SEASONAL BEHAVIOR OF *XYLELLA FASTIDIOSA* CAUSING ALMOND LEAF SCORCH DISEASE UNDER FIELD CONDITIONS AND DETECTION OF THE BACTERIA BY MEANS OF ARRAY-PCR

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ABSTRACT

Diseases caused by *Xylella fastidiosa* (*Xf*) have re-emerged as a serious threat to several economically important crops, such as grape and almond, in the San Joaquin Valley of California. Knowledge of the bacterial behavior in plant hosts under field condition is important for disease control. This research characterized populations of *Xf* almond leaf scorch disease (ALSD) strains in almond orchards. In 2006, two almond orchards were selected based on known history of ALSD. One ALSD tree and one non-ALSD tree were identified from each orchard. The branch pattern of each tree was mapped. Samples were taken every month from each scaffold at both distal and proximal positions. Presence of leaf scorch symptoms were monitored, bacteria were isolated from different regions of the trees, and genotypes were determined by PCR analysis of both conserved housekeeping genes and the hypervariable *psp*B locus. We reported the population dynamics of ALSD bacteria and the pattern of leaf scorching symptoms in 2006, and continued the study in 2007. In 2007, the earliest occurrence of leaf scorching symptoms was in July, almost one month later than that in 2006. In both 2006 and 2007, PCR detected *Xf* in plant tissue one month ahead of symptom development. PCR was slightly more sensitive than cultivation method for early bacterial detection. However, uneven bacterial distribution and random sampling errors may contribute to the differences among the assays. Correlation between cultivation and PCR detection was over 90%. Analyses of tandem repeat numbers (TRNs) at the *psp*B locus showed that three TRN genotypes existed in the same almond tree, although one genotype predominated. To reduce PCR error that result from large volume sample processing, we developed an array-PCR protocol using primers from seven housekeeping genes. This array-PCR significantly improved detection accuracy.

INTRODUCTION

Xylella fastidiosa (*Xf*) is the causal agent for several economically important diseases including Pierce's disease of grapevine (PD) and almond leaf scorch disease (ALSD) in the San Joaquin Valley of California. The bacterium has received extensive laboratory characterization in the past few years, leading to the completion of genome sequencing of six *Xf* strains (9a5c, Temecula-1, Dixon, Ann-1, M12, and M23). Among them, three (Dixon, M12 and M23) are ALSD strains. More research in genomics, molecular genetics and phenotypic characterizations are underway. However, knowledge of bacterial behavior under field condition is limited. This information is important not only for the bacterial biology research, but also for effective disease management programs.

In this project, we characterized the population of *Xf* ALSD strains in almond orchards in Fresno County of California. In 2006, two almond orchards were selected based on the previously known history of ALSD. One ALSD tree and one non-ALSD tree were identified from each orchard. The branch pattern of each tree was mapped. Samples were taken every month from each scaffold at both distal and proximal positions. Presence of leaf scorch symptoms were monitored, bacteria were isolated from different sections of the trees, and genotypes were determined by PCR using primers designed from 16S rDNA sequence directly from freeze-dried pulverized tissue (FDPT). The population dynamics of ALSD bacteria in 2006 was reported previously (Chen et al., 2006). We continued to study disease development in these same trees in 2007. In addition to the use of FDPT as PCR template, petiole sap mixtures (PSM) derived from isolation experiment (Chen et al., 2005, 2007) were also used.

To reduce PCR errors that result from large volume sample processing, the concept of multiple PCR was employed. Using the 96-well microplate format, we developed an array-PCR for *Xf* detection. In this protocol, multiple primer sets were designed based on different housekeeping genes identified from the genome sequence of strains Temecula-1 (causing PD) and Dixon (causing ALSD). By placing each well with a different primer sets along with appropriate controls, a row of 12 wells was used for simultaneous PCR for every sample. Comparing to the previous one-well-one-sample PCR, the 12-well-one-sample PCR was designed to tolerate occasional failures in PCR amplification. Results from multiple primer sets allow a conclusion on a sample detection to be drawn with high confidence.

Since sequences of housekeeping genes are highly conserved, they are reliable in detecting *Xf* at the species level. Multiple alignment sequence analyses can identify single nucleotide polymorphisms in housekeeping gene that differentiate A- and G-genotype ALSD strains. However, DNA polymorphisms in housekeeping genes are not sensitive enough to detect intra-pathotype variation. To further increase detection sensitivity in *Xf* population, the *psp*B locus was used. The *psp*B gene encodes a serine protease and contains a hypervariable tandem repeat region. Specific primer set flanking the tandem repeat

region was designed. Tandem repeat number (TRN) from each strain could be determined from PCR amplicons and used as a parameter to describe the bacterial population.

OBJECTIVES

1. To study the seasonal leaf scorch symptom expression patterns in the orchards and its association with pathogen detection.
2. To study the distribution and variation of *Xf* in almond trees under the field conditions.
3. To develop a PCR–array protocol for better detection accuracy of *Xf*.

RESULTS

ALSD symptom expression in 2007 was one month later than that of 2006 (Figure 1). However, the patterns of symptom appearance in both years were similar, i.e. the disease severity showed a pattern of gradual increase. In contrast, patterns of the rates of bacterial isolation, PSM-PCR and FDPT-PCR showed a much higher level of fluctuation during the growing season, although the trend of overall increases were still be seen (Figure 1). Isolation of *Xf* from different sections of the same almond petiole showed difference in bacterial titer. The inconsistent detection rate in both culture and isolated methods was at least in part related to the uneven distribution of *Xf* cells *in planta*.

