Development of a Molecular Assay for the Detection of a Phytoplasma Associated With Almond Brown Line Disease in California

Mysore R. Sudarshana[1*], Saul Gonzalez[1], Franz J. Niederholzer[2], and Jerry K. Uyemoto[1]
[1] USDA-ARS, Dept. of Plant Pathology, University of California, One Shields Av., Davis, CA 95616, USA
[2]University of California Cooperative Extension, Yuba City, CA, USA
*Corresponding author: mrsudarshana@ucdavis.edu , Ph:530-752-3621

ABSTRACT

Polymerase chain reaction (PCR) using primers specific to 16S-23S rRNA amplified a 1.6 kb product from nucleic acid extract (NA) obtained from leaves collected from almond trees affected by almond brown line disease (ABL). Sequence analysis of the amplified product indicated the presence of a phytoplasma related to Pear decline phytoplasma (PDP; *Candidatus* Phytoplasma pyri) and Peach yellow leafroll phytoplasma (PYLRP). However, primers specific to the non-rDNA region of PDP failed to amplify a product from NA from leaves of trees with ABL. A new set of primers were designed to detect Almond brown line phytoplasma (ABLP), PDP, and PYLRP. Detection of ABLP is generally difficult in NA from leaves of trees in advanced stages of the disease, and detection is only possible in late summer and fall. We are currently developing a real-time PCR assay to detect ABLP.

INTRODUCTION

Almond brown line (ABL) was first reported in 1992 in California by Uyemoto et al (1992). Affected trees were stunted with pale green foliage, and necrotic unions (Fig. 1 and 2). Disease symptoms developed only in almond trees on plum rootstock 'Marianna 2624'. Almond scions displaying symptoms included cvs. Carmel, Peerless, and Price. Based on anecdotal evidence the etiological agent was considered to be Peach yellow leafroll phytoplasma (PYLRP), the causal agent of yellow leafroll and decline in peach trees. The PYLRP strain in California is genetically related to Pear decline phytoplasma (PDP) based on the DNA sequence homology of the 16S ribosomal RNA and 16/23S spacer regions (Kirkpatrick et al.,1997). Incidentally, pear psyllids transmit both PYLRP and PDP.

Shriveled kernel disease is another disease caused by a phytoplasma on almond trees grown on peach rootstock (Uyemoto et al., 1999). In graft inoculation experiments, it was found that this phytoplasma also caused ABL in almond trees on Marianna 2624. However, the association of PYLRP with ABL in natural infections has not been demonstrated by "direct" assays. In Spring 2008, an outbreak of ABL occurred in Sutter County, CA. Here we report on the molecular detection of a phytoplasma associated with ABL and development of primers to detect this phytoplasma, PDP and PYLRP by PCR.

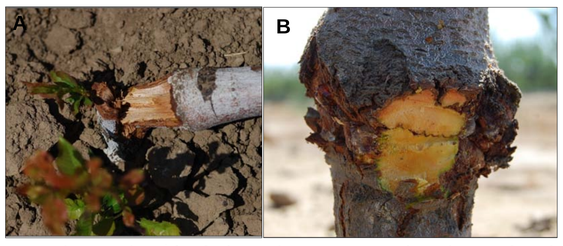

Fig. 1. Diseased 2nd-leaf almond tree snapped at the graft union due to development of brown line (A) . Graft union of a 3rd-leaf almond tree showing brown line disease (B).

Fig. 2. Diseased almond tree (Right) showing stunted growth and pale green foliage. Note extensive suckering of rootstock.

RESULTS AND DISCUSSION

Detection of phytoplasma association: PCR assays using general primers O-P1 and Tint , specific to apple proliferation phytoplasma group, amplified a 1.6 kb product from nucleic acid (NA) extract of leaves from diseased trees, but not from healthy trees. The amplified product was similar in length to the one obtained from a tree inoculated with PYLR P. However, in Yr. 2008, the1.6 kb product was obtained from only one of six ABL-trees (Fig. 3). Incidentally this PCR positive tree was partially girdled. In 2009 also, only one of 12 trees with ABL was positive in PCR assays. It was apparent that the agent was difficult to detect in "girdled" trees. Similarly, Uyemoto et al. (1992) were unable to graft-transmit collections of ABL sources to healthy almond trees. They however, effectively reproduced ABL symptoms using inocula from peach with PYLR disease.

Identification of the phytoplasma: Cloning and sequence analysis of the PCR product indicated that the amplified product had 99% homology with the 16S/23S region of Pear decline phytoplasma (PDP) also known as *Candidatus* Phytoplasma pyri) from Germany and Peach yellow leafroll phytoplasma (PYLRP) in California in blast searches of NCBI database. It is likely that ABLP is a strain of PDP.

