CONTINUED ASSESSMENT OF *XYLELLA FASTIDIOSA* FIMBRIALADHESINS AS IMPORTANT VIRULENCE FACTORS IN PIERCE'S DISEASE: INFLUENCE OF XYLEM SAP

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ABSTRACT
Specific biological characteristics of *Xylella fastidiosa* (*Xf*) Temecula were investigated in microfluidic flow chambers *in vitro* by examining the influence of xylem saps from Pierce's disease (PD) susceptible *Vitis vinifera* and resistant *V. champinii* and *V. smalliana* grapevines on *Xf* motility. Compared to that observed in *V. vinifera* sap, type IV pili mediated twitching motility of *Xf* was significantly reduced in saps from both resistant grapevines and is associated with down-regulation of type IV pili associated genes. The *Xf* response in resistant sap mirrors observations in citrus sap: down-regulation of type IV pili associated genes, lack of twitching motility, and resistance to pathogenesis by *Xf* Temecula. Overall the findings suggest that PD resistance in certain grape cultivars is associated with sap that blocks motility through gene regulation, limits aggregation, and lessens production of biofilms by *Xf.*

LAYPERSON SUMMARY
Cells of *Xylella fastidiosa* (*Xf)* aggregate, form biofilms, and occlude the host's vascular (xylem) system, resulting in Pierce's disease symptoms in grapevine. Colonization of grapevine xylem by *Xf* involves migration of individual cells through a process of twitching motility by which hair-like type IV pili are repeatedly extended from the cell, attach to the xylem surface, and are retracted, pulling the cell forward. Using microfluidic 'artificial' chambers through which xylem sap from highly susceptible and resistant grapevines is flowing, the biological behavior of *Xf* in these saps was assessed. Toward this we observed reduced motility in sap from a resistant grapevines as well as a reduction in formation of cell aggregates and biofilms.

INTRODUCTION
This project continues efforts toward understanding the biological relationship between *Xylella fastidiosa (Xf)* cells and the xylem environment, and specifically the roles of fimbrial adhesins (type I and type IV pili, and associated proteins) in *Xf* virulence, motility, cell aggregation, and biofilm development. The research targets the functional biology of *Xf* in xylem sap. It tests and explores traits of sap and xylem vessels from resistant and susceptible grapevines, as well as that of citrus, that may inhibit or promote *Xf* cell activities associated with *pil* and *fim* gene products.

Previous observations describing roles for fimbrial adhesins (type I and type IV pili) in *Xf* virulence, motility, aggregation, and biofilm development have provided insight into their genetic mechanisms and regulation (De La Fuente, 2007; 2008). Studies on *Xf* motility and biofilm formation under natural conditions viz., *in planta*, have been hindered in part by optical inaccessibility of vascular tissue. Recent studies have shown the importance of xylem sap chemistry on growth, aggregation, and attachment of *Xf* cells, highlighting the establishment of stable cultures in 100% xylem sap (Andersen, 2007; Zaini, 2009). Studies with *Vitis riparia* and *V. vinifera* cv. Chardonnay sap (100%) in either microfluidic chambers or in culture tubes have shown that the pathogen responds to this more natural chemical environment differently than it does in rich artificial media such as PD2 (Zaini, 2009). Aggregation and biofilm development are enhanced (Zaini, 2009), and early indication is that twitching motility is also greater—in both the number of *Xf* cells and in rate of movement. It was reported that xylem sap from Pierce's disease (PD) resistant *V. rotundifolia* maintained *Xf* in a planktonic state, whereas the bacteria were more likely to form aggregates when incubated in xylem sap from susceptible *V. vinifera* cultivars (Liete, 2004). Those directed the attention to a more natural environmental system for *Xf*—one that will greatly enhance the value and significance of information generated in studying *Xf* in an *in vitro* system: the inclusion of xylem sap and xylem vessel tissue.

