Differential expression of genes of Xylella fastidiosa in xylem fluid of citrus and grapevine

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Abstract

Xylella fastidiosa causes a serious Pierce's disease (PD) in grapevine. Xylella fastidiosa cells from a PD strain were grown in a pure xylem fluid of a susceptible grapevine cultivar vs. xylem fluid from citrus, which is not a host for this strain of X. fastidiosa. When grown in grapevine xylem fluid, cells of the PD strain formed clumps and biofilm formed to a greater extent than in citrus xylem fluid, although the PD strain did grow in xylem fluid of three citrus varieties. The differential expression of selected genes of a PD X. fastidiosa strain cultured in the two xylem fluids was analyzed using a DNA macroarray. Compared with citrus xylem fluid, grapevine xylem fluid stimulated the expression of X. fastidiosa genes involved in virulence regulation, such as gacA, algU, xrvA, and hsq, and also genes involved in the biogenesis of pili and twitching motility, such as fimT, pilI, pilU, and pilY1. Increased gene expression likely contributes to PD expression in grapevine, whereas citrus xylem fluid did not support or possibly suppressed the expression of these virulence genes.

Introduction

Xylella fastidiosa is a fastidious, xylem-limited, nonflagellated bacterium that causes many economically important plant diseases, including Pierce’s disease (PD) in grapevine (Purcell, 1981). In southern California, several plant species were identified as hosts of a PD strain of X. fastidiosa (Costa et al., 2004), but some were symptomless. A citrus strain of X. fastidiosa causes citrus variegated chlorosis (CVC) in South America, but this strain is not known to be present in North America and appears to be distantly related to the California PD strain (Simpson et al., 2000; Van Sluys et al., 2003). In southern California, this bacterium is vectored mainly by a leafhopper, the glassy-winged sharpshooter (GWSS), Homalodisca vitripennis. In the Temecula Valley, where PD has caused catastrophic losses to wine grapevine, the major crop hosts for GWSS are grapevine and citrus. Vineyards and citrus groves are often in close proximity in that region. PD infection is most severe when the grapevines are adjacent to citrus. There are no X. fastidiosa-caused disease symptoms in citrus, although GWSS feeds and moves back and forth between nearby citrus and grapevine plants (Perring et al., 2001). This evidence suggested that while grapevine are susceptible to the PD strain of X. fastidiosa, citrus trees are resistant or tolerant, but could be a reservoir harboring the pathogen, allowing increased GWSS acquisition.

We previously investigated the mechanisms of host plant resistance/susceptibility in the Temecula Valley agro-ecosystem by examining the in vitro effect of the mixture (1 : 1) of PD3 medium and xylem fluid from grapefruit, orange, lemon, and grapevine on the growth, aggregation, and attachment of an X. fastidiosa Temecula1 (PD) strain isolated from grapevine in the region (Costa et al., 2004; Bi et al., 2007). We showed that the mixture of PD3 medium and xylem fluid from grapefruit, orange, and lemon trees supported bacterial cell growth and aggregation, but inhibited biofilm formation, whereas the mixture of PD3 medium and xylem fluid from grapevine supported both cell growth and biofilm formation (Bi et al., 2007). In the present study, we cultured X. fastidiosa in a pure xylem fluid from these host plant species and detected differential expression of
X. fastidiosa genes involved in transcriptional and post-transcriptional virulence gene regulation, as well as differential regulation of genes related to X. fastidiosa attachment, biofilm formation, and twitching motility.

Materials and methods

Preparation of xylem fluid

Xylem fluid of grapevine in commercial vineyards and grapefruit, lemon, and orange shoots in commercial orchards in proximity to those vineyards in the Temecula Valley, CA, were collected in April 2008 using a pressure chamber apparatus as described previously (Andersen et al., 1992; Bi et al., 2007). Xylem fluid was stored at −80 °C until final use.

Bacterial growth, cell aggregation, and biofilm formation

Cells of X. fastidiosa strain A05 (isolated from infected grapevine in the Temecula Valley, CA) (Costa et al., 2004) were cultured at an OD600 nm of 0.05 in 3 mL of xylem fluid from citrus and grapevine in borosilicate glass culture tubes, which were placed on a rotary shaker under constant agitation at 28 °C for 20 days. Xylem fluid without the bacteria and the bacteria inoculated in PD3 broth or sterile water was used as a control. All tubes were covered with a black cardboard box.

