ENHANCING CONTROL OF PIERCE'S DISEASE BY AUGMENTING PATHOGEN SIGNAL MOLECULES

Principal Investigator:
Steven E. Lindow
Dept. Plant & Microb. Biol.
University of California
Berkeley, CA 94720
icelab@berkeley.edu

Cooperator:
Michael Ionescu
Dept. Plant & Microb. Biol.
University of California
Berkeley, CA 94720

Cooperator:
Clelia Baccari
Dept. Plant & Microb. Biol.
University of California
Berkeley, CA 94720

Cooperator:
Rodrigo Almeida
Dept. Env. Sci., Pol., & Mgmt
University of California
Berkeley, CA 94720

Cooperator:
Aline da Silva
University of California
Berkeley, CA 94720

Cooperator:
Nabil Killiny
University of California
Berkeley, CA 94720

Cooperator:
Ellen Beaulieu
University of California
Berkeley, CA 94720

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ABSTRACT

Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusitble signal factor (DSF) that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzymes involved in degrading pit membranes and hence movement between vessels, DSF accumulation suppresses virulence of *Xf* in grape. We thus are exploring different ways to elevate DSF levels in plants to achieve disease control via "pathogen confusion." Plants expressing *rpfF* from *Xf* produce low levels of DSF and are highly resistant to Pierce's disease (PD). Chloroplast targeting of RpfF apparently substantially increases DSF production. *Xf* moved much less rapidly in *rpfF*-transformed grape, colonized many fewer xylem vessels, and achieved a much lower population size indicating that elevated DSF levels suppressed movement within the plant. As exogenous sources of DSF applied in various ways to grape suppressed pathogen mobility and hence virulence we have further studied the chemical identity of DSF. Preliminary evidence suggests that DSF is comprised of three closely related fatty acid molecules. One component is 2-Z-tetradecenoic acid (hereafter called C14-cis) while a second compound termed C12-cis is apparently also produced. The chemical identity of a third component is as yet undetermined and is being investigated. We are currently determining the relative activity of these forms of DSF and if such molecules cooperate in regulating gene expression in *Xf*. The various forms of DSF may preferentially affect different behaviors of *Xf*. Since some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggests that DSF produced by rootstocks can somewhat move to scions and confer disease control the control of disease, grafted plants are being made that have a relatively large rootstock to test the hypothesis that increased supply of DSF to the scion will be associated with a larger rootstock. Naturally-occurring endophytic bacteria within grape are being assessed for DSF production; only about 1% of the endophytic bacteria in grape produce DSF and these are being tested for their ability to move within plants after inoculation. As studies of pathogen confusion will be greatly facilitated by having an improved bioassay for the DSF produced by *Xf*, we have been developing several immunological and biochemical means to assay for the presence of DSF using *Xf* itself as a bioindicator. Bioassays based on immunological detection of the cell surface adhesin XadA and EPS have been developed. Gene expression in *Xf* exposed to various levels of DSF can also be directly assessed using *phoA* reporter gene fusions. Xanthomons campestris-based biosensors in which Rpf components have been replaced by those form *Xf* also selectively detect the DSF produced by *Xf*. The adherence of mutants of *Xf* to grape vessels is predictive of their virulence, indicating that adhesiveness is a major facor affecting the ability of *Xf* to cause disease. Such adhesive assays should enable us to more rapidly screen transgenic plants for their resistance to PD as well the efficacy of chemical analogs of DSF to induce resistance. The adherence of WT strains of *Xf* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* was much higher than non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation.

LAYPERSON SUMMARY:

Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease (PD). We have investigated both the role of DFS-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. Elevating DSF levels in plants erduces is movement in the plant. We have found naturally-occurring bacterial endophyte strains that can produce large amounts of DSF; we are testing them for their ability to move within plants and to alter the abundance of DSF sufficiently to reduce the virulence of *Xf*. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of the pathogen to enable DSF production in plants; such grape plants produce

at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control. The chemical composition of DSF itself is being determined so that synthetic forms of this signal molecule can be made and applied to plants in various ways. We have found that the adherence of *Xf* to grape tissue is much more tenacious in the presence of DSF, and we thus have developed assays to more rapidly screen transgenic plants for their resistance to PD as well the efficacy of chemical analogs of DSF to induce resistance.

INTRODUCTION

We have found that the virulence of *Xylella fastidiosa (Xf)* is strongly regulated in a cell density-dependent fashion by accumulation of a signal molecule called diffusible signal factor (DSF) encoded by *rpfF* and involving signal transduction that requires other *rpf* genes. We now have shown that the pathogen makes at least one DSF molecule that is recognized by *Xanthomonas campestris pv. campestris (Xcc*) but slightly different than the DSF of *Xcc* (**Figure 1**).

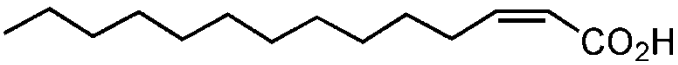


Figure 1 Structure of DSF: C14-cis.

Our on-going work suggests that it also makes other, closely related signal molecules as well. In striking contrast to that of Xcc, *rpfF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DFS-deficient mutants of *Xf* are consistent with the role of this density-dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged pathogen are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf.* Importantly, rpfF- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that the pathogen has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow to excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of *Xf* we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of Rpf regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf.* In this period we have extensively investigated both the role of DFS-production by the pathogen on its behavior within plants, the patterns of gene regulation mediated by DSF, the frequency with which other endophytes can produce signal molecules perceived by *Xf*, have further characterized the behavior of the pathogen in grape genetically transformed to produce DSF, and explored other means to alter DSF abundance in plants to achieve Pierce's disease (PD) control. We have particularly emphasized the development of various methods by which DSF abundance in plants can be assessed so that we can make more rapid progress in testing various ways to modulate DSF levels in plants, and have also developed more rapid means by which the behavior of *Xf* in plants can be assessed that does not require the multi-month PD assay. Lastly, we have developed better methods to assess DSF-mediated changes in phenotypes in the pathogen itself.