Previous observations reported that both type I and type IV pili are involved in aggregation and biofilm development (Li, 2007), and that type IV pili of *Xf* are involved in twitching motility within the xylem vessels of grapevine (Meng, 2005). Citrus is often grown adjacent to vineyards in California and may be considered a potential reservoir for PD *Xf* (Bi, 2007). Xylem sap from commercial citrus plantings in Temecula (grapefruit, orange, lemon) did not support *Xf* biofilm development while at the same time grapevine xylem sap obtained from adjacent vineyards supported thick biofilms (Shi, 2010). Citrus

xylem sap did not support the induction of a number of *pil* and *fim* genes, such as *pilT*, a gene that encodes for type IV pilus retraction (necessary for twitching motility), *pilY1*, a gene encoding a type IV pilus tip adhesion protein, *pilI, pilU,* and *fimA* that encodes the type I pilus subunit (Shi, 2010). The significant reduction in *pil* and *fim* gene expression in citrus sap is notable for at least two reasons: i) it may explain, in part, why the PD strain of *Xf* is not symptomatically expressed in citrus i.e. it does not move from the sites of introduction (no twitching motility), nor does it form biofilms, and ii) it may provide valuable clues into what chemical factors from citrus sap may be exploited in grape to reduce or inhibit similar gene product expression. Based on those data, it may be that in xylem sap from Pierce's disease resistant grapevines that pili function is suppressed.

OBJECTIVES

Objectives covered in this report include:

1. Establish a baseline of *Xf* activity *in vitro* for grapevine sap. This will include temporal and spatial activities for pili-associated functions—motility, cell aggregation, and biofilm formation (reported in 2010 PD symposium).
2. Assess pili-associated functions in grapevine sap from *Vitis vinifera* cultivars and *Vitis* species expressing distinct PD resistance and susceptibly. (preliminary report in 2010 PD symposium).
3. Assess pil and fim gene expression for conditions in Objective 2 that exhibit significant differences in functional *Xf* activities.
4. Compare pili-associated functions in grapevine vs. citrus sap

RESULTS

***Xf* motility in various grapevine saps**

Xf cells were assessed for motility in microfluidic flow chambers. Most *Xf* cells exhibited twitching motility in PD2 broth (**Figure 1**). After a period of adjustment from growth on PD media to sap *Xf* cells in *V.vinifera* sap from NY also showed nearly complete motility (96%) (**Figure 1**). In *V. champinii* and *V. smalliana* saps from NY, relatively few *Xf* cells attached to the chamber surface; furthermore, few (3% and 1%, respectively) *Xf* cells exhibited twitching motility (**Figure 1**). Similar responses were observed for saps obtained from grapevines grown in Davis, CA (**Figure 1**). These results suggest that twitching is either upregulated in PD-susceptible sap or suppressed in PD-resistant sap.

