accessing vessels connected only by bordered pits because pit membranes do not readily allow passage of objects 20 nm or larger (Choat et al. 2003). Considerable evidence suggests that *Xf* degrades pit membranes to traverse bordered pits. *Xf* has been shown to express genes predicted to encode pit membrane degrading enzymes such as cellulases and pectinases *in vitro*. Furthermore, a mutant blocked in production of polygalacturase (pectinase) was unable to move within grape and was avirulent. In addition, transgenic grapes expressing a pear polygalcturonase inhibiting protein (PGIP) exhibited more resistance to *Xf* than did untransformed plants (Aguero et al. 2005). Work from our lab, using a gfp-marked strain of *Xf* reveals that it could be seen transiting the pit of grape xylem (Newman et al. 2003) (Figure 2).

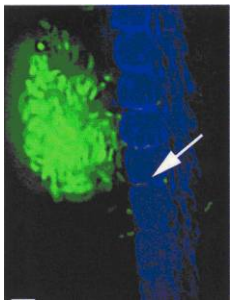

Figure 2. Image of xylem vessel.

While the movement of *Xf* has been recognized as an important trait necessary for disease, the process is still poorly understood. It is generally agreed that symptoms of PD do not occur until a large number of vessels are colonized by *Xf*. Studies by Newman et al. (2003) found a very high correlation between incidence of highly colonized vessels and symptom development in grapes. Thus, *Xf* must move through many (perhaps hundreds of) different xylem cells if such high levels of colonization are to occur. *Xf* attains higher population in susceptible cultivars than in more resistant species and cultivars of grapevines. More recent studies have shown that systemic infections occur in both susceptible and tolerant genotypes of grape. However, susceptible genotypes were characterized by higher cell populations especially in the stem internodes (Krivanek and Walker 2004). It is also known that in resistant grapes varieties, as well as other plant species, *Xf* can have a systemic infection with relatively low populations in greenhouse conditions (Fry and Milholland 1990a, Fry and Milholland 1990b). Krivanek and Walker (2004) note that the mechanism of resistance to *Xf* is localized within the stem xylem and is not fully functional or absent in the xylem of petioles and leaf blades. They observed little difference in the colonization of these tissues as opposed to those of the stem xylem. They speculate that a more constitutive resistance mechanism is present in stem xylem based on nutritional or structural differences between resistant and susceptible stem xylems.

OBJECTIVES

1. Study the process of movement of *Xf* cells between xylem vessels and through plant by determining the changes in proportion of genetically distinct strains of the pathogen initially inoculated into plants at an equal proportion with distance and time from point of inoculation.
2. Determine if bottlenecks in movement of cells of *Xf* from xylem vessel to xylem vessel is more extreme in resistant plants than in susceptible plants and whether this phenomenon can be exploited as a tool to screen germplasm for resistance to *Xf*.

RESULTS

In these past few months, we have been propagating plant material from naturally resistant species such as *Vitis rotundifolia* and the highly susceptible *Vitis vinifera* species to conduct comparative experiments. The representative resistant cultivars used are Tampa and Roucaneuf, both of which are field resistant. The susceptible cultivars we are working with include Cabernet Sauvignon, also used in our initial study. In a preliminary experiment, we inoculated a large number of Cabernet Sauvignon plants with a mixture of two isogenic and highly virulent strains of *Xf* strains (Temecula and KLN61) via petiole needle inoculation. These isogenic strains could be distinguished by the fact that KLN61, but not Temecula, was resistant to kanamycin. When plants were sampled 50 cm from the inoculation point several weeks after inoculation, 46% were found to be infected with only strain KLN61 and 20% were infected only with strain Temecula while the remaining 34% of the plants were not infected with either strain. While both strains were initially found at the site of inoculation, in none of the plants were both of the strains found at distal sampling sites. We interpret these results to suggest that anatomical features of the plant greatly limit the number of cells of *Xf* that can move from one infected xylem vessel to an adjacent uninfected vessel,

and that sequential passage of *Xf* cells through such a series of physical "bottlenecks" characterizes the process of plant colonization. If only a few cells were transferred to adjacent xylem vessels when moving to an adjacent vessel as suggested by the microscopy analyses of Newman et al. (2003) (Figure 2), then with time it is likely that only one genotype of an originally mixed genotype inoculum would be present after many such "bottlenecks" that are encountered during movement in the plant. A cartoon illustrating this process is shown in Figure 3.

