

逆転写-polymerase chain reaction(RT-PCR)を用いたホップ潜在ウイルス(LVd)の検出

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	畑谷,達児 日影,勝幸 須田,成志 永田,龍哉 季,世訪 糸賀,裕 谷越,時夫 四方,英四郎
発行元	日本植物病理學會
巻/号	58巻5号
巻号補足	
掲載ページ	p. 677-684
発行年月	1992年12月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター

Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Detection of Hop Latent Viroid (HLVd) Using Reverse Transcription and Polymerase Chain Reaction (RT-PCR)*

Tatsuji HATAYA**, Katsuyuki HIKAGE**, Narushi SUDA**,
Tatsuya NAGATA**, Shifang LI**, Yutaka ITOGA***,
Tokio TANIKOSHI*** and Eishiro SHIKATA**

Abstract

A viroid-like RNA was detected in low molecular weight RNAs extracted from virus-free hops by two-dimensional polyacrylamide gel electrophoresis (PAGE). It migrated considerably faster than hop stunt viroid in 5% PAGE containing 8 M urea under a denaturing condition. A cDNA fragment was amplified from the low molecular weight RNAs containing the viroid-like RNA by reverse transcription and polymerase chain reaction (RT-PCR) using a pair of hop latent viroid (HLVd) specific primers. The amplified cDNA product of expected size was detected by agarose gel electrophoresis. The full-length cDNA of the viroid-like RNA in RT-PCR products was cloned and sequenced. The established nucleotide sequence of the inserted cDNA was completely identical to that previously reported for HLVd. The total nucleic acids were extracted from hops cultivated in Japan, and the presence of HLVd were examined by dot blot hybridization and RT-PCR. In a hop sample, HLVd was not detected by dot blot hybridization, but detected by RT-PCR. The result indicates that RT-PCR is potentially more sensitive than dot blot hybridization using a radioactive probe. HLVd was detected in all mother cultivars tested. However, the two virus-free clones obtained by meristem culture showed negative reaction in both dot blot hybridization and RT-PCR detection methods. It is also shown that HLVd probably can be eliminated by meristem culture as indicated in several plant viruses and viroids.

(Received February 22, 1992)

Key words: hop latent viroid, hop, polymerase chain reaction, hybridization, detection, meristem culture.

INTRODUCTION

Viroids are low molecular weight, single-stranded circular RNA molecules causing several important diseases in higher plants. An agent of hop stunt disease, designated as hop stunt viroid (HSVd), which had 297 nucleotides and was infectious to fruit trees such as grapevine, citrus, plum, peach and pear²¹⁾, has been reported in Japan¹⁸⁾. Recently, a new viroid, consisting of 256 nucleotides, was found in hops and named hop latent viroid (HLVd)¹³⁾. HLVd is widely distributed in hops cultivated in the

* This work was supported in part by a Grant-in-Aid for Developmental Scientific Research from the Ministry of Education, Science and Culture of Japan, a Grant-in-Aid for Special Scientific Research on Agriculture, Forestry and Fisheries from the Ministry of Agriculture, Forestry and Fisheries of Japan, and a research grant from the Brewers Association of Japan.

** Department of Botany, Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060, Japan 北海道大学農学部植物学教室

*** Hokkaido Center, Plant Bioengineering Research Laboratories, Sapporo Breweries Ltd., 3-5-25 Honcho, Kamifurano, Sorachi, Hokkaido 071-05, Japan サッポロビール株式会社植物工学研究所北海道センター