OBJECTIVES

1. Using novel, improved biosensors for the DSF produced by *Xf*, identify naturally-occurring endophytic bacteria which produce *Xf* DSF, and evaluate them for biological control of Pierce's disease after inoculation into plants in various ways.
2. Evaluate plants with enhanced production of DSF conferred by co-expressing RpfB, an ancillary protein to DSF biosynthesis, along with the DSF synthase RpfF for disease control as both scions and as rootstocks.
3. Optimize the ability of DSF-producing in rootstocks to confer resistance to Pierce's disease in the scion.
4. Determine the movement and stability of synthetic DSF and chemical analogs of DSF applied to plants in various ways to improve disease control.

RESULTS AND DISCUSSION

Characterization of DSF made by *Xf*.

While the molecule C14-cis is one component of DSF made by *Xf*, further support for the possibility that more than one fatty acid signal molecule is made by RpfF was obtained by the use of a Thin Layer Chromatography (TLC) method to assess the fatty acids produced by *Xf*. In this method, acidified ethyl acetate extracts of culture supernatants of a wild-type *Xf* strain and an RpfF- mutant and a RpfB mutant were subjected to TLC and fatty acids visualized by iodine vapors. Interestingly, three different fatty acids were visualized in the wild type strain, while these were largely missing in an RpfF- mutant, with only very small amounts of two other putative fatty acids present (**Figure 2**). It also was of interest to see that the RpfB mutant produced an altered pattern of putative fatty acids, with the major chemical species produced by the WT strain missing, and much larger amounts of one of the other species produced. The top-most spot observed in extracts of a WT strain of *Xf* co-migrates with C14-cis, a chemical form of DSF that we have previously characterized. Interestingly, the middle band found in the WT strain, which migrated similarly to the topmost, and most abundant band seen in the rpfB mutant, co-migrates with C12-cis. It is noteworthy, that a re-examination of the DSF species produced by *Xanthomonas campestris* pv. campestris using different methods have revealed that this species also produces C12-cis. The most prominent, lower, band seen in the WT strain is not observed in the *rpfB* mutant. This supports the model that RpfB, a putative long-chain fatty acyl CoA ligase, serves to produce suitable substrates for RpfF, the DFS synthase. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. It is interesting to note that *rpfB* mutants have an altered behavior compared to *rpfF* mutants and WT strains of *Xf*. While *rpfF* mutants are hyper-virulent in grape, *rpfB* mutants were nearly as virulent as WT strain. In contrast, while rpfF mutants are non-transmissible by sharpshooters, the *rpfB* mutants exhibit only a slight decrease in their transmission suggesting that they are retained by sharpshooters more efficiently than the rpfF mutant. Given that the *rpfB* mutant appears to make C12-cis but not C14-cis, this suggests that C12-cis is sufficient to enable signaling that leads to insect transmission but does not greatly affect virulence. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants expressing both *rpfB* and *rpfF* transgenic plants can better complement the virulence of the non-pathogenic *rpfF* mutant of *Xcc*. Based on this and other data, we are expressing both RpfF and RpfB simultaneously in transgenic plants for optimum production of suitable DSF molecules. We thus are preparing genetic constructs to transform grape with these two genes to further enhance DSF production.

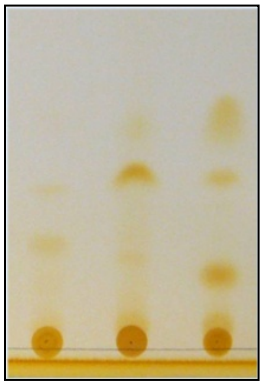


Figure 2. Fatty acids resolved by TLC from a RpfF mutant of *Xf* (left lane) a RpfB mutant (center lane) and a wild type strain of *Xf* (right lane). Fatty acids were visualized after exposure to iodine vapor.

Studies of adhesion of *Xf* to grape

Our studies have suggested strongly that adhesion of *Xf* to plant tissues inhibits movement of the pathogen through the plant, and hence tends to reduce the virulence of the pathogen. RpfF- mutants of the pathogen that do not produce DSF adhere to glass surfaces and to each other much less effectively than WT strain that produce DSF. This is consistent with the apparent rpfF-regulation of adhesins such as HxfA, HxfB and XadA etc. To better correlate levels of DSF in the plant and the stickiness of the *Xf* cells we have developed a practical assay to measure and compare stickiness of *Xf* cells in grapes infected with *Xf gfp-Wt* and *Xf* mutants. In this assay, the release of cells of *Xf* from stems and petioles tissue from grape infected with *Xf* wild type Tem and gfp-rpfF mutant were compared. Tissues from infected Thompson seedless grapes were surface sterilized. From the sterile tissues, 5mm stem or petiole segments were cut and placed individually in sterile buffer and shaken gently for 20 minute. After 20 minutes the number of cells released from the cut end of the segment were estimated by dilution plating on PWG. To determine the total number of cells in a given sample (the number of cells that potentially could have been released by washing) the washed segment was macerated and *Xf* populations again evaluated by dilution plating. Total cell populations were calculated by summing the cells removed by washing and those retained in the segment. The ratio of easily released cells to the total cells recovered in the samples was termed the release efficiency. In both stems