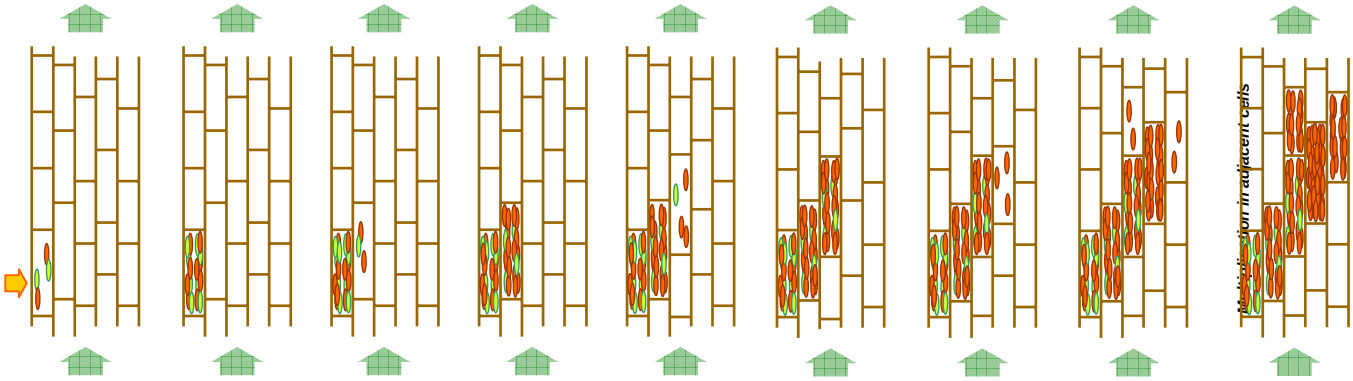

Figure 3. *Xf* movement in the plant.

In another preliminary experiment, ten rooted cuttings of a susceptible Cabernet Sauvignon variety were stem-inoculated with a suspension (10^7 cells/ml) of equal populations of Temecula and KLN61. Ten rooted cuttings were inoculated only with Temecula and ten with only KLN61. Inoculums of suspensions were prepared from two-week old plates of *Xf* in SCP buffer. Concentrations of *Xf* cells were estimated with a spectrometer and adjusted to approximately equal cell densities. The population of each *Xf* strain in suspension was estimated following inoculation by dilution plating on PWG media, followed by counting colonies on PWG and PWG+Kan media. A stem of each vine was inoculated with 5µl of suspension. The inoculation site was marked with tape. Each plant was sampled at five internodes location: 0 (point of inoculation), 10, 20, 30, 60 cm from the inoculation site plus a proximal petiole to the inoculation site. Six plants were sampled at week one, two, four, for a total of 18 plants, and 12 plants were sampled at week ten. The proportion of Temecula and KLN61in the plant part sampled was calculated for each sample as mentioned above. Twenty-seven out of the thirty plants (90%) at 10 weeks were showing symptoms and proved to be infected when assayed by culturing. At week 10, out of the four plants co-inoculated with both strains (plants numbered seven, eight, nine, ten) one plant, number seven, had nearly all Tem strain when cultured at the sampling location, and plant number nine had nearly only KLN61. The other two plants still had a mixture of the two strains in different ratios, indicating that it takes more than a 10 week period for a single strain to completely occupy the plant stem. This was also confirmed from the first experiment where culturing was done at 14 to 18 week post-inoculation. Over time, only one of the two strains ultimately dominates in infecting the test grapes. We found that both strains were still present in the petioles proximal to the point of inoculation at week 10 in plant numbers seven and eight (petiole samples from plant numbers nine and 10 were contaminated), while just one of the two strains continued to invade the rest of the stem, as the infection and resulting symptoms (disease) progressed.

CONCLUSIONS

Our preliminary results indicate that the segregation of the mixed inoculums initiate within two to four weeks of inoculation in susceptible grape varieties and is evident in samples within 10 to 20 cm of the point of introduction. Thus it would appear that the process of movement of *Xf* through plants is a stochastic one, which is characterized by growth in a given xylem vessel where it is introduced, followed by "active escape" of at most a few cells into adjacent uncolonized vessels, and then further multiplication of the cells which starts the process anew.