world. From its electrophoretic mobility, HLVd is very closely related to a viroid-like RNA fast (HV-f) detected from hops in Spain¹²⁾, which seems to be identical to HLVd. As HLVd was detected in symptomless hop cultivars^{1,13)}, it was most likely that the viroid was latently infected in hop cultivars. However, Barbara *et al.*²⁾ reported that HLVd decreased total cone yields, individual cone weights, and alpha-acid content of cones in two cultivars of hop cultivated in the UK. They suggested that HLVd was the potentially serious pathogen, therefore should be eliminated. At present, no host plant of HLVd was found except for hop plants (*Humulus lupulus* L.) and *H. japonicus*^{1,13)}, and thus HLVd was detected only by biochemical procedures such as return-gel electrophoresis¹³⁾ and dot-blot hybridization^{1,13)}. Recently, the revolutionary method for *in vitro* DNA amplification, the polymerase chain reaction (PCR)¹⁵⁾, has been used for the detection of plant RNA viruses^{6,8,14,23,25)} and viroids⁴⁾ from infected plants. In this paper we report the detection of HLVd in the cultivated hops in Japan using reverse transcription and polymerase chain reaction (RT-PCR), and the possibility to eliminate HLVd from hops by meristem culture.

MATERIALS AND METHODS

Hop samples. Five samples of hop plant used in this experiment were grown in the field of Hokkaido University. They are virus-free (VF), hop latent virus (HLV) infected, apple mosaic virus (ApMV) infected hop (cv. Kirin II), a hop plant (cv. Kirin II) showing stunt symptom and hop mosaic virus (HMV) infected hop cv. Brewers Gold. It was ascertained that the VF—cv. Kirin II hop was also free from HSVd by inoculation to cucumber plants. Twelve samples of hop, designated as sample No. 1 to 12, were grown in the greenhouse or fields of Sapporo Breweries Ltd. in Hokkaido Prefecture. They include six mother cultivars and six virus-free clones derived from each mother cultivar: cv. Shinsyu Wase (No. 1 and 2), cv. Furano Ace (No. 3 and 4), cv. Sorachi Ace (No. 5 and 6), cv. Saaz YG (No. 7 and 8), cv. Tettnang YG (No. 9 and 10) and cv. Saaz Osvald Clone 72 (No. 11 and 12). The even numbered cultivars were virus-free clones derived from the shoot tip cultures of the odd numbered cultivars. Each shoot tip (0.2 mm) was grown on Murashige and Skoog (MS) basal medium¹⁰⁾ supplemented with 2% sucrose, 0.8% agar, 2 mg/l of 6-benzyladenine and 0.2 mg/l of gibberellic acid for 40 days, and transferred to the agar medium supplemented with 0.5 mg/l of 3-indolebutyric acid to stimulate rooting. After 14 days, rooted cultures were potted in the greenhouse. The absence of ApMV and HLV was ascertained by enzyme linked immunosorbent assay (ELISA).

Low molecular weight RNAs extraction from hops. Low molecular weight RNAs were extracted from fresh or frozen leaves using the method described by Uyeda *et al.*²²⁾ with a slight modification. Hop leaves (4 g) were homogenized in TESLP buffer (130 mM Tris-HCl, pH 8.9, 17 mM EDTA, pH 7.0, 0.83% sodium dodecyl sulfate (SDS), 1M LiCl, 5% polyvinylpyrrolidone (PVP)) instead of 1 M K₂HPO₄. After phenol extraction, low molecular weight RNAs were purified by following procedures: 2-methoxyethanol extraction, cethyltrimethylammonium bromide precipitation, 2 M LiCl fractionation, and DNase I (Takara Shuzo Co., Ltd.) digestion. Extracted low molecular weight RNAs were dissolved in 1 ml of distilled water.

Polyacrylamide gel electrophoresis. The extracted low molecular weight RNAs were analysed by two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE)¹⁹⁾ or 15% PAGE under a non-denaturing condition and 5% PAGE containing 8 M urea under a denaturing condition as described previously¹⁶⁾. The gel was stained with silver²⁰⁾. The following viroid RNAs were used as molecular weight standards. Citrus exocortis viroid (CEVd: 371 nucleotides³⁾)—kindly provided from Dr. H.L. Sanger, Max Planck Institute, Germany—purified from *Gynura aurantiaca* DC., chrysanthemum stunt viroid (CSVd: 354 nucleotides) purified from chrysanthemum cultivated in Japan⁹⁾, apple scar skin viroid (ASSVd: 330 nucleotides⁸⁾)—Kindly provided from Dr. H. Koganezawa, Morioka Branch, Fruit Tree Research Station, Japan; present address: International Rice Research Institute, the Philippines—purified from apple tree, and hop stunt viroid (297 nucleotides) isolated from pear tree (HSVd-pear)²¹⁾ and purified from cucumber.