and petioles the release efficiency of the *rpfF* mutant was much higher than that of the WT strain (**Figure 3**). There was a very strong inverse relationship between the adhesiveness of the cells to grape and their ability to cause disease (**Figure 3**). This very striking difference in the adhesiveness of the *Xf* cells experiencing different levels of DSF in the plant suggest that this release efficiency assay will be valuable for rapidly assessing the susceptibility of grapes treated in various ways. For example, the adhesion of cells could be measured within a couple of weeks after inoculation of WT *Xf* cells into transgenic plants harboring various constructs designed to confer DSF production in plants, or in plants treated with DSF producing bacteria or topical application of chemicals with DSF-like activity. Such an assay would be far quicker than assays in which disease symptoms must be scored after several months of incubation, and could be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

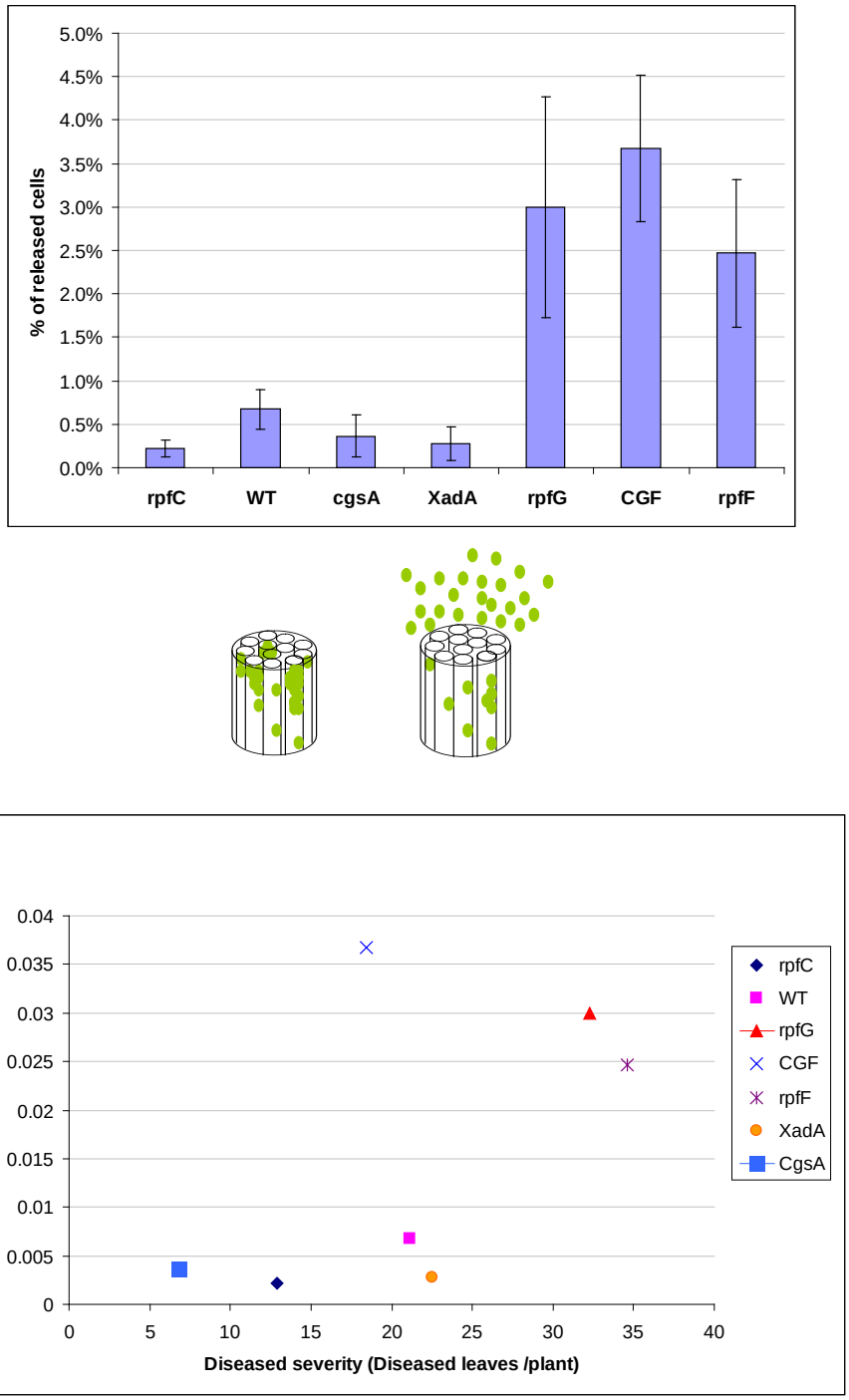


Figure 3. (top) Proportion of total cells of various mutants of *Xf* that were released during gentle washing of grape stem segments in buffer as depicted (middle). The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment. (bottom) Relationship between proportions of various *Xf* mutants released from tissues and the virulence of those strain in Thompson seedless grape.