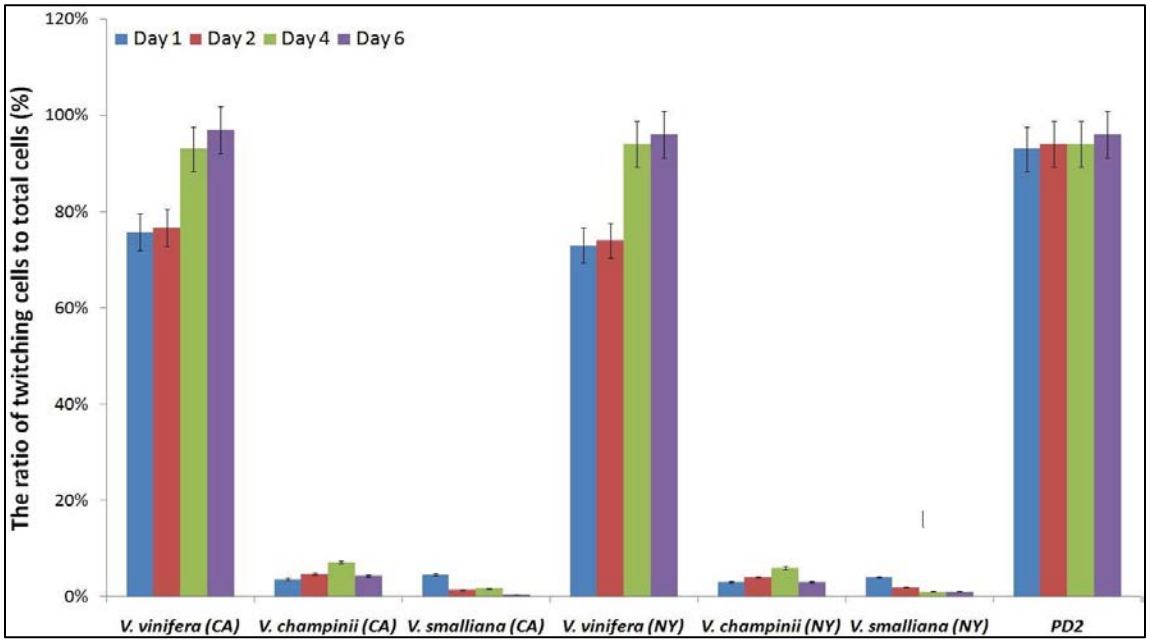

Figure 1. Twitching motility of *Xf* in various saps. The ratio of twitching cells to total cells in PD2 broth and saps of *V. vinifera*, *V. champinii*, and *V. smalliana* in microfluidic flow chambers was assessed over 6 days.

***Xf* response following exchange of sap types**

To determine if *Xf* behavior in saps could be modified under different sap conditions, we acclimated cells in microfluidic chambers to PD-susceptible *V. vinifera* sap and then exchanged it with a PD-resistant sap. *Xf* cells in PD-susceptible *V. vinifera* sap were motile and developed large cell aggregates [**Figures 2A(a) and (g)**]. After four days growth in *V. vinifera* susceptible sap. the sap was replaced with PD-resistant *V. smalliana* sap. After eight hours, the cell aggregates began to disperse and twitching was reduced [**Figure 2A(b)**]. In an adjacent *V. vinifera* perfused sap control chamber the cells continued to exhibit motility and form aggregates [**Figure 2A(h)**]. By day five *Xf* cells in *V. smalliana* sap were uniformly distributed within the chamber and only 5% were motile [**Figure 2A(c)**], while ca. 85% of the *Xf* cells in *V. vinifera* sap

continued to twitch and form aggregates [**Figure 2A(i)**] (**Figure 4**). From day five to eight, cells in *V. smalliana* sap formed a thin biofilm layer within the chamber [**Figures 2A(c)-(f)]** whereas cells in the control chamber developed a robust biofilm [**Figures 2A(i)-(l)**].