A new set of primers, CPPrRNAf609 and CPPrRNAr1135 were designed to specifically amplify a 530 bp product from the 16S/23S rDNA spacer region of ABLP, PDP, and PYLRP. This primer set consistently amplified ~530 bp product from NA from almond and peaches inoculated with PYLRP and pear infected with PDP (Fig. 4). However, ABLP was not always detected in NA from diseased almond trees in 30 cycles. A 40-cycle amplification was able to amplify a product of expected size in more trees with ABL, but higher number of cycles also generated some non-specific products (Fig. 5).

Real-time PCR for the detection of ABLP: Using the primers that amplified a 530 bp product in conventional PCR, quantitative real-time (RT) PCR was attempted to detect ABLP DNA. Cloned plasmid DNA was used to spike NA extracts obtained from healthy almond tree RT-PCR was performed using SYBR®Green. Preliminary results indicated that as low as 10 pg of cloned DNA could be detected and NA from pears infected with PDP, and almond and peach infected with PYLRP were positive (Table 1; Fig. 6). The dissociation constants indicated that amplified products from PYLR and PDP were nearly identical.

To determine if ABLP was genetically identical to PDP and PYLRP, a primer pair specific to a non-ribosomal region of PDP (Lorenz et al., 1995) was used to amplify ~ 1.5 kb product. PCR assays failed to amplify expected size product. Our results conclude association of a phytoplasma in almond trees with ABL, but the identity of the phytoplasma remains to be established.

ACKNOWLEDGEMENTS

This project was supported by USDA-ARS project No. 5306-22000-014-00D. Authors acknowledge finacial support from the California Almond Board and the Fruit Tree, Nut Tree, and Grapevine Improvement Advisory Board, California Department of Food and Agriculture. The authors appreciate technical assistance of Saul Gonzalez, CPGR, USDA-ARS.

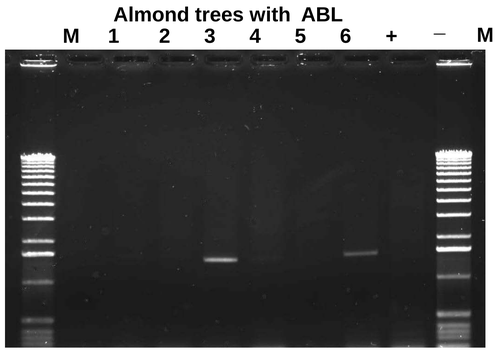

Fig. 3. Agarose gel showing PCR products amplified from almond trees affected by brown line disease using O-P1 and Tint primers. M= 1 kb DNA ladder ; + = almond tree inoculated with Peach yellow leafroll phytoplasma; H = healthy almond

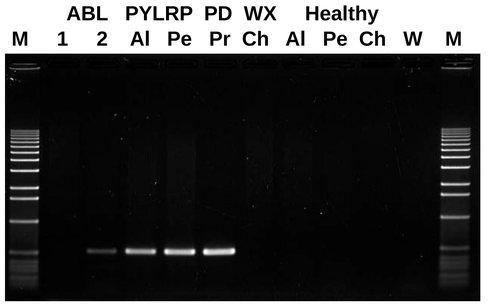

Fig. 4. Agarose gel showing amplified products from almond trees affected by brown line disease using primers CPPrRNAf609 and CPPrRNAr1135 after 30 cycles of amplification. Al = almond; Ch = Bing Cherry; Pe = Peach; Pr = Pear; M = 1 kb Ladder; Wa = water; ABL= almond trees with brown line; PD = Pear deline; PYLRP = Peach yellow leafroll phytoplasma; WX = Western X phytoplasma.

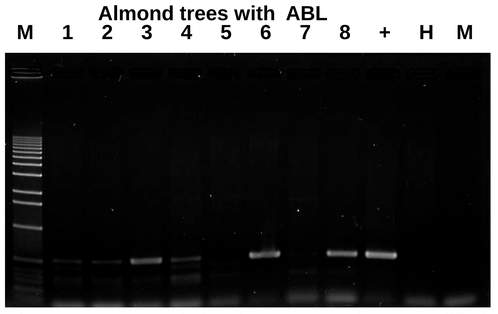

Fig. 5. Agarose gel showing PCR products amplified from almond trees exhibiting brown line disease using primers CPPrRNAf609 and CPPrRNAr1135 after 40 cycles of amplification. M = 1 kb ladder; + = almond on peach rootstock inoculated with PYLRP; H = healthy almond.

Table 1. Real-time qPCR assay on nucleic acid extracts from pear and stone fruit trees infected with phytoplasmas.