To further address the usefulness of the cell release assay to assess treatments designed to limit the movement of *Xf* in plants to achieve disease control, we tested the adherence of WT strains of *Xf* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* compared with that to non-transformed plants. Plants were inoculated with a gfp-marked wild type strain of *Xf* and petioles were removed from plants at a distance of about 20 cm from the point of inoculation, and the percent of the cells removed during a brief washing step measured as above. The adherence of WT strains of *Xf* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* was much greater than that of cells in the non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation. That is, the percentage of cells that was released from *rpfF*-expressing plants was from two to three-fold less than that of control plants (**Figure 4**). As seen before, cells of an *rpfF* mutant that does not produce DSF exhibited about three-fold higher percentage of cells released from a normal Thompson grape compared to the WT strain in the non-transgenic grape (**Figure 4**). These results suggest strongly that DSF production presumably has occurred in the *rpfF*-expressing plants, and that the enhanced adhesiveness of these cells is associated with their reduced ability to spread through the plant and cause disease. These results suggest that the release efficiency assay should be a useful tool to rapidly assess treatments designed to control PD.

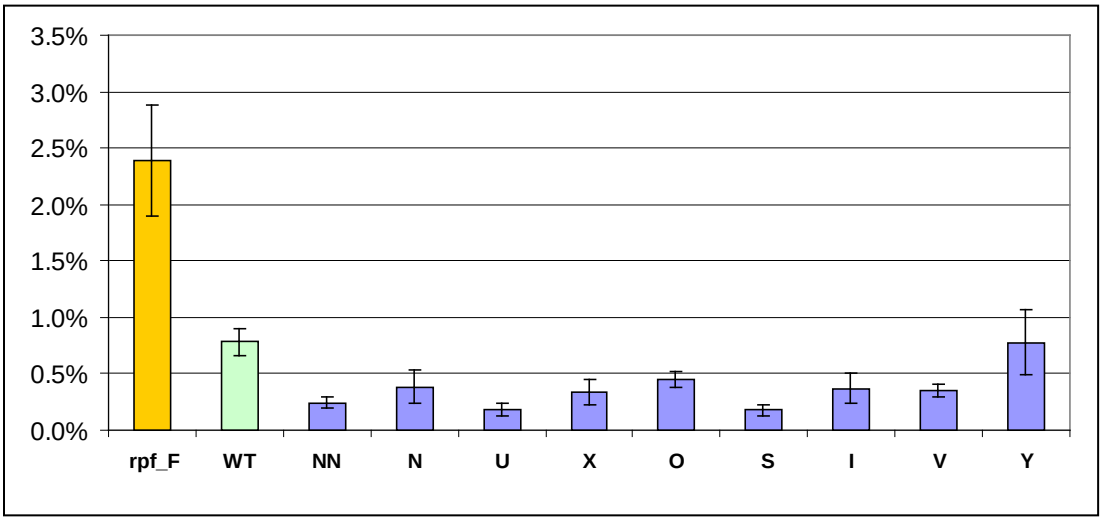


Figure 4. Percentage of total cells of a gfp-marked WT strain of *Xf* (blue and green) and a gfp-marked rpfF mutant of *Xf* (orange) in petioles of non-transformed Thompson (Orange and Green) or of transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* (blue) that were released during gentle washing of the segments in buffer. The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment.

While the movement of *Xf* has been recognized as an important trait necessary for disease, the process is still poorly understood. Other studies we have performed in our lab are strongly supportive of a model of progressive and sequential colonization of a large number of xylem vessels by *Xf* after inoculation of a single vessel. Furthermore, we believe that the process of movement of *Xf* through plants is a stochastic one which is characterized by growth in a given xylem vessel into which 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. We thus are exploiting the use of mixtures of phenotypically identical strains of *Xf* differing by only one or two genes 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 are major factors conferring resistance in plants. *Xf* must move from one xylem vessel to another dozens or hundreds of times to be able to move longitudinally down a vine as well as laterally across the vine to achieve the extensive colonization of the stem that are typical of diseased vines. If, at each step in this movement process only a few cells are transferred then, by chance, xylem cells distal to the inoculation point will receive by chance only one of the two genotypes of the pathogen. 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. We now have identified differently marked *phoA* mutants as having identical behavior in grape as the WT strain, enabling us to examine the process of spatial segregation of cells of *Xf* during colonization of grape. The population size of the WT strain of *Xf* was similar to that of the *phoA* mutant whether inoculated singly or in a mixture. More importantly, the proportion of cells of the two strains that were recovered from different locations within a given plant differed greatly between plants (**Figure 5**). For example, eight plants were inoculated with an equal mixture of the WT and *phoA* mutant, yet in some plants all of the cells recovered from locations either 10 cm or 120 cm form the point of inoculation were either one strain or the other; seldom was a mixture of both strain found, and a similar fraction of the plants harbored one strain or the other, suggesting that the two strains had an equal likelihood to move within the plant, but that stochastic processes determined the movement. We hypothesize 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. We thus are currently further exploring the spatial dependence of this segregation process in different grape varieties that differ in resistance to PD. Plants have been inoculated and assessment of the ratios of cells of the two strains in the mixture are currently being made. Not only should this provide considerable insight into the process of movement which, while central to the disease process, remains very poorly understood, but it should also provide new tools for screening grape germplasm for resistance to *Xf*.