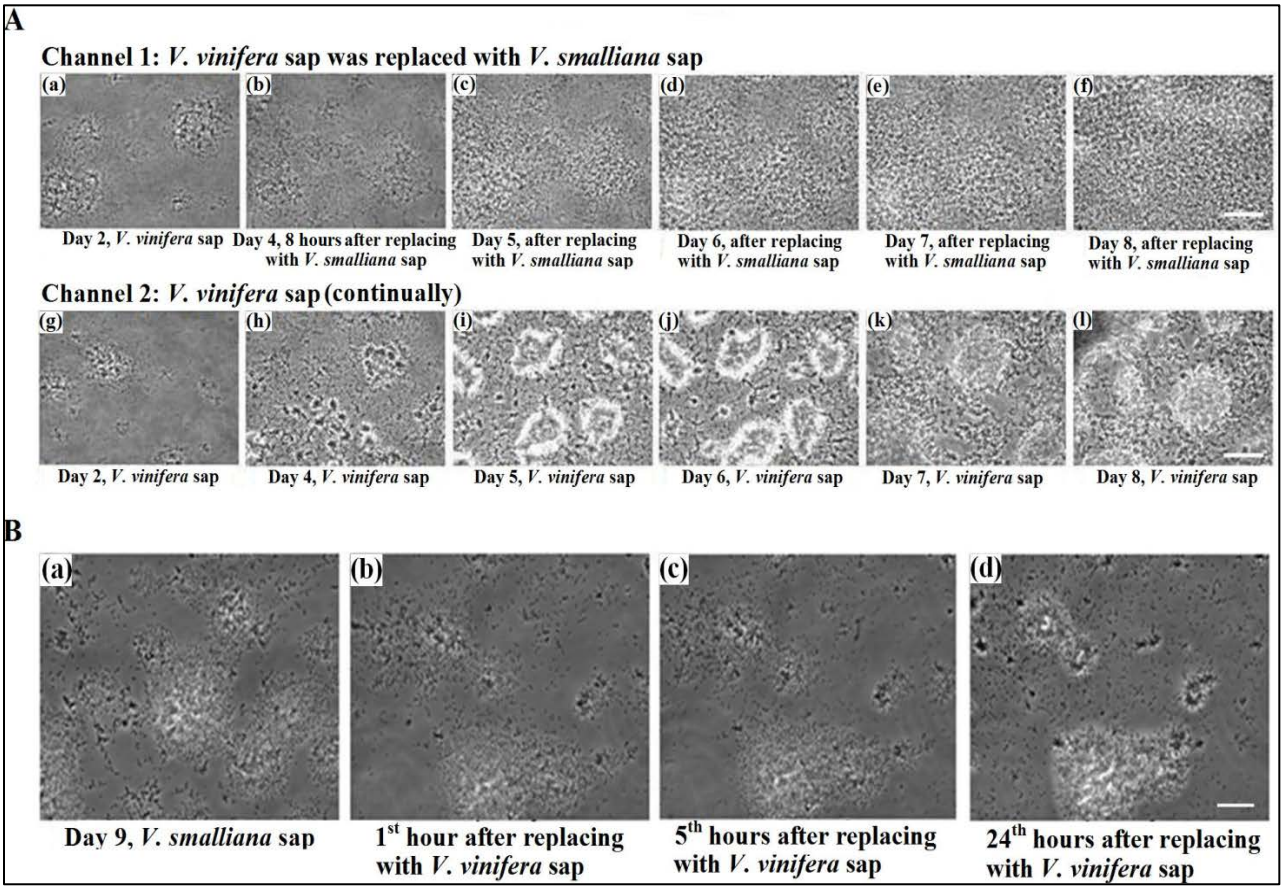

Figure 2. Twitching motility of *Xf* in PD-susceptible and PD-resistant saps. **(A)** Images taken before and after *V. vinifera* sap was exchanged with *V. smalliana* sap. In one microfluidic chamber *Xf*cells were continually exposed to PD-susceptible *V. vinifera* sap for eight days l (g-l). In another chamber *Xf* cells the*V. Vinifera* sap was changed to PD-resistant *V. smalliana* after day four (a-f). **(B)** *Xf* cells that had been grown in *V. vinifera* and changed to *V. smalliana* sap were exchanged back to *V. vinifera* sap on day nine (a-d). Scale bar equals 50 µm.

To determine if the *Xf* cells could return to the twitching phenotype, the PD-resistant *V. smalliana* sap in the chamber was replaced with *V. vinifera* sap on day nine [**Figure 2B(a)**]. After one to five hours the cells began to twitch and aggregates began to form [**Figures 2B(b) and (c)**], and by 24 hours large aggregate appeared [**Figure 2B(d)**]. When quantified, 3% of *Xf* cells expressed twitching motility in PD-resistant *V. smalliana* sap, whereas, 75% of the *Xf* cells were motile after *V. smalliana* sap replaced the PD-susceptible *V. vinifera* sap (**Figure 3**). Similar observations were made when *Xf* cells were grown in microfluidic chambers in *V.vinifera* and then replaced with *V. champinii* sap and back to *V. vinifera* sap (data not shown). These data suggest that either a) PD-susceptible saps induce the function or expression of type IV pili, resulting in twitching motility or b) PD-resistant saps inactivate the function or expression of type IV pili, resulting in the loss of twitching motility. The results of reduced motility are consistent with previous reports that *Xf* spreads faster in xylem vessels of PD-susceptible compared to PD-resistant or tolerant grapevines (Hopkins, 1984; Fry and Milholland, 1990).

