A qPCR assay for the Detection of Phytoplasmas causing Almond brownline, Peach yellow leafroll, and Pear decline diseases in California

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INTRODUCTION

In California, almond (*Prunus dulcis*) production is affected by diseases caused by phytoplasmas such as almond brown line (ABL) and shriveled kernel disease (ASKD; Uyemoto et al., 1992; Uyemoto et al., 1999). While a phytoplasma association has been clearly established in the case of ASKD, it is yet to be proven in the case of ALB (Sudarshana et al., 2009; Uyemoto et al., 1992).

Peach yellow leafroll phytoplasma (PYLR-P) was first reported in California as the causal agent of declining in peach trees. One predominant strain was found related to pear decline phytoplasma (PD-P; = *Candidatus* phytoplasma pyri) based on the sequence homology of the 16S ribosomal RNA and 16/23S spacer regions (Kirkpatrick et al.,1997). Incidentally, pear psylla vectors both PYLR-P and PD-P (Blomquist et al., 2002). While PD-P is present in Europe and North America, PYLR-P is believed to be restricted to North America and hence is quarantined in Europe. This quarantine status is based on the assumption that it is related to Western X disease phytoplasma (WX-P) present only in North America despite evidence stating the genetic relatedness of PYLR-P to PD-P (Kison et al., 1997). Detection of PYLR-P and several other phytoplasmas is generally done by PCR using P1 and Tint Primers or other primers specifically targeting the 16S-23S rDNA spacer region (Smart et al.,1996).

Here we describe a qPCR for the detection of ABL-P, PYLR-P and PD-P in DNA extracts from almond trees affected by brown line disease, almond and peach trees infected by PYLR-P and pear trees infected with PD-P (Fig. 1 to 3).

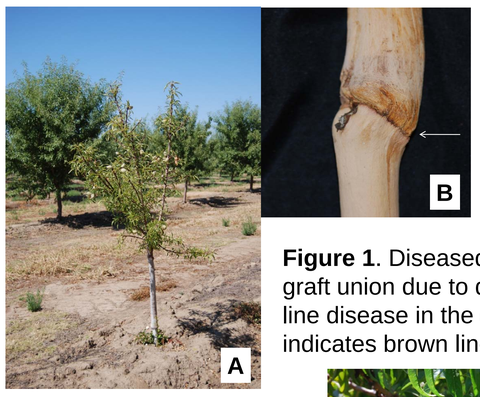


Figure 1. Diseased tree snapped at the graft union due to development of brown line disease in the orchard (A). The arrow indicates brown line at the union (B).

Figure 2. Peach cv. Fey Elberta inoculated with peach yellow leafroll phytoplasma (Right). A healthy tree is on the left.

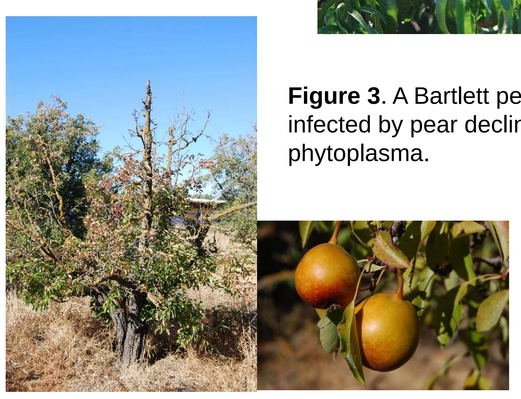
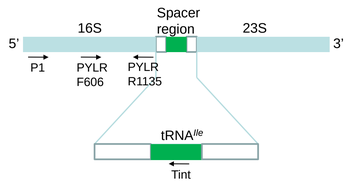
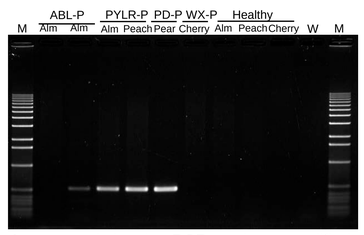


Figure 3. A Bartlett pear tree infected by pear decline phytoplasma.