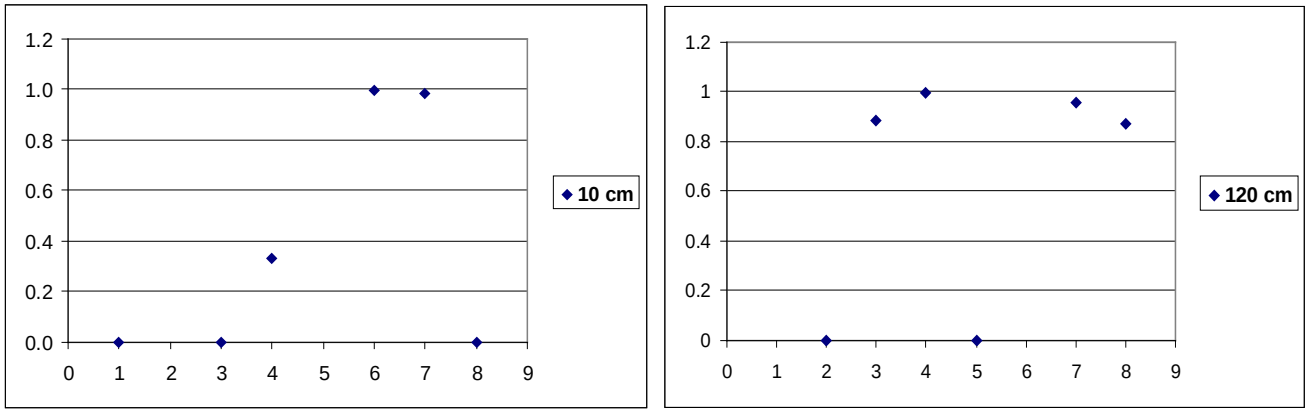


Figure 5. Proportion of cells recovered at 10 cm (left panel) or 120 cm (right panel) from the point of inoculation from individual plants that were inoculated with an equal mixture of a gentamycin-resistant PhoA mutant strain of *Xf* and a kanamycin–resistant PhoA mutant (abscissa) that were the kanamycin-resistant strain.

Graft transmissibility of DSF

To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing Freedom grape transformed with the *rpfF* gene of *Xf* are used as rootstocks to which normal Cabernet Sauvignon grape were green-grafted as a scion. As a control, normal Freedom was also used as a rootstock. These plants were inoculated with *Xf* to test whether normal scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization as a rootstock than as a scion. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto an *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock. Thus, like in the studies of the *rpf*F-expressing tobacco, it appears that DSF production in the scion is more efficacious for disease control than is the expression of *rpfF* in the rootstock. We are repeating these grafting experiment both with the non-targeted *rpfF* Freedom as a rootstock as well as the chloroplast-targeted *rpfF* Thomson seedless as a rootstock. We are testing the hypothesis that increasing the size of the rootstock will increase its potential to distribute DSF to the scion. We thus are producing wild-types scions grafted to rootstocks of differing sizes. This is proving difficult because the normal process is root the root stock at the same time that the scion is grafted onto the top. Establishing a large rootstock before grafting has made establishment of the grafted scion more difficult. Some success has now been achieved in these plants will soon be inoculated and their disease susceptibility will be related to the size of the rootstock.

Disease control with endophytic bacteria

The severity of PD can be reduced when DSF-producing bacteria such as rpfF-expressing *E. coli* and *E. herbicola* and certain *Xanthomonas* strains are co-inoculated with *Xf* into grape. Hoowever, these bacteria do not spread well within the plant after inoculation. Presumably to achieve control of PD by endophytic bacteria where *Xf* might be inoculated at any point in the plant by insect vectors it will be important to utilize endophytic bacteria that can colonize much of the plant in order that DSF be present at all locations within the plant. Naturally-occurring endophytic bacteria that produced the DSF sensed by *Xf* and which might move extensively within the plant would presumably be particularly effective as such biological control agents. For that reason we have initiated a study of naturally-occurring bacterial endophytes for their ability to produce DSF. This objective was possible since much effort devoted in the last two years has resulted in the development of better biosensors for the DSF produced by *Xf* (C14-cis and related molecules) (discussed below). We now have several highly sensitive assays for *Xf* DSF. Our new biosensor, however has allowed us to screen large numbers of bacteria recovered from blue-green sharpshooter insect heads and grape plants for *f* DSF production. We are executing this part of the project using two approaches; the first approach is building our own endophyte library using mainly endophytes isolated from insect head and wild grapes. The second approach involved screening an existing large grape endophyte library which was kindly made available to us by Dr. B. Kirkpatrick. Our initial results reveal that 0.9% of the endophytes recovered from either insect mouthparts or from grape xylem produce either a DSF detected by the *Xcc*-specific DSF biosensor, or the *Xf* DSF-specific biosensor or both. More than twice as many strain produce a DSF detected by the *Xcc*-specific DSF biosensor, suggesting that the DSF produced by *Xf* is not as common as that produced by other bacteria such as environmental *Xanthomonas* strains

etc. Many more strains remain to be tested as a relatively large library of DSF-producing bacteria has been assembled. Those strains found to produce the same DSF as *Xf* are then being assessed for their ability to grow and move within grape plants as well as their ability to reduce symptoms of PD when co-inoculated and pre-inoculated into grape before *Xf*.