The bacterial cell concentration in the tubes was determined by measuring the OD600 nm at 10 and 20 days after culture. The cells in the tubes were dispersed by repeated pipetting and vortexing. For cell aggregation analysis, the cell concentration in the tubes was measured by determining the OD540 nm (OD5). The tubes were then kept without shaking for 1 h to allow bacterial cells to clump and settle. The OD540 nm of supernatants of the tubes (ODt) was measured again. The relative percentage of cell aggregation was measured using the following formula: % aggregated cells = (ODt − OD5)/(OD5) × 100 (Burdman et al., 2000). Clumped cells in the bottom of the tubes were photographed at 20 days. Cells from the tubes were cultured on PD3 medium plates and incubated at 28 °C for 10–20 days to determine the growth of the cells. At 20 days, the cells were collected from the plates and confirmed to be X. fastidiosa using primer-specific PCR (Minsavage et al., 1994). This procedure was repeated three times after the initial incubation.

For measurements of biofilm formation, X. fastidiosa cells were first cultured in PD3 broth and incubated at 28 °C without shaking for 4–6 days. The bacterial cells were then collected, rinsed, and adjusted in the xylem fluid of grapefruit, lemon, orange, and grapevine, respectively, to an OD600 nm of 0.05. One hundred fifty microliter aliquots of each cell suspension were added to 96-well microtiter plates, respectively. The negative control consisted of xylem fluid or PD3 without bacteria. Plates were incubated at 28 °C without shaking. At 10 and 20 days after incubation, biofilm formation on the wall of the wells was determined using a crystal violet staining method (Leite et al., 2004). Each treatment had three replications, and the resulting data were averaged.

DNA macroarray preparation, RNA preparation, macroarray analysis, and reverse transcription (RT)-PCR

DNA macroarray membranes were prepared with 111 selected genes with putative roles in X. fastidiosa virulence, as well as others involved in the metabolism of nucleic acids and proteins, and cellular transport and stress tolerance, based on the genome sequences of X. fastidiosa 9aSc (a CVC strain) (Simpson et al., 2000) and X. fastidiosa Temecula1 (a PD strain) (Van Sluys et al., 2003). Several unknown function genes that up- and down-induced in xylem fluid from grapevine were also included (Bi et al., 2007; Shi et al., 2008). DNA fragments (average 600 bp) of the ORF of the 111 genes were individually amplified by specific PCR from the genomic DNA of X. fastidiosa Temecula1, purified, and spotted onto nylon membranes (Hybond, Amersham Pharmacia Biotech Inc., NJ) using a manual 384-pin replicator (V&P Scientific Inc., CA). Spotted DNA was denatured with 0.4 M NaOH, neutralized with standard saline phosphate EDTA, UV cross-linked, and boiled in 0.1% sodium dodecyl sulfate (Hernandez-Martinez, 2005).

Total X. fastidiosa A05 RNA was extracted from the cultures grown in grapevine and citrus xylem fluid as described above at an OD600 nm of 0.15 using a Qiagen RNAeasy mini kit (Qiagen, CA). After extraction, total RNA was DNase-treated using Turbo DNA-free (Ambion, TX) and purified again using a Qiagen RNAeasy mini kit (Qiagen). To ensure that the RNA preparation was DNA free, an aliquot of 1 μL of RNA (50 ng μL−1) was then used to amplify the ORF of tolC with specific primers. The result was negative. The qualities of isolated prokaryotic RNA were determined by denaturing RNA formaldehyde gel electrophoresis (Chuang et al., 1993). cDNA was synthesized and digoxigenin-labeled by RT from storage DNA-free total RNA according to the manufacturer’s protocol (Roche Applied Science, IN).

DNA macroarray nylon membranes were hybridized with digoxigenin-labeled cDNA probes following the manufacturer’s instructions (Roche Applied Science). Signal intensities of spots on the membranes were analyzed using QUANTITY ONE® software (Bio-Rad, CA). One-way ANOVA of the expression values was used to select differentially expressed genes among mRNA samples. The expression levels...
of 111 genes under treatment (grapevine xylem fluid) and the control (citrus xylem fluid) were analyzed (Gusnanto et al., 2005). The hybridization signal intensity obtained from RNA extracted from X. fastidiosa grown in grapevine xylem fluid and citrus xylem fluid was normalized according to total signal strength. The normalized hybridization signals were log plot analyzed for reliability (Gusnanto et al., 2005) and were statistically analyzed for differential expression using Student’s t-test (P < 0.001). The normalized signal intensity from X. fastidiosa grown in grapevine xylem fluid was divided by that of citrus to calculate the grapevine/citrus (G/C) ratio. The G/C ratios obtained from individual hybridization experiments were averaged to yield the final G/C ratio. Genes having ≥1.5 or ≤0.66 final G/C ratios were selected as upregulated or downregulated in grapevine, respectively. In this experiment, mRNA was prepared from three biological replicates of each xylem fluid culture and had three hybridizations in the macroarray.

