SCREENING OF APPLE MOSAIC VIRUS IN HOP CULTIVARS IN THE CZECH REPUBLIC BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

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Summary. – Thirteen cultivars of hop (*Humulus lupulus* L.) were tested by reverse transcription-polymerase chain reaction (RT-PCR) for the presence of apple mosaic virus (ApMV). The virus was detected in various amounts in all tested cultivars. Control hop clones derived from tissue cultures, treated by thermotherapy and maintained in greenhouse were virus-free. The procedure for sample preparation and RT-PCR of ApMV is described in detail.

Key words: apple mosaic virus; detection; direct-binding polymerase chain reaction; primers

Introduction

ApMV (genus *Ilarvirus*) is world-wide distributed virus which occurs naturally in hop (Bock, 1967; Sano *et al.*, 1985; Wong and Horst, 1993). Its incidence in hop varies significantly: ApMV occurs in 1% of plants in the Pacific Northwest area of the USA, where the American hop latent carlavirus is predominant (Klein and Husfloen, 1995), while ApMV is the predominant virus in Europe with almost 100% incidence (Polák, 1994).

The virus infection has a dramatical influence on the yield and quality of producing hop (Svoboda and Kopecký, 1996). Hop is a vegetatively propagated perennial and the health control during the propagation of root stock material is necessary. Tissue cultures from meristem tips combined with thermotherapy is the only way for production of high quantities of a virus-free material. Therefore the availability of highly sensitive test for virus indexing in the earliest stages of multiplication is of econocomical and phytoquarantine importance.

ApMV is a member of the genus *Ilarvirus* which consits of extraordinary unstable viruses loosing their infectivity in crude sap in few hours. Even the purified virus particles are easily deformed (Fulton, 1972) and poorly immunogenic (Wong and Horst, 1993). The only routine serological method available, enzyme-linked immunosorbent assay (ELISA), is unreliable and of low sensitivity for detection of minute amounts of ApMV present in tissue culture samples.

The genome of ApMV consists of three molecules of single-stranded RNA (RNA 1-3). RNA 3, containing genes for the movement protein and coat protein of the virus was sequenced recently (Shiel *et al.*, 1995), and thus gave us an opportunity to develop a more sensitive detection method based on RT-PCR. In this paper we describe a method for detection of ApMV in hop plants and present results of ApMV screening in the majority hop cultivars in use in the Czech Republic.

Abbreviations: ApMV = apple mosaic virus; ArMV = arabis mosaic virus; EDTA = ethylenediamine tetraacetic acid; ELISA = enzyme-linked immunosorbent assay; HLV = hop latent virus; HMV = hop mosaic virus; PCR = polymerase chain reaction; PEG = polyethylene glycol; PRNV = prunus necrotic ringspot virus; PVP = polyvinylpyrrolidone; RT = reverse transcriptase

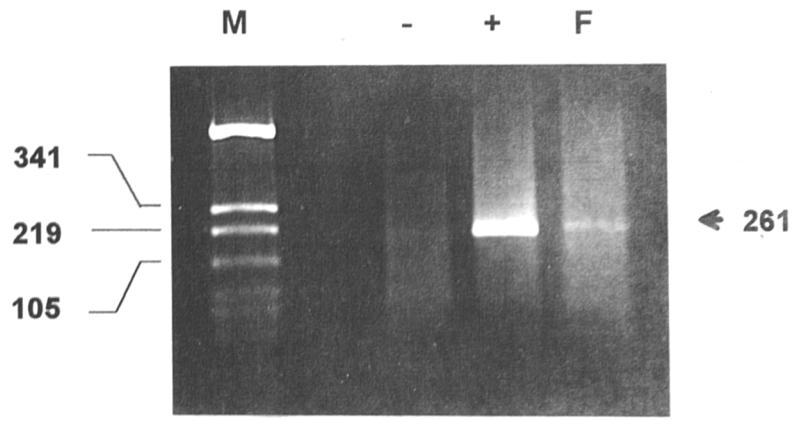

Fig. 1
Agarose gel electrophoresis of RT-PCR amplification products
Healthy control (lane (–), ApMV-infected control (+) and field (lane F) samples. pBS/*Dpn*I size markers (lane M) and their size in bp. Arrow: the 261 bp RT-PCR product. Ethidium bromide staining.

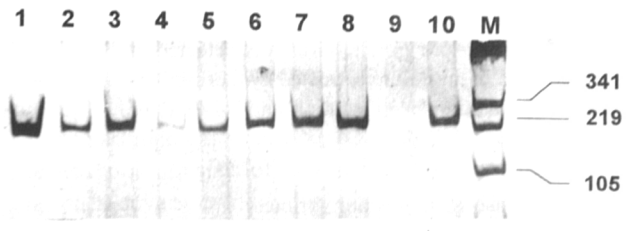

Fig. 2
PAGE od RT-PCR amplification products of field samples
Field samples (lanes 1-10). Size markers (lane M) and their size in bp. Silver staining.