Development of *Xcc*-based biosensors efficient in detecting *Xylella* DSF

For many of the objectives of this project, an improved bioindicator for DSF would be very valuable. Until recently we have used an *Xcc*-based biosensor in which the *Xcc* endoglucanase gene is linked to a GFP reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a much lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. We constructed two independent *Xcc* -based DSF biosensors specific to the DSF produced by *Xf* RpfF and which is sensed by *Xf* RpfC. In the first sensor, we replaced the Rpf-DSF detection system of *Xcc* with that of homologous components from *Xf*. An *Xcc* mutant in which both *rpfF* and *rpfC* was deleted was transformed with a pBBR1MCS-2 based plasmid harboring *Xf rpfC* and *rpfF* genes. A second *Xcc*-based *Xf* DSF sensor was constructed that is composed of an *Xcc rpfF* and *rpfC* double mutant into which *Xf rpfF* and *rpfG* and a hybrid *rpfC* allele composed of the predicted trans-membrane domain of *Xf* RpfC and the cytoplasm domain of the *Xcc* RpfC has been added. We named the three different sensors based on their DSF sensing element, *Xcc*-rpfC, *Xf*-RpfC and *Xf-Xcc* chimeric RpfC. The *eng'::gfp* transcriptional fusion in *Xcc* was inactive in the absence of DSF but was strongly induced by ≥0.1 μM 2-*Z*- tetradecenoic acid. We compared the activity of these two *Xf*-DSF specific biosensors to the activity of our original *Xcc*-DSF specific biosensor (*Xcc rpfF* mutant harboring an *eng'::gfp* reporting fusion) in response to diluted DSF extracts obtained from *Xcc* and from *Xf*. The three *Xcc*-based DSF biosensors (*Xcc*RpfC (also called pKLN55), *Xf*RpfC (also called GCF), and *Xcc*chimericRpfC (also called Chimeric)) exhibited similar sensitivity to the DSF containing extract from *Xcc* but in sharp contrast, *Xcc*RpfC exhibited much lower sensitivity to the DSF containing extract from *Xf* than the two *Xf*-DSF specific biosensors(**Figure 6**). These results suggest that *Xf*-RpfC can interact with a wider range of molecules then *Xcc*-RpfC. In addition, these two sensors were found to become activated even in the presence of dilutions of the *Xf* extract that contain less than the fraction contributed by a single PWG plate from which the DSF was originally extracted.

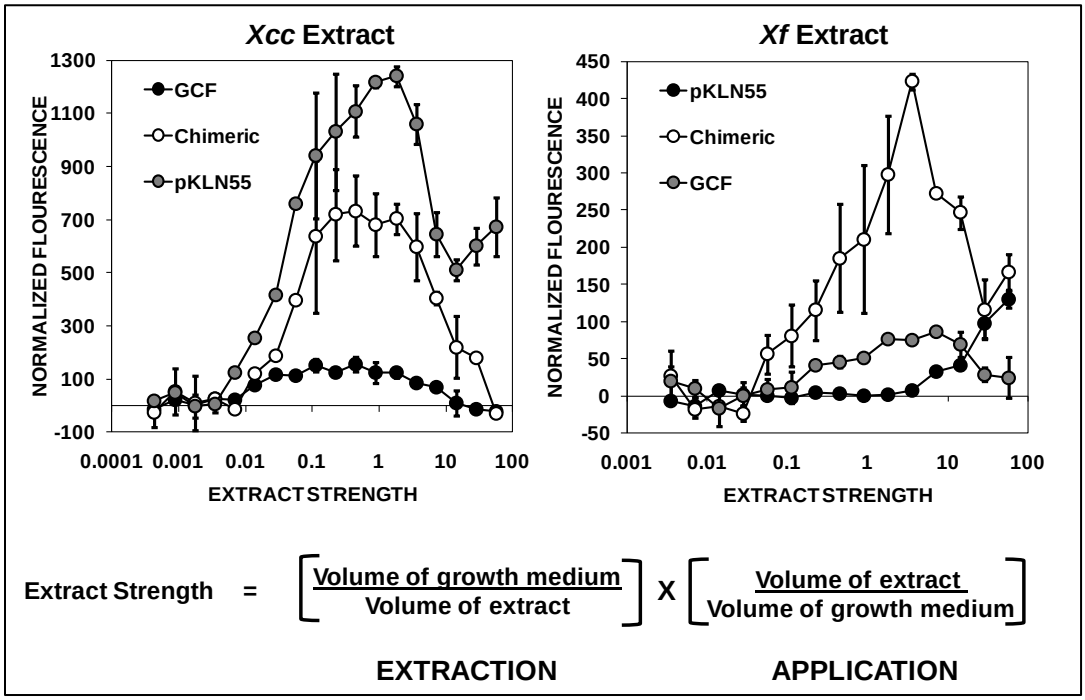


Figure 6. GFP fluorescence of different DSF biosensors to increasing concentrations of DSF from culture extracts of *Xcc* (left panel) or from *Xf* (right panel).

To test the specificity of the DSF biosensors we compared the activity of those sensors to a panel of pure DSF analogues. Quantification of the response of the three biosensors to various fatty acid molecules revealed a substantial difference in their selectivity to compounds of different chain length. The *Xf*RpfC and *Xcc*ChimericRpfC biosensors, based on the use of an *Xf* RpfC receptor were clearly more responsive than the XccRpfC biosensor for fatty acid molecules greater than 12 Carbon atoms long (**Figure 7**). Given that the DSF produced by *Xcc* is 12 Carbons in length, while at least one of the molecules made by *Xf* is apparently 14 Carbons in length, it appears that the RpfC receptor has evolved to bind fatty acid signal molecules of a particular length. This also suggests that while *Xcc* may be relatively unaffected by exposure to DSF made by *Xf*. The converse is probably not the case. The two *Xf* DSF specific DSF biosensors, particularly the *Xf-Xcc* RpfC biosensor

is much more responsive to C14-cis, and hence will be far more useful that the original *Xcc* RpfC biosensor for assessing DSF levels in plants and bacterial cultures.