RT-PCR was used to validate the differential expression of genes obtained in the macroarray analysis. cDNA was amplified from stored DNase-cleaned RNAs using the AccessQuick RT-PCR system, following the instructions of the manufacturer (Promega, WI). The equal amount of cDNA was used for PCR with specific primers designed to amplify the internal regions of the ORFs of the selected genes according to the manufacturer’s instructions (Promega). Ten microliters of the reaction mixture was run in agarose gels, and the products were stained and visualized with ethidium bromide.

Results

Xylella fastidiosa cell densities in the pure xylem fluid from citrus and grapevine were low compared with those obtained in PD3 medium (Fig. 1), but the cells appeared as visible clumps at 10–20 days after they were introduced into the fluids (Fig. 2). Xylella fastidiosa cell densities in grapevine xylem fluid were higher than those in the other tested xylem fluids by 20 days after inoculation (Fig. 1), but the cell densities increased by 20 days in all xylem fluids. Bacterial cells grown in each xylem fluid were then inoculated to PD3 medium and confirmed to be X. fastidiosa species by specific PCR (data not shown). These data showed that X. fastidiosa can grow in the pure xylem fluid of citrus and grapevine in vitro.

The percentage of aggregated cells of X. fastidiosa in grapevine xylem fluid was similar to that in PD3 medium, but significantly higher than that seen in citrus xylem fluid (Fig. 3). The bacterial cells aggregated to form tight clumps in the xylem fluid of grapefruit, orange, and lemon. In contrast, bacterial cells were loosely clumped in grapevine xylem fluid (Fig. 2). Bacteria cells were more loosely clumped in PD3 medium than in the xylem fluids (Fig. 2).

After 20 days of culturing, X. fastidiosa cells in the grapevine xylem fluid formed more biofilm than those in the citrus xylem fluid (Fig. 4).

Of 111 selected genes from X. fastidiosa tested in a DNA macroarray, 27 genes were differentially expressed in grapevine xylem fluid vs. citrus xylem fluid (Table 1). Most had a higher expression in the grapevine xylem fluid, but two genes had a higher expression in the citrus xylem fluid. Using RT-PCR, several genes putatively involved in virulence were validated based on differential expression in the xylem fluid of grapevine vs. citrus (Fig. 5). rRNA was detected at similar levels in bacteria grown in each of the
The expression of genes of *X. fastidiosa* in xylem fluid

In the present study, xylem fluid of citrus supported the growth of a PD strain of *X. fastidiosa*, although this strain does not cause disease in citrus. This supports the hypothesis that citrus may serve as an asymptomatic reservoir for *X. fastidiosa* in southern California (Perring et al., 2001; Bi et al., 2007).

Biofilm formation is a major factor in *X. fastidiosa* virulence (Marques et al., 2002), and our measurements of enhanced biofilm formation in grapevine xylem fluid are consistent with the recent report of Zaini et al. (2009). The observation that more biofilm was formed in the grapevine xylem fluid than in the citrus xylem fluids (Fig. 4) would be compatible with the observation that infections of citrus species by *X. fastidiosa* are asymptomatic in southern California.

Differences in biofilm formation and aggregation by *X. fastidiosa* in xylem fluids from grapevines of varying susceptibility to PD have been correlated with specific differences in the nutritional components of the xylem fluid (Andersen et al., 2007). We were interested in the underlying genetic basis of the differential responses of *X. fastidiosa* to differences in xylem chemistry in different hosts. Therefore, we began an analysis of the effects of xylem fluid, from the grapevine host of a PD strain and from nonhost citrus species, on the expression of *X. fastidiosa* genes. Genes predicted to be involved in virulence regulation, such as the virulence regulator *xadA*, transcriptional regulator *algU*, two-component regulator *hsq*, and post-transcriptional regulator *gacA*, were expressed at greater levels in grapevine xylem fluid vs. citrus xylem fluid (Table 1, Fig. 5). The regulatory genes *algU* and *gacA* were previously shown to play roles in controlling several potential virulence factors in *X. fastidiosa*. An *algU* defective mutant (Shi et al., 2007) and a *gacA* defective mutant (Shi et al., 2009) had decreased cell aggregation, biofilm formation, and pathogenicity in grapevine compared with the wild type. *Hsq*, an RNA-binding protein, may indirectly affect biofilm formation in *X. fastidiosa* through a complex *hsq*/*rsmA*-mediated system (Shi et al., 2007).

Genes predicted to be involved in surface structures and attachment components, such as *PD0312*, *hsf*, and *xadA*, were expressed more vigorously in the xylem fluid of grapevine than that of citrus (Table 1, Fig. 5). *hsf* of *X. fastidiosa* is similar to the adhesion gene *hsf* in *Haemophilus influenzae*, and *xadA* encodes a putative afimbrial outer membrane protein involved in adhesion. An *xadA* defective mutant in *X. fastidiosa* is surface adhesion-deficient, which reduces *X. fastidiosa* adhesion in the early stages of attachment to the surface of its host (Feil et al., 2007). The expressions of *hsf* and *xadA* were increased in grapevine xylem fluid, likely contributing to an enhanced ability to adhere to xylem vessel walls. In this study, the lower percent aggregation of *X. fastidiosa* cells and lower biofilm formation in citrus xylem fluid might be related to decreased xylem fluids. No RNA was detected in the water and pure xylem fluid controls.