Table 1. List of hop cultivars tested

Cultivar	Country of origin	Tested/infected
Aromat	CR	3/3
Blšanka	CR	3/2
Bor	CR	3/3
Os.clone 31	CR	3/3
Os.clone 72	CR	3/3
Os.clone 114	CR	3/3
Os.clone 126	CR	3/2
Podlešák	CR	3/2
Premiant	CR	3/2
Sifem	CR	3/3
Sládek	CR	3/2
Zlatan	CR	3/3
Cascade	USA	1/1
Hallertau	Germany	1/1
Northern Brewer	England	1/1
Bačka	Yugoslavia	1/1
Kirin II	Japan	1/1

CR = Czech Republic

Materials and Methods

Hosts plants. All samples from producing hop cultivars (Table 1) were collected in September 1996 from symptomatic as well as symptomless leaves in the lower third of hop shoots and stored at -20°C. Positive and negative controls were a part of the ApMV ELISA kit (Löwe Biochemica, Germany). Healthy controls originated from plants derived from meristem tip cultures treated by thermotherapy and found negative by ELISA for the presence of ApMV, prunus necrotic ringspot virus (PNRV), hop latent virus (HLV), hop mosaic virus (HMV) and arabis mosaic virus (ArMV) (Svoboda and Kopecký, 1996).

Oligonucleotide primers were designed from the sequence ApMV RNA 3 (EMBL database accession number U15608). Primers ApMV1 and ApMV2 with the sequences 5′ TGG ATT GGG TTG GTG GAG GAT3′ and 5′ TAG AAC ATT CGT CGG TAT TTG3′ specified the region of nt 1504-1765 of the coat protein gene.

Sample preparation. One leaf disc 0.8 cm in diameter (approx. 5 mg of fresh tissue) was homogenized in 200 µl of the extraction buffer of Nolasco *et al.* (1993) (0.5 mol/l Tris-HCl pH 8.3, 150 mmol/l NaCl, 0.05% Tween 20, 2% polyvinylpyrrolidone (PVP), 1% polyethylene glycol (PEG) 6000, 3 mmol/l sodium azide, 1 mmol/l ethylenediamine tetraacetic acid (EDTA) and centrifuged at 13,000 x g for 5 mins. A direct binding method was used to bind viruses present in the extracts to the walls of microcentrifuge tubes non-specifically and to remove plant substances inhibiting the enzymes of the RT-PCR assay. Sterile 0.2 ml PCR tubes (Perkin Elmer, Norwalk, USA) were loaded with 100 µl of the supernatant and incubated at 37°C for 1 hr. After removing the supernatant and two washes of the tube with 200 µl of the extraction buffer, the cDNA synthesis was performed in the same tube.

RT-PCR. The Access RT-PCR System (Promega, Madison, USA) containing the AMV RT and thermostable *Tfl* DNA polymerase was used according to the manufacturer's protocol. The reaction mixture (20 µl) contained 2 U of AMV, 2 U of DNA

polymerase, 0.2 mmol/l each of dNTPs, 1 mmol/l $MgSO_4$, 20 nmoles of each primer and the AMV/*Tfl* reaction buffer. RT-PCR was performed in GeneAmp PCR System 9600 Amplifier (Perkin Elmer) as follows: 45 mins at 42°C, 2 mins at 95°C, and 35 cycles of 30 secs at 94°C, 1 min at 53°C and 2 mins at 68°C. One third of the product was separated by 1% agarose or 6% polyacrylamide gel electrophoresis in 1 x TBE buffer and stained with ethidium bromide or silver.

Results and Discussion

The field samples were collected at the end of the season, when the virus concentration in plants decreases and its detection by ELISA is less reliable (Polák and Svoboda, 1989). We used the direct binding procedure, trapping virus particles to the tube wall, for preparation of samples for RT-PCR. It is slightly less effective than the immunocapture method (Wetzel *et al.*, 1992) but sufficient for ApMV detection.

We tested three independent series of seventeen cultivars and clones of hop, which represented the whole sortiment of hop in the Czech Republic.

The RT-PCR detection of ApMV resulted in demonstration of the predicted 261 bp amplification product. This product was the only one visible on gels (Fig. 1) and its nature was confirmed by *Hinf*I and *Mse*I restriction cutting (Fig. 3). Occasionally, an additional product of about 300 bp was observed. Its amount was about one fifth to one half of that of the 261 bp product as estimated from the band intensity. It occured unpredictably in various samples possibly as a results of low stringent amplification conditions and/or low amount of the template.

All Czech hop cultivars grown in the Czech Republic as well as all foreign cultivars were susceptible to ApMV (Table 1). The ApMV concentration in some samples was at the detection limit of the RT-PCR, and we observed faint bands only (Fig. 2, lane 4). To confirm the ApMV presence in these samples we increased the number of amplification cycles to 40. Then in all so far dubious samples we clearly confirmed the presence of the virus. On the other hand, samples from the meristem cultures after the thermotherapy were ApMV-free. ApMV-free samples were observed in cultivars Blšanka, Podlešák, Premiant and Sládek, and in the Osvald's clone 126, too.

To our knowledge, the only report dealing with PCR detection of ApMV so far published is that by Rowhani *et al.* (1995). We employed the primers published there, but probably due to different plant source and/or low concentration of ApMV in our samples we did not obtain the predicted amplification product of 700 bp. According to our experience, shorter products about 300 bp are more suitable for detection of viruses, especially in case of unstable viruses, when viral nucleic acid is quickly unpacked from virions and exposed to degradation enzymes in the cell cytoplasm. In addition, the probability of forming secondary structures and knots at low stringency conditions, which could influence the annealing of primers and the yield of a PCR product is lower with a 300 bp target sequence than with a 700 bp one.

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