Affect of sap on type IV pili-related

A number of mechanisms could be envisioned as to how a component in sap activates or inhibits motility such as altering gene expression of pili-associated genes. To explore gene expression, we analyzed the mRNA levels of representative genes *pilI, pilJ, pilG, pilA, pilQ,* and *pilR* involved in type IV pili biogenesis and regulation (**Figure 4**). The *pilI, pilJ,* and *pilG* gene encode proteins in the chemotaxis-like operon, Pil-Chp (Cursino, 2011). Chemosensory-like proteins have been implicated in motility, pili formation, transcriptional regulation, and exopolysaccharide production (Kirby, 2009). While the exact function of Pil-Chp is unknown, we previously found that disruption of the operon blocks twitching motility without inhibiting type IV pili biogenesis (Cursino, 2011). The PilQ protein is predicted to be a multimeric protein that forms the pore through which the type IV pili thread. We previously found that mutations in *pilQ* prevented type IV pili formation and motility (Meng, 2005). The PilR protein is predicted to belong to a two-component regulatory system, PilR/PilS, in which

PilS activates PilR, which in turn regulates *pilA* transcription; PilA is the major pilin protein of the type IV pilus (Winther-Larsen and Koomey 2002). PilA mutants of *P. aeruginosa* have reduced virulence (Comolli, 1999), and *pilA* mutants of *R. solanacearum* cause less severe wilting symptoms in tomato plants (Kang, 2002). Disruption of *pilR* leads to *Xf* without type IV pili and incapable of twitching motility (Li, 2007). In this study we found that *Xf* grown in *V. smalliana* and *V. champinii* saps, but not *V. vinifera* sap, fail to express *pilI*, *pilQ*, and *pilR*. These findings suggest that sap from resistant plants fail to support type IV pili production, block twitching motility, and therefore limit the pathogen spread within the plant. Given that multiple genes are down regulated in the resistant saps (or upregulated in susceptible sap), a key chemical component in sap may target an upstream regulator gene for type IV pili.

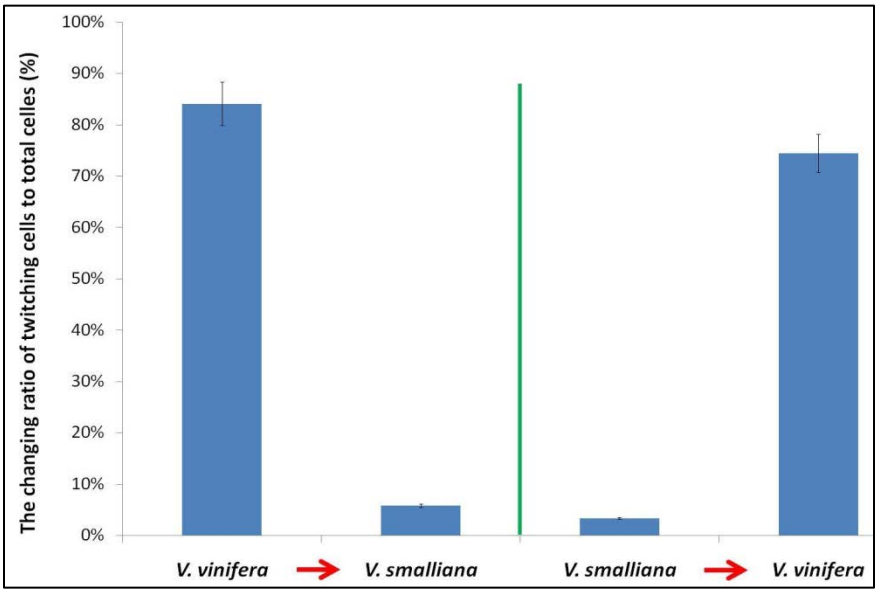

Figure 3. The changing ratio of twitching cells to total *Xf* cells after *V. vinifera* sap were replaced with *V. smalliana* sap and back to *V. vinifera* sap.

