Bulletin of Insectology ***60*** *(2): 123-124, 2007*
ISSN 1721-8861

Detection and identification of phytoplasmas in *Ribes rubrum*

Milan NAVRÁTIL[1], Jaroslava PŘIBYLOVÁ[2], Pavla VÁLOVÁ[1], Renata FIALOVÁ[1], Dana ŠAFÁŘOVÁ[1], Josef ŠPAK[2], Darina KUBELKOVÁ[2], Karel PETRZIK[2], Renata KAREŠOVÁ[3], Vlastimila ŠPAKOVÁ[2]
[1]*Faculty of Science, Palacký University in Olomouc, Olomouc-Holice, Czech Republic*
[2]*Biology Centre of the Academy of Sciences, Institute of Plant Molecular Biology, České Budějovice, Czech Republic*
[3]*Research and Breeding Institute of Pomology, Holovousy, Czech Republic*

Abstract

Using PCR, RFLP and sequence analysis in red and white currant, aster yellows (ribosomal group 16SrI) and apple proliferation (16SrX) phytoplasma 16S ribosomal sequences were obtained and submitted to GenBank from 12 *Ribes* samples. The use of different primer sets for PCR, RFLP, and sequencing was validated for reliable phytoplasma detection and identification in *Ribes* and allow a substantial revision of previously published data.

Key words: red currant, Apple proliferation phytoplasma, Aster yellows phytoplasma, PCR, sequencing.

Introduction

There are only a few conclusive data concerning the phytoplasma occurrence in *Ribes* species. Navrátil *et al.* (2001) first detected phytoplasmas of aster yellows and apple proliferation groups in *Ribes rubrum* using nested PCR and RFLP analysis. The objective of this study was to elucidate problems with the detection and identification of phytoplasmas that were found infecting red and white currant (*R. rubrum*) in a previous study (Navrátil *et al*., 2004).

Materials and methods

Forty two samples of red and white currant cultivars were repeatedly collected from branches and flower stalks in several locations of the Czech Republic during the flowering seasons and in the early autumn from 1999 to 2004 (table 1). The plants exhibited flower deformities, rubbery wood and decumbent aspect. Currant seedlings (Jonkheer van Teets and Baldwin) raised from seeds were included in the study as negative controls. Positive controls in periwinkle were apple proliferation (kindly provided by E. Seemüller-IPO Dossenheim, D) and aster yellows phytoplasmas (Přibylová *et al.*, 2001).

The isolation of total DNA was performed according to an enrichment procedure (Ahrens and Seemüller, 1992). DNA samples were subjected to PCR analysis with phytoplasma primer pairs in 16S rRNA gene. Nested-PCR assays were performed with primers pairs R16F1/R0 followed by R16F2/R2 (Lee *et al*., 1998) or fU5/rU3. PA2F/R primer pair followed by NPA2F/R (Heinrich *et al*., 2001) was also used. The amplicons obtained with primers fU5/rU3 (table 1) were digested with *Rsa*I (Promega), *Mse*I (New England Biolabs), *Bfm*I (MBI Fermentas), and *Alu*I (Promega) restriction endonucleases at 37 °C overnight.

PCR products were directly cloned using TOPO-TA (Invitrogen) or pGEM-T (Promega) cloning kits, following the instructions of the manufacturers. The nucleotide sequence was established on automated DNA ABI PRISM 310 sequencer (Perkin Elmer Applied Biosystems, Lincoln). Sequence data were read and analyzed by DNASTAR programme package (Lasergene). The nucleic acid sequences of PCR products obtained from the selected samples were analyzed by pairwise comparison with phytoplasmal sequences available in GenBank, using the BLAST algorithm at http://www.ncbi.nlm.nih.gov/.

Results

In 12 out of 42 examined bushes the presence and identity of phytoplasmas was confirmed by PCR/RFLP and sequence analysis (table 1). Expected length bands were obtained in PCR with R16F2/R2 (1,239 bp), fU5/rU3 (876 bp), NPA2F/R (845 bp). No amplification was observed in samples of healthy currant seedlings or water controls. The primer pair fU5/rU3 enabled amplification of the target DNA from all samples (table 1), but also amplified bacterial 16SrDNA. The other primer pairs yielded a low number of specific products or failed to amplify. Obvious PCR products without smear background and non-specific amplification were cloned and sequenced. RFLP analyses of fU5/rU3 PCR products confirmed identity of the 6 apple proliferation isolates. Within the aster yellows phytoplasma RFLP analysis classified LS 11/6B/860 and Mulka 866 strains in subgroup 16SrI-B, whereas Blanka 5, Blanka 6, Vitan 2, and *R. rubrum* strains were placed in subgroup 16SrI-C. The identity of phytoplasma isolates obtained from 12 bushes was confirmed also by sequence analysis of PCR products (table 1) confirming their classification with the aster yellows and apple proliferation ribosomal groups (Lee *et al*., 1998). Sequence analysis of Blanka 5, Blanka 6, Vitan 2, and *R. rubrum* isolates revealed high identity (99%) with clover phyllody phytoplasma (AF222066) classified as 16SrI-C. False positive detection of phytoplasmas in 7 red currant bushes was most likely due to amplification of non-phytoplasma bacterial 16S rDNA as revealed by sequencing (table 1).

Table 1. Detection and identification of phytoplasmas infecting red and white currants in the Czech Republic.