In this study we are exploiting the use of mixtures of phenotypically identical but genetically distinct strains of *Xf* to better understand the process of progressive movement of *Xf* through plants. We hypothesize that anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem) limit the number of *Xf* cells that can transit from one vessel to another and represent important factors conferring resistance in plants. It would is expected that the stochastic processes that tend to segregate cells of one strain from another during progressive movement would increase the degree of segregation with distance from the point of inoculation (with increasing numbers of vessels the cells had to traverse to get from one part of the stem to another given that each vessel in grape is an average of only about 10 cm long). Thus, for a given plant inoculated with a mixture of cells, the proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the "bottleneck" that it faced while moving from one vessel to another. This is depicted in Figure 4. While at the point of inoculation of an equal mixture of cells into the stem, the ratio of the two strains would be 1.0, the proportion of strain A in a mixture with strain B would decrease to 0 for some plants or increase to 1.0 in others. The departure from a ratio of 1.0 should increase with distance for a given plant, and when considered over many plants the variance in the proportion of the strains in a mixture should increase with distance.

This is depicted in Figure 5. Furthermore, we hypothesize (as speculated by Walker) that resistant grape varieties harbor anatomical differences from susceptible varieties that limit the movement of *Xf* from vessel to vessel. Such plant would thus present a more extreme "bottleneck" to *Xf* at each movement event and hence we would expect a more rapid segregation of mixtures of *Xf* at a given point away from inoculation.

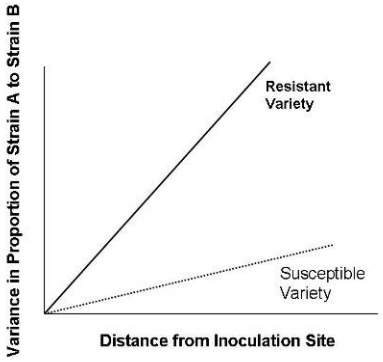

Figure 4. The proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the "bottleneck" that it faced while moving from one vessel to another

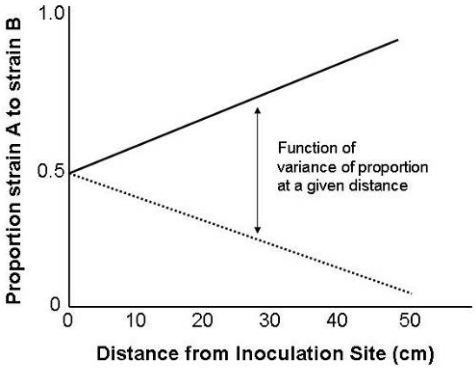

Figure 5. The departure from a ratio of 1.0 should increase with distance for a given plant, and when considered over many plants the variance in the proportion of the strains in a mixture should increase with distance.

We expect to test this model of movement of *Xf* in grapevines of varying susceptibility to PD. This study will provide considerable insight into the process of bacterial movement, which is central to the disease process though it remains poorly understood. Moreover, it will provide new and necessary tools for screening grape germplasm for resistance to *Xf*. As Walker (Krivanek and Walker 2004) has noted, since "resistant" grape varieties still support the growth and movement of *Xf*, albeit at a lower level than susceptible varieties, qualitative measures of *Xf* presence, such as by PCR, are not suitable for screening germplasm. Furthermore, difficult quantitative measures, such as culture plating or optimized ELISA, are required to distinguish resistant varieties. We expect that the segregation of mixed inoculums in "resistant" varieties will be rapid as suggested by the relationships described in figures 4 and 5, and that a simple estimation of the presence or absence of segregation of mixed inoculums near the site of inoculation would provide an accurate yet quick and easy method for assessing resistance levels.