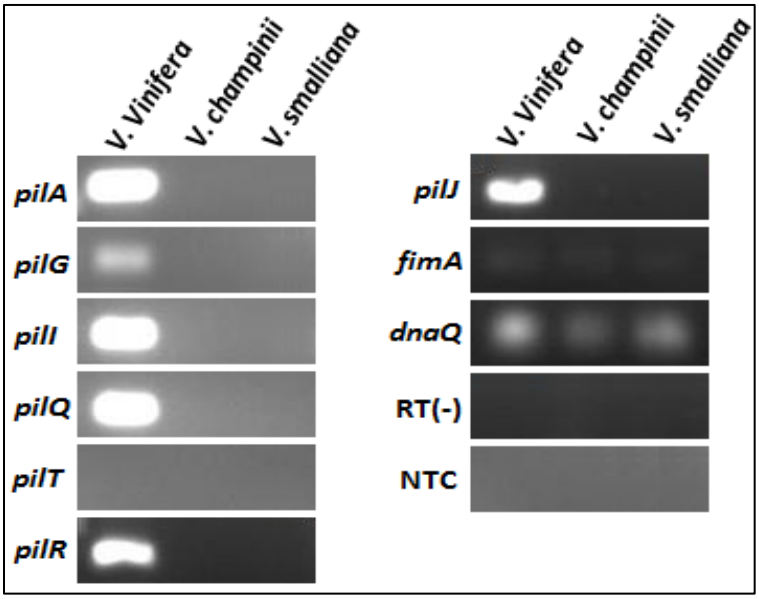

Figure 4. The detection of differential expression of type IV pili related genes *Xf* in saps of *V. vinifera*, *V. champinii*, and *V. smalliana* by reverse transcription polymerase chain reaction (RT-PCR). Positive control was DanQ expression. Negative control wasRT (-) negative control: all RT reactioncomponents but no RT reverse enzyme, and NTC: all RT reaction components but no RNA template.