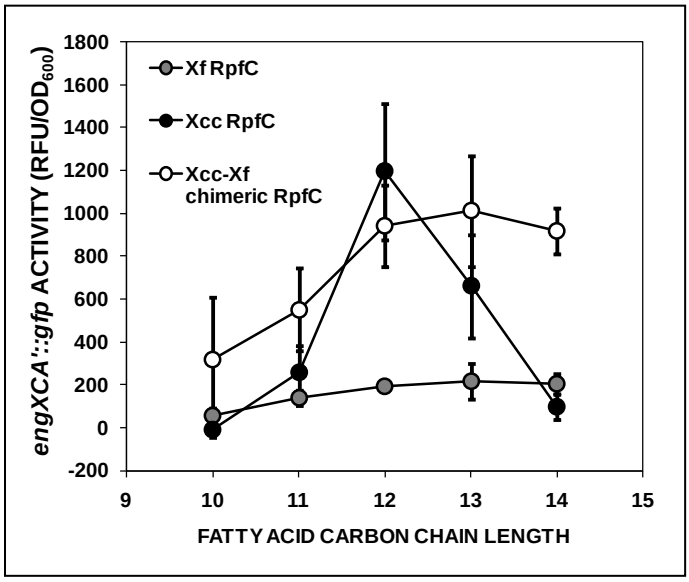


Figure 7. Relative responsiveness of three different DSF biosensors to unsaturated fatty acids of different chain lengths.

Since the various DSF sensors appeared to have high specificity and sensitivity for DSF, they are suitable for large-scale testing of DSF molecules and transgenic plants as well as for DSF produced by antagonistic bacteria. Methods were therefore tested to ascertain the most expedient way in which these biosensors could be deployed for the detection of DSF. A broth culture assay in which the biosensors were suspended in xylem sap of transgenic plants expressing RpfF from *Xf* successfully detected DSF. We were pleased to find that GFP fluorescence and hence DSF responsiveness of the biosensor could be easily monitored in such a high throughput manner.

Development of a *Xf*-based bioreporter for DSF

We have developed methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. Previous attempts to establish *gfp* or *inaZ*-based transcriptional fusions in *Xf* failed, presumably due to its incapability to express foreign genes properly we have successfully use the endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in *Xf*. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF (**Figure 8**). We are currently conducting extensive tests of this *Xf*-based biosensor to screen various compounds produced by *Xf* to determine those that are active as DFS signaling molecules.

We also examined the phenotypes of an *rpfF*- mutant and WT strain of *Xf* exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. The DSF-induced behaviors of *Xf* were found to be strongly dependent on the culture medium that the cells were grown in. All strains of *Xf* are much more responsive when cells are grown in a minimal medium such as PIM6 or PDA than in media such as PWG containing BSA. For example, cells of the WT strain which are not adherent in culture, and thus which do not form cell-cell aggregations became much more adherent to each other when DSF was added to shaken broth cultures. The increased adherence is readily visualized as an enhanced ring of cell-cell aggregates that forms at the liquid-air interface of shaken cultures (**Figure 9** left). The attachment of *Xf* cells to tubes or wells is readily measured by estimating the number of attached cells by their ability to bind crystal violet. The amount of cells bound to the surface of tubes increased with increasing concentration of C14-cis above about 1 uM. The concentrations of C14-cis at which cell binding was increased as estimated by this assay was similar to that as measured in an *Xcc*-based bioassay (**Figure 9** right). Cells of *Xf* exposed to DSF in PIM6 minimal medium also appear to adhere to each other more than those in the medium with added DSF. The DSF-medated increase in adhesiveness is readily apparent as cells that clump together when visualized microscopically (**Figure 10**). Clearly, exogenous DSF stimulates an adhesive state in *Xf*. The *Xf*-based cell binding assay therefore appears to be a very valuable and rapid method by which DSF response in *Xf* can be assessed.

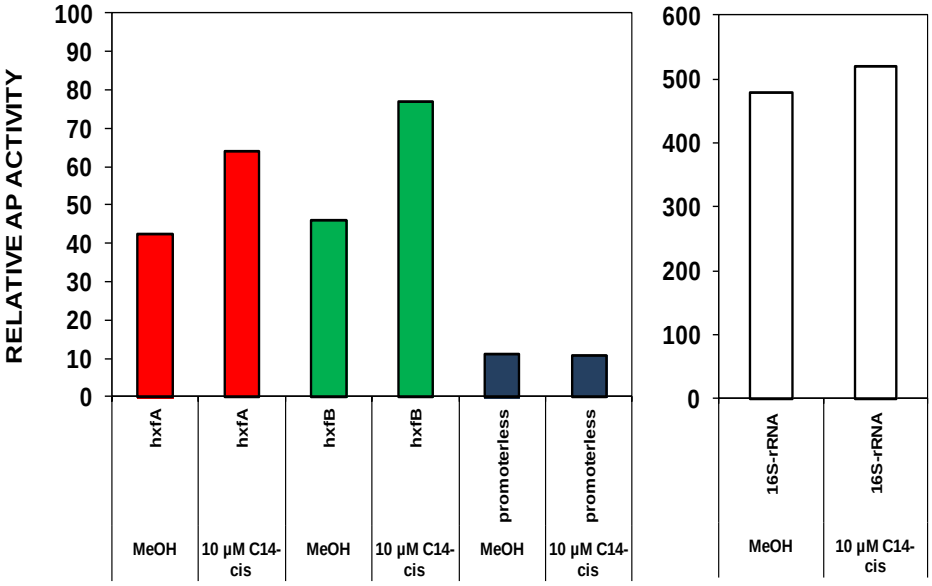
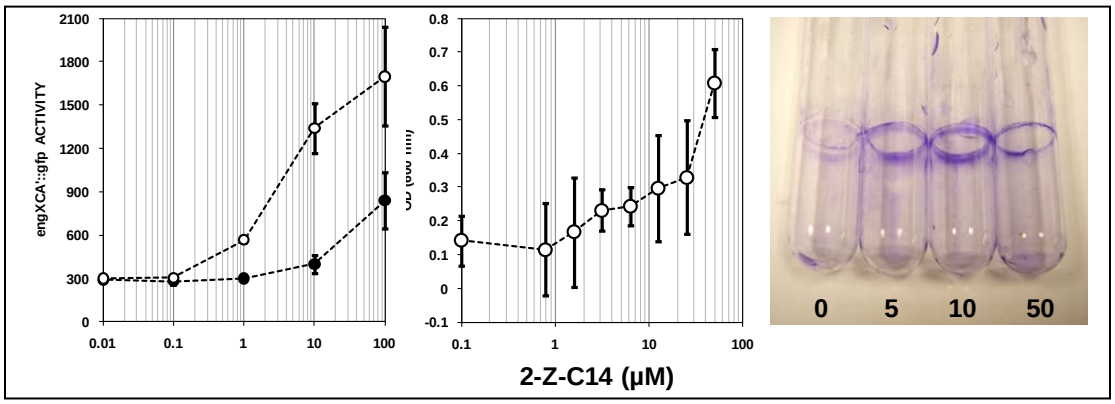