RESULTS

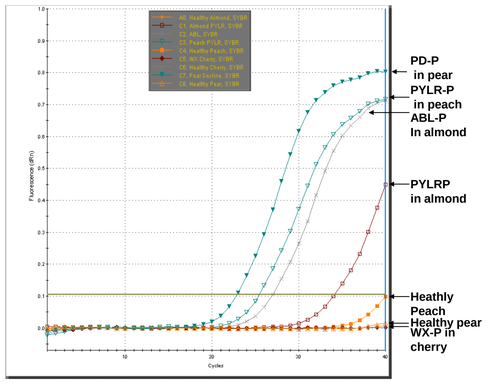


Ribosomal DNA region of phytoplasmas was targeted for amplification by primers PYLRrRNAF606 and PYLRrRNAR1135.



Agarose gel showing PCR products amplified from almond trees affected by brown line disease using primers CPPrRNAf609 and CPPrRNAr1135 in 30 cycles of amplification.

In PCR, primers PYLRrRNAF606 and PYLRrRNAR1135 amplify ~530 bp product from DNA extracts from ABL-P, PYLR-P and PD-P, but not from Western X phytoplasma (WX-P) and healthy almond (Alm) , cherry and peach trees. Samples from ABL affected almond trees generally needed 40 cycles to show the expected size product in agarose gels.



In qPCR, primers PYLRrRNAF606 and PYLRrRNAR1135 can detect in real-time ABL-P, PYLR-P and PD-P, but not Western X phytoplasma (WX-P).

DNA	Mean Ct	Tm ° C
a) **Cloned DNA**		
10^6	19.9±0.14	81.9
10^5	21.9±0.07	81.9
10^4	25.5±0.04	81.9
10^3	23.4±0.05	81.9
10^2	22.8±0.15	81.9
b) **Phytoplasmas**		
1) <u>In diseased trees</u>		
ABL-P	26.4±0.34	81.9
PYLR-P in almond	29.3±0.24	82.3
PYLR-P in peach	27.2±0.26	82.3
PD-P pear	25.1±0.14	82.3
2) <u>In dormant buds</u>		
PYLR-P Almond buds	39.8±0.41	82.3
PYLR-P Peach buds	34.6±0.14	82.3
c) **Negative controls**		
WX-P Cherry	No Ct	
Healthy almond	No Ct	
Healthy peach	No Ct	
Healthy pear	No Ct	
Water	No Ct	

Ct = cycle threshold (value Ct) from three replicates; Tm = dissociation constant.

A minimum of 100 copies (equivalent to 50 to 100 phytoplasmal cells) was sufficient to obtain test result. qPCR assays were also able to detect PYLR-P in dormant buds of almond and peach.

MATERIALS AND METHODS

<u>Primers for PCR:</u> The sequences of 16S-23S rDNA regions of ABL-P, PYLR-P and PD-P, were aligned and two new primers CPPrRNAF606 (5'-TGC TAT AGA AAC TGT TTG ACT AGAGT-3') and CPPrRNAR1135 (5'-AAT TTA TCA TTG GCA GTC TCAC-3') specific to these phytoplasmas were designed.

<u>PCR and qPCR analysis</u>: Total genomic DNA was extracted from symptomatic almond and peach leaves from trees affected by ABL and PYLR and endocarp of pear fruits from a Bartlett tree affected by pear decline using a plant DNeasy kit (Qiagen Inc., Valencia, CA).

A 530 bp PCR product was amplified by PCR from ABL-P, cloned into a plasmid vector and used as a standard to spike nucleic acid extracts from a healthy almond tree to optimize qPCR conditions using SYBR®green on a Stratagene Mx3000P smart cycler (Agilent Technologies, Inc., La Jolla, CA).

Detection and quantitation of phytoplasmal DNA was achieved by qPCR using one cycle of 95 C for 10'; 40 cycles of 95 C for 30 sec, 60 C for 1 min, and 72 C for 1 min 40 sec; and one cycle of 72 C for 3 min. Dissociation constants were obtained by melting at 95 C for 1 min followed by incubation at 55 C for 30 sec and 95 C for 30 sec.

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