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CONTRIBUTION OF *XYLELLA FASTIDIOSA* GENES UNIQUE TO GRAPE STRAINS TO ITS VIRULENCE TO GRAPE AND UTILITY IN SPECIFIC DETECTION OF GRAPE STRAINS BY DNA-BASED METHODS

Project Leader:
Steven E. Lindow
Dept. of Plant and Molecular Biology
University of California
Berkeley, 94720-3112
icelab@socrates.berkeley.edu

Cooperator:
William S. Feil
Dept. of Plant and Molecular Biology
University of California
Berkeley, 94720-3112
bhfeil@nature.berkeley.edu

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ABSTRACT
Xylella fastidiosa (*Xf*) is a group of genetically similar strains that infect a wide range of plants. We hypothesized that discrete genetic factors among the very similar strains determine the ability of a strain to infect a particular host plant. To better understand what makes grape a good host for all grape strains but not for strains such as oleander and almond that cannot colonize grape, we conducted experiments to look for host specific genes of the grape strain. Through our microarray and *in silico* genomic studies, we have identified 20 potential *Xf* grape strain virulence genes. Of these, we have focused on 10 genes. We excluded 10 genes based on criteria such as they are phage related, DNA modification genes, part of a repeated gene complex, or are predicted house keeping genes, and thus not likely to have a role in plant virulence. It was clear from our studies that the microarray studies have produced fewer unique genes (genes present in one strain and lacking in another) to grape strains than expected, indicating that the identity between *Xf* 'Temecula' and other non-grape strains must be closer than expected. Our *in silico* comparisons also revealed a high level of identity between grape and non-grape strains of *Xylella*. Because of this, we are now using dual labeling with our microarray studies to determine even small differences in gene sequence rather than simple lack of a particular gene. This is a more sensitive way to determine qualitative differences between the strains. We have now made knock-out mutants for seven of the 10 genes unique to grape strains that we expect to be most likely involved in virulence to grape. We used constructs that have a Kanamycin gene inserted near the 5' end of the gene for optimum efficiency in knocking out a given gene while preventing partial transcripts to be made in such knockout strains. Inoculation studies with grape have shown that several of these genes confer the ability to move within grape and thus to incite disease at sites away from the point of inoculation. The growth of these mutant strains in grape near the point of inoculation was not usually impaired, suggesting that such traits are involved specifically in other aspects of movement and symptom development in grape.

INTRODUCTION
Xylella fastidiosa (Xf) is a group of genetically similar strains that infect a wide range of plants. A particular strain often has a relatively small and distinct host range when compared to other strains. Some strains of *Xf* originating from host plants other than grape do not sustain viable populations or are not virulent in grape. In particular, many of the strains of *Xf* isolated from almond do not infect grape (Almeida and Purcell 2003). This strongly suggests that differing genetic factors among the strains determine the ability of a strain to infect a particular host plant. Other studies provide evidence for host specificity among the *Xf* strains (Chen et al. 1992; Chen et al. 1995; Pooler and Hartung 1995;Hendson et al. 2001; Bhattacharyya et al. 2002a, 2002b; Doddapaneni et al. 2006). Cross inoculations in greenhouse studies showed that the oleander and grape strains of *Xf* were not pathogenic to citrus and that the almond strain was not pathogenic to oleander (Feil et al. *unpublished*). In California, three distinct strains of *Xf* as designated by their host range are recognized; the grape strain, the almond strain, and the oleander strain.

To better understand the underlying genetics of *Xf* as it relates to pathogenesis, several strains have been sequenced. The *Xf* '9a5c', a citrus strain, was fully sequenced in Brazil (Simpson, 2000). The draft-genome sequences of the almond and oleander strains of *Xf*, 'Dixon' and 'Ann1', respectively, are also publicly available. We used this information to identify a list of genes present in the grape strain genome but missing in other strains that do not sustain viable colonies in grape. We also developed a DNA microarray based on the sequence of the Temecula grape strain to interrogate the genomes of other strains by a process of DNA-DNA hybridization. We tested the ability of target DNA from non-grape strains to hybridize to probes designed from the reference strain, *Xf* 'Temecula', which were affixed to epoxy slides. During this process, we determined that most strains are highly identical to each other, having genes that are at least similar in sequence to reference genes in strain Temecula; very few genes were found in the Temecula strain that were lacking in other *Xf* strains. We thus have used a more sensitive approach to identify unique genes of the grape strain that is based on competitive hybridization of mixed DNA samples to the DNA microarray. Using this method, as well as *in silico* and other single strain hybridization results we have now obtained a very short list of genes that were found in all grape strains of *Xf* but are lacking, or substantially divergent in non-grape strains of *Xf*. The goals of this project thus was to determine the role of such genes in the virulence of *Xf* to grape and other plants, and to determine if such genes would be useful in distinguishing grape strains of *Xf* from all other strains in PCR-based detection schemes.