Figure 8. Increased alkaline phosphatase activity of cells of *Xf* harboring a fusion of *phoA* and *hxfA* and exposed to C14-cis (left box) and constitutive expression of *phoA* when fused to *rrnB* (right box).



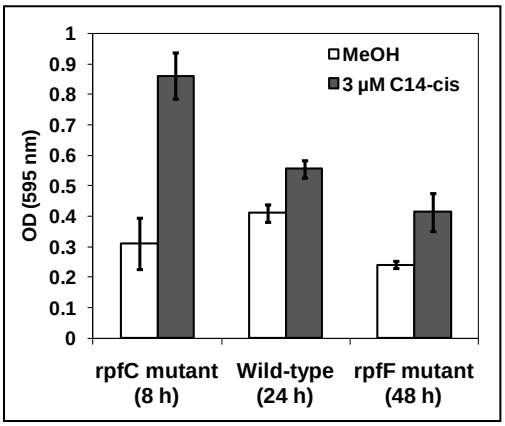


Figure 9. (top 3 panels). Binding of cells of *Xf* to the walls of glass tubes at the air-medium interface in cells grown in PIM6 medium containing up to 50 micromolar C14-cis as visualized by crystal violet staining (right panel). The quantification of cell binding as measured by crystal violet absorption is shown in the center panel for the cells. The responsiveness (gfp fluorescence) of an *Xcc*-based biosensor grown in PIM6 medium containing up to 50 micromolar C14-cis is shown in the left panel. (bottom panel). Quantification of attachment of cells of the RpfC mutant, wild type strain, and RpfF mutant of *Xf* to the walls of glass tubes to which 3 uM C14-cis had been added when assayed with a crystal violet binding procedure.

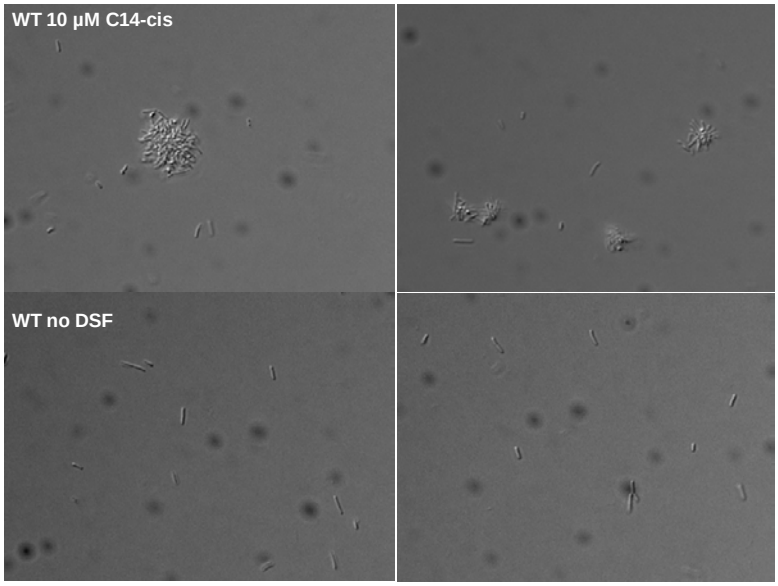


Figure 10. Microscopic depiction of cells of wild type *Xf* grown in PIM6 medium for three hours to which 10 uM C14-cis had been added (top panels) and cells grown in PIM6 medium without added DSF (bottom). Note the cellular aggregates found in the top panels in the presence of DSF.

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion." Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, and transgenic DSF-producing plants appear particularly promising and studies indicate that such plants provide at least partial protection when serving as a rootstock instead of a scion. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion. The tools we have developed to better detect the specific DSF molecules made by *Xf* will be very useful in our on-going research to test the most efficacious and practical means to alter DSF levels in plants to achieve disease control. We are still optimistic that chemically synthesized DSF molecules might also ultimately be the most useful strategy for controlling disease. The presence of more than one DSF base signal molecule suggests that perhaps more than one molecule might be needed to achieve changes in pathogen behavior. Our major advances in the development of biosensors to detect the responsiveness of *Xf* to signal molecules is a major breakthrough that hopefully will allow us to make rapid progress in ascertaining those transgenic plants most capable of altering pathogen behavior as well as in formulating synthetic molecules suitable for use in disease control. The biological sensors also have proven useful in screening naturally occurring bacteria associated with grape that might also be exploited to produce signal molecules

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