DNA	Ct	Tm ° C
a) Plasmid DNA		
10,000 pg	10.6	83.8
1,000 pg	15.2	83.8
500 pg	14.4	83.8
100 pg	19.0	82.3
50 pg	18.3	83.8
10 pg	18.7	83.8
b) Phytoplasmas		
ABLP - almond	27.0	81.8
PYLRP - almond	34.1	82.2
PYLR P- peach	25.5	82.2
PDP - pear	22.9	82.2
Western X - cherry	-	59.1
c) Negative control		
Healthy almond	-	70.5
Healthy cherry	-	70.0
Healthy peach	-	81.8
Healthy pear	-	81.8
Water	-	76.9

Ct= cycle threshold value; Tm ° C = dissociation Constant; ABLP = almond brown line phytoplasma; PYLRP = Peach yellow leafroll phytoplasma; PDP= Pear Decline phytoplasma

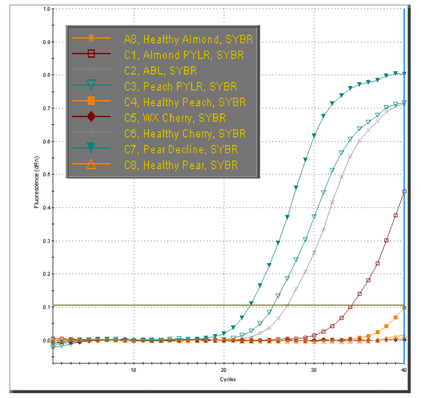

Fig. 6. Real-time monitoring of accumulation of amplified products

MATERIALS & METHODS

Analysis of 16/23S spacer regions: Total genomic DNA was extracted from leaves using a plant DNAeasy kit (Qiagen Inc., Valencia, CA). Extracted DNA was subjected to polymerase chain reaction (PCR) using O-P1 and Tint primers (Smart et al., 1996). PCR conditions were 1 cycle of 94 C for 2'; 40 cycles of 94 C 30"; 62 C for 30"; 72 C for 2'; and 1 cycle of 72 C for 5', on an Eppendorf Mastercycler epgradient S Thermo cycler. Nucleic acid extracts from healthy and PYLRP-inoculated almond trees from the USDA/UCD disease garden were used as positive and negative controls for PCR assays.

After analysis on 1% agarose gel, PCR products were cloned using a TOPO TA cloning kit (Invitrogen Inc., Carlsbad, CA). Randomly selected colonies were sequenced using M13-reverse and T7 primers and the sequence obtained was subjected to BLAST search at the NCBI web site.

New Diagnostic Primers for Molecular Detection: Two new primers, CPPrRNAF606(5'-TGC TAT AGA AAC TGT TTG ACT AGA GT-3') and CPPrRNAR1135 (5'-AAT TTA TCA TTG GCA GTC TCAC-3') were designed to amplify a 530 bp product from ABLP, PDP, and PYLRP. PCR reactions were carried out using 30 cycles on NA obtained from almond trees showing ABL. The conditions were 1 cycle 94 C -2'; 30 cycles 94 C 30 sec, 55 C 30 sec, 72 C 1 min; one cycle 72 C 5min.

Real-time qPCR for the detection of ABLP: A real-time qPCR was attempted to evaluate detection of genomic DNA from ABLP. The 530 bp product obtained from ABLP was cloned and various amounts of plasmid DNA was spiked into nucleic acid extracts from almond leaves. Amplification and simultaneous quantitation was done using a master mix with SYBR®green on Mx3000P thermal cycler (Agilent Tech., Inc., Santa Clara, CA). The conditions were 1 cycle 95 C -10'; 40 cycles 95 C 30 sec, 55 C 1', 72 C 1.5 min; and one cycle 72 C 3 min. The dissociation constants were obtained by denaturation at 95 ° C for 1', annealing at 55 ° C for 30 sec and denaturation at 95 ° C for 30 seconds. The ramp rate from 55 to 95 ° C was 0.2 ° C/sec.

LITERATURE CITED

1. Blomquist, C.L. and Kirkpatrick, B.C. 2002. Plant Dis., 86:759-763.
2. Kirkpatrick, B.C., Purcell, A.H., Gao, J.L., Fisher, G.F., and Uyemoto, J.K. 1993. Phytopathology 83:1341.
3. Lorenz, K.H., Schneider, B., Ahrens, U., and Seemuller, E. 1995. Phytopathology 85:771-776.
4. Smart, C.D., Schneider, B., Blomquist,C.L., Guerra, L.J, Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemuller, E., and Kirkpatrick, B.C. 1996. Appl. Environ. Microbiol., 62:2988-2993.
5. Uyemoto, J.K., Asai, W.K., and Kirkpatrick, B.C. 1999. New Zealand J. Crop and Hort . Sci., 27:225-228.
6. Uyemoto, J.K., Connell, J.H., Hasey, J.K., and Luhn, C.F. 1992. Ann. App. Biol., 120:417-424.