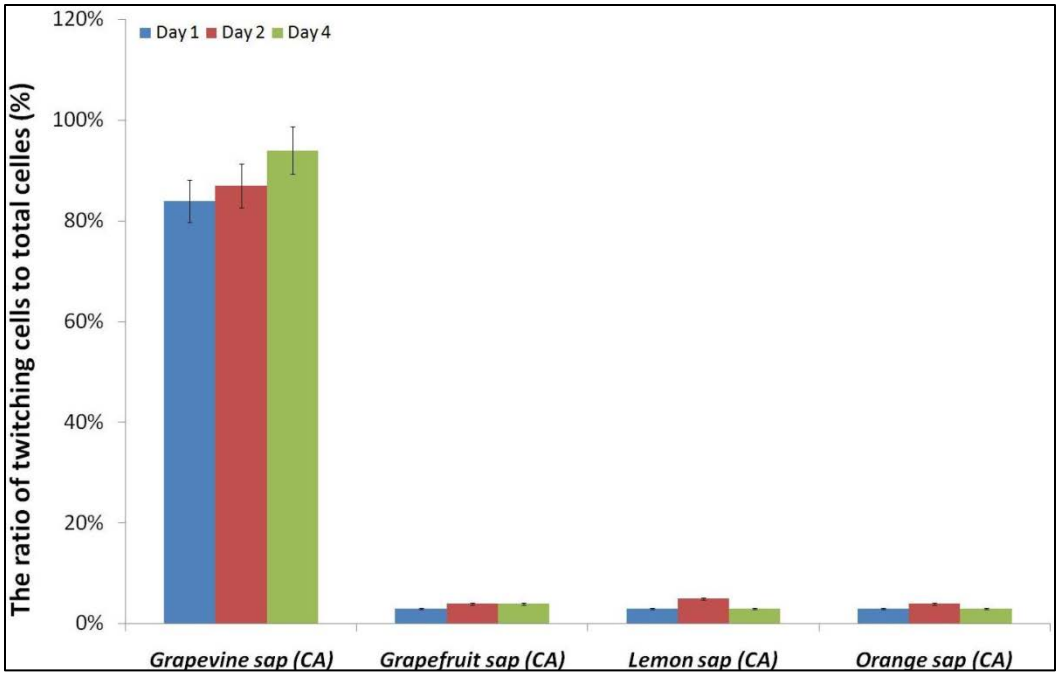

Figure 5. Twitching motility of *Xf* in various saps. The ratio of twitching cells to total cells in saps of grapevine (*V. vinifera*) and citrus (grapefruit, lemon, and orange) from CA in microfluidic flow chambers was assessed.

***Xf* motility in citrus sap**

Xf gene expression observed in PD-resistant saps mirrors findings when *Xf* is grown in citrus sap (Shi, 2010), and *Xf* Temecula does not produce disease in citrus plants (Perring, 2001). Therefore we wanted to determine if *Xf* is also non-motile in sap from citrus plants. *Xf* was found to be twitching impaired in saps of grapefruit, lemon, and orange (**Figure 5**). *Xf* cells in sap of grapevine (*V. vinifera*) from CA showed high levels of motility (94%+/-4). In saps of grapefruit, lemon, and orange from CA, relatively few (4%+/-2, 3%+/-2, and 3%+/-2, respectively) cells exhibited twitching motility (**Figure 5**). These results suggest that twitching of *Xf* cells is suppressed in citrus sap.

Comparison of responses in PD-resistant and citrus sap

The finding that grape sap composition alters gene expression of type IV pili associated genes is particularly interesting in light of resistance in non-grape plants. Citrus plants infected with *Xf* strain 9a5c exhibit a PD-like response known as citrus variegated chlorosis (CVC) (Chang, 1993; Hartung, 1994; Purcell & Hopkins, 1996). However, the *Xf* Temecula strain does not exhibit CVC even when PD-infected plants are found next to citrus orchards (Perring, 2001; Bi, 2007). Why Temecula does not produce disease is unknown. When examining twitching motility, we found that both PD-resistant and citrus saps fail to support mobility. Additionally, just as we found with PD-resistant sap, *Xf* grown in citrus sap is known to result in down regulation of type IV pili biogenesis and regulatory genes (Shi, 2010). While additional resistant mechanisms unique to each plant may play key roles in preventing *Xf*-induced disease, our findings suggest potential universal mechanisms for disease regulation.

CONCLUSIONS

The symptomatic development of PD in grapevine is related to biological features of the *Xf* pathogen and how it interacts with its host. By establishing more natural features of xylem vessels environment to study the motility and aggregation of *Xf*, we hope to provide a better understanding of the biological features of the *Xf* in natural xylem sap. The long-distance directional upstream migration of *Xf* might enhance intraplant spread of the bacteria and colonize grape xylem vessels from the initial site of infection. The chemical differences among saps of susceptible and resistant grapevine are highly desired to understand the biochemical mechanisms of host resistance to *Xf*. The present results suggest that the inhibition of twitching motility of *Xf* by chemical components in *V. smallaina* sap may limit the spread of *Xf* in xylem vessels in PD-resistant grapevines, resulting in the restriction of *Xf* to fewer xylem vessels and less proportion of *Xf* colonized vessels, which results in a limitation of systemic infection and no PD development in resistant grapevine.

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