Currant cultivar Bush number	Location	R16F1/R0 R16F2/R2	R16F1/R0 fU5/rU3	PA2F/R NPA2F/R	RFLP (*Rsa*I, *Mse*I, *Bfm*I)	Sequence analysis	Accession number
LS 11/6B/756	Kozov	-	+	+	AP	AP	AY900008
LS 11/6B/860	Kozov	+	+	-	AY-B	AY	AY900011
Vitan 758	Kozov	+	+	-	AP	AP	AY900009
Vitan 859	Kozov	-	+	-	AP	AP	AY900007
Vitan 3	Lhenice	-	+	-	AP	AP	AY669060
Vitan 7	Lhenice	-	+	-	AP	AP	AY669061
Vitan 8	Lhenice	-	+	-	AP	AP	AY669062
Ribes rubrum	Rožmitál	-	+	+	AY-C	AY	AY669063
Mulka 866	Velké Losiny	-	+	-	AY-B	AY	AY900010
Blanka 5	Lhenice	-	+	+	AY-C	AY	AY669064
Vitan 2	Lhenice	+	+	+	AY-C	AY	AY966889
Blanka 6	Lhenice	-	+	+	AY-C	AY	AY966890
Vitan 1214	Kamenice	+	+	-	NSC	*Sphing.*	
Vitan 1217	Kamenice	+	+	-	NSC	*Sphing.*	
Vitan 857	Kozov	+	-	-	NSC	*Sphing.*	
LS 11/6B/869	Velké Losiny	+	+	-	NSC	N.C.B.	
LS 13/6/880	Velké Losiny	+	+	-	NSC	*Alpha pr.*	
Rondom 1772	Kamenice	+	+	+	NSC	*Sphing.*	
Vitan 1782	Kamenice	+	+	-	NSC	*Sphing.*	

+, positive; -, negative; AP, apple proliferation; AY-B, aster yellows (16SrI-B); AY-C, clover phyllody (16SrI-C); NSC, non specific cutting; *Sphing*., *Sphingomonas*; *Alpha pr*., *Alpha proteobacterium*; N.C.B., non culturable bacterium.

Discussion

Previous findings of phytoplasma presence and identity in red currant were substantially extended and revised. Several phytoplasmas were identified (Navrátil *et al*., 2001; 2004), however, in some cases no amplification or weak signals not of the expected size were obtained. Aster yellows phytoplasma isolates were confirmed by further RFLP analysis as well as sequence analysis. The reported presence of European stone fruit yellows and pear decline phytoplasmas was not confirmed while new isolates of apple proliferation phytoplasma were identified at the Kozov locality.

Despite wide acceptance of PCR for detection of low amounts of target DNA, homology to ribosomal RNA of other bacteria as well as to the DNA of host plant cell organelles is still a bottle neck in detection assays (Skreczkowski *et al*., 2001; Heinrich *et al*., 2001). Using primers fU5/rU3, R16F2/R2 Skreczkowski *et al*. (2001) reported non-specific amplification of *Pseudomonas* and *Staphylococcus* in dormant budwood of different stone and pome species. The R16F2/R2 and fU5/rU3 primers are able to preferentially amplify the bacterial 16S rRNA operons: Amplicon cloning sequencing several clones reliably confirms phytoplasma detection by PCR.

Acknowledgements

Granted GACR No.522/02/0040 and AV0Z50510513. We thank A. Bertaccini, E. Seemüller, J. Fránová for kind providing of reference phytoplasma strains.

References

AHRENS U., SEEMÜLLER E., 1992.- Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene.- *Phytopathology,* 82: 828-832.

HEINRICH M., BOTTI S., CAPRARA L., ARTHOFER W., STROMMER S., HANZER V., KATINGER H., BERTACCINI A., LAIMER DA CAMADA MACHADO M., 2001.- Improved detection methods for fruit tree phytoplasmas.- *Plant Molecular Biology Reporter,* 19: 69-179.

LEE I.-M., GUNDERSEN-RINDAL D. E., DAVIS R. E., BARTOSZYK I. M., 1998.- Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences.- *International Journal of Systematic Bacteriology,* 48: 1153-1169.

NAVRÁTIL M., VÁLOVÁ P., FIALOVÁ R., ŠPAK J., PŘIBYLOVÁ J., 2001.- First attempt at identification of phytoplasma associated with full blossom of red and white currant in the Czech Republic.- *Acta Horticulturae,* 551: 51-54.

NAVRÁTIL P., VÁLOVÁ P., FIALOVÁ R., PŘIBYLOVÁ J., ŠPAK J., KUBELKOVÁ D., KAREŠOVÁ R., 2004.- Occurrence of phytoplasmas in red and white currant in the Czech Republic.- *Acta Horticulturae*, 656: 119-124.

PŘIBYLOVÁ J., ŠPAK J., FRÁNOVÁ J., PETRZIK K., 2001.- Association of aster yellows subgroup 16SrI-B phytoplasmas with disease of *Rehmannia glutinosa* var. purpurea.- *Plant Pathology,* 50: 776-781.

SKRZECZKOWSKI L. J., HOWELL W. E., EASTWELL K. C., CAVILEER T. D., 2001.- Bacterial sequences interfering in detection of phytoplasma by PCR using primers derived from the ribosomal RNA operon.- *Acta Horticulturae,* 550: 417-424.

Corresponding author: Josef ŠPAK (spak@umbr.cas.cz), Biology Centre of the Academy of Sciences, Institute of Plant Molecular Biology, 37005 České Budějovice, Czech Republic.