**Discussion**

The observation that *X. fastidiosa* cells growing in a pure xylem fluid from citrus and grapevine and appearing as visible clumps at 20 days after introduction into the fluid was consistent with previous studies using a mixture (1:1) of PD3 and xylem fluid (Bi et al., 2007). *Xylella fastidiosa* cells have been reported elsewhere to grow in 100% grapevine xylem fluid (Andersen et al., 2007; Zaini et al., 2009), and in the present study, xylem fluid of citrus supported the formation of biofilm and aggregation of *X. fastidiosa* cells in grapevine (grape), citrus (lemon, grapefruit, and orange) xylem fluid, and PD3 medium. Xylem fluid and PD3 without bacteria were used as control. *Xylella fastidiosa* was inoculated into the xylem fluid of grapevine and citrus at different letters indicate significant differences (Student’s t-test, *P* < 0.05) between the treatment means.

**Fig. 3.** Quantitative assessment of biofilm formation of *Xylella fastidiosa*. Three replicates were used in each experiment. For each assay time, different letters indicate significant differences (Student’s t-test, *P* < 0.05) between the treatment means.

**Fig. 4.** Clumping of *Xylella fastidiosa* cells in grapevine (grape), citrus (lemon, grapefruit, and orange) xylem fluid, and PD3 medium. Xylem fluid and PD3 without bacteria were used as control. *Xylella fastidiosa* was inoculated into the xylem fluid of grapevine and citrus at OD$_{600nm}$ of 0.05 in borosilicate glass culture tubes, which were placed on a rotary shaker at 28°C. Three weeks after the inoculation, the clump of *X. fastidiosa* was photographed from the bottom of each glass culture tube.
expression of adhesion-related genes, such as *hsf* and *xadA*. In contrast, increased expression of *hsf* and *xadA* in grapevine may be related to the higher biofilm formation and percent aggregation of cells. In addition, we reported previously that *xadA* and *hsf* were positively regulated by *gacA* in *X. fastidiosa* (Shi et al., 2009), suggesting that these adhesion functions are influenced by the *gacA* regulatory pathway.

Genes involved in the biogenesis and of type I and IV pili in *X. fastidiosa*, such as *fimT*, *fimA*, *pilT*, *pilU*, *pilI*, *pilG*, *pilZ*, and *pilH*, showed a higher expression in the xylem fluid of grapevine than of citrus (Table 1, Fig. 5). It was reported that type I pili function in attachment, cell aggregation, and biofilm formation, whereas type IV pili are involved in twitching motility within the xylem vessels of host plants (Meng et al., 2005). A mutation in *fimA* (type I pilus) resulted in a biofilm-deficient and twitching-enhanced phenotype, which increased *X. fastidiosa* motility within the xylem vessels of grapevine (Meng et al., 2005). A *pilY1* mutant had a twitching-reduced phenotype (Meng et al., 2005). The expression of genes, such as *fimT* and *fimA* encoding type I pili, was increased in grapevine xylem fluid, likely contributing to an enhanced ability to attach and form a biofilm within the xylem vessels of grapevine. The higher expression of the type IV pili genes *pilT*, *pilI*, *pilU*, *pilY1*, *pilE*, *pilG*, *pilZ*, and *pilH* in grapevine xylem fluid suggested that *X. fastidiosa* could enhance the migration and colonization of the xylem system of grapevine. In contrast, the lower expression of type IV pili genes in the xylem fluid of citrus (Table 1) suggests that *X. fastidiosa* remains in relatively few xylem vessels and has less motility within the xylem system of citrus. These results are consistent with reports that the severity of disease symptoms is positively associated with a higher proportion of *X. fastidiosa* colonized vessels in coffee and plum, but not in citrus (Alves et al., 2004).

The increased expression of *secG*, a secreted protein in the type II secretion system (Simpson et al., 2000), in grapevine xylem fluid, is consistent with a role for the type II system in the...
environments within the xylem of plants, and host range may in part be determined by differential regulation of virulence genes in different host xylem environments. Host plant resistance has been recognized as the most cost-effective and environmentally safe method for controlling many major microbial pathogens of economic plants. Understanding the underlying biochemical mechanisms of host resistance may lead to the development of resistant varieties or anti-X. fastidiosa chemicals useful in preventing disease in established grapevine. Identification of specific chemical components of citrus xylem fluid that influence the expression of virulence genes in X. fastidiosa is underway.

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References