A total of 312 samples were collected from both almond orchards in 2007, 46 % of the samples showed symptoms, 37 % were culture positive, 35% were SM-PCR positive, and 50 % were FDPT-PCR positive. The correlation between ALSD symptom appearance and bacterial detection was from 80% to 100% as of September 2007. Similar to 2006, PCR detection of *Xf* in 2007 was also one month earlier than symptom expression. PCR was slightly more sensitive than cultivation method for early bacterial detection. However, uneven bacterial distribution and random sampling errors might contribute to the differences among the assays.

Results of array PCR on 12 FDPT samples are tabulated in Table 1. Primers used in this study were checked by BLAST analyses using each primer set as query against the current GenBank database and they were specific to *Xf*. As expected, 10 out of the 12 samples had 1- 3 unsuccessful PCR amplifications that could lead to false negative conclusions, should there be a one-well-one-sample PCR was performed. We considered these variations as random experimental errors. The use of array-PCR minimized such errors.

Observation of TRN variation in the two Fresno almond orchards are shown in Table 2. Contrasting to the limited variations in the conserved genomic loci, at least three TRN genotypes, ranging from 12 to 14, were found in both orchards. TRNs in *psp*B seemed to decrease as the growing season proceeded.

CONCLUSIONS

1. The occurrence of ALSD symptoms is heavily influenced by environmental factors, as shown by the one month delay of ALSD symptoms, as well as *Xf* detection, in 2007. However, we do not know what exactly those environmental factors are.
2. ALSD symptom expression is related to the increase of *Xf* titer. Regardless of the variation in symptom expression, *Xf* cells were detected consistently one month ahead of symptom expression.
3. Because of the uneven distribution of *Xf* cells in the affected almond tree, there exists a large margin of sampling error for pathogen detection. Such sampling errors could lead to false negative conclusion in disease diagnosis. Array-PCR significantly improved detection accuracy.
4. An almond tree can harbor multiple *psp*B TRN genotypes. The role of these genotypic variations in ALSD development remains to be studied in the future.

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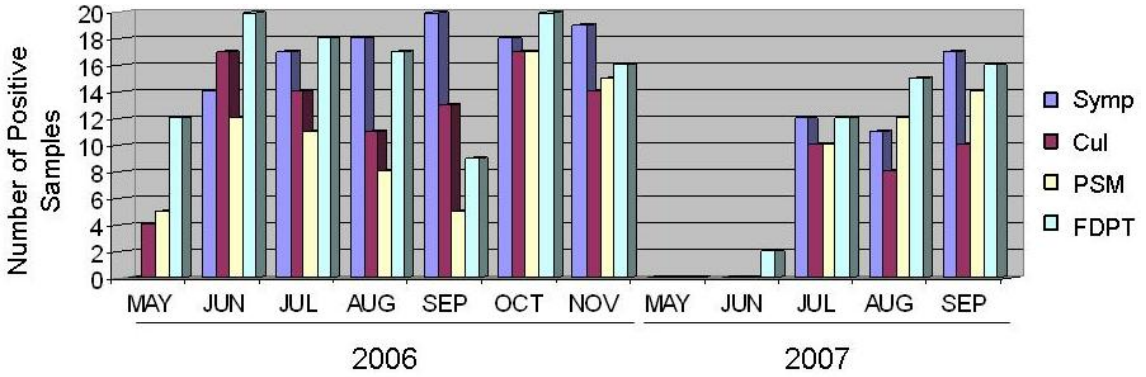

Figure 1. Temporal development of almond leaf scorch disease from May 2006 to September 2007. SYMP-leaf scorch symptom observed; Cul-cultivation of *Xf* on PW-G medium; PSM-petiole sap mixture as template for PCR; and FDPT-freeze-dried pulverized tissue as template from PCR.

Table 1. A summary of array-PCR on the detection of almond leaf scorch disease samples.

		Genomic loci									
NO	SAMPLES	GYR	MDH	DAS	GLT	T-D	16SG	16SA	PspB	ACON	ICD
1	MC-asym	+	+	+	+	+	+	+	-	+	+
2	MC-symp	+	+	+	+	+	+	+	+	+	+
3	MC-asym	+	+	+	+	+	+	+	+	+	+
4	MC-symp	+	+	+	+	+	+	+	-	+	+
5	MC-asym	+	+	+	-	+	+	-	-	+	+
6	MC-asym	-	+	+	+	+	-	-	+	+	+
7	MC-symp	+	+	+	+	+	+	-	+	+	+
8	MC-asym	+	+	+	+	+	+	-	+	+	+
9	MC-symp	+	-	+	+	+	+	-	+	+	+
10	FS-asym	+	-	+	+	+	+	-	-	+	+
11	FS-symp	+	+	+	+	+	+	-	+	+	+
12	FS-symp	+	+	+	+	+	+	-	+	+	+
13	Teme DNA	+	+	+	+	+	+	+	+	+	+
14	Teme Cells	+	+	+	+	+	+	+	+	+	+
15	No DNA	-	-	-	-	-	-	-	-	-	-

Table 2. Variation of tandem repeat numbers in *psp*B locus observed from two locations in Fresno County.

Location	Month	Number of tandem repeats
MC	May	13
	June	14
	July	14
	August	13
	September	13
	October	13
	November	12
FS	May	14
	June	14
	July	14
	August	14
	September	13
	October	12
	November	12