Reverse transcription and polymerase chain reaction. A pair of HLVd specific synthetic

oligonucleotide primers for PCR, designated as HLVD-1P and HLVD-1M, was designed based on the nucleotide sequence of HLVD¹³, and synthesized using an Applied Biosystems model 380B DNA synthesizer. First strand cDNA was synthesized in 20 μ l of RT reaction mixture containing 4 μ l of nucleic acids of hop sample, 1 μ g HLVD-1M primer (20-mer: 5' TAGTTTCCAACCTCCGGCTGG 3'; calculated T_m value²⁴) = 62°C) complementary to HLVD sequence No. 192-173¹³, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM each dNTP, 100 U M-MLV reverse transcriptase (Bethesda Research Laboratories). The mixture was incubated for 1 hr at 37°C. A half (10 μ l) of the RT reaction mixture were added to 40 μ l of PCR premixture containing 1 μ g HLVD-1P primer (20-mer: 5' GGATACAACCTCTTGAGCGCC 3'; calculated T_m value = 62°C) homologous to HLVD sequence No. 199-218¹³, 1 μ g HLVD-1M primer, 7.5 mM Tris-HCl (pH 8.9), 81.25 mM KCl, 1.125 mM MgCl₂, 0.375 mg/ml bovine serum albumin (BSA), 0.075% sodium cholate, 0.075% TritonX-100, 187.5 μ M each dNTP, 1 U *Tth* DNA polymerase (Toyobo Co., Ltd.). The addition of MgCl₂ and KCl was deduced, because of high concentration of MgCl₂ and KCl in RT reaction mixture. In this PCR reaction mixture, final concentration of MgCl₂ and KCl were prepared to 1.5 mM and 80 mM, respectively. The PCR reaction mixture was overlaid with one drop of mineral light oil (Sigma Chemical Company). The PCR amplification proceed through a cycle of denaturation for 1 min at 94°C (in only first cycle for 5 min), annealing for 2 min at 57°C (calculated T_m value of each synthetic oligonucleotide primer -5°C), and primer extension for 3 min at 72°C (for 10 min to ensure completion of the amplified product in the last cycle) for a total cycle of 25 by using a Program Temp Control System PC-700 (Astec Co., Ltd.).

The PCR products (10 μ l or 5 μ l) were removed and analysed by electrophoresis in a 3% agarose gel which was subsequently stained by 0.5 μ g/ μ l ethidium bromide. To decide a cross-contamination occurred through PCR⁷, a negative control was prepared by an addition of distilled water instead of nucleic acids of hop sample in RT reaction mixture.

Molecular cloning and sequencing. The cDNA of HLVD in RT-PCR product was digested with *Bam*HI and ligated into pUC119 using a ligation kit (Takara Shuzo Co., Ltd.). The ligated DNAs were cloned into *Escherichia coli* JM 109 cells. Alkaline-denatured recombinant DNAs were sequenced using a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp.).

Total nucleic acids extraction from hops. The total nucleic acids from hops were extracted by the following procedure. Fresh leaves (0.5 g) were homogenized in 1 ml of TESLP buffer containing 5% 2-mercaptoethanol, transferred into 2 ml volume of microcentrifuge tube, and centrifuged for 5 min at 15,000 *g*. The aqueous phase was then treated with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)-saturated phenol:chloroform (1:1) mixture. After centrifugation, the nucleic acids in aqueous phase were precipitated with 0.6 vol. of isopropyl alcohol. The nucleic acids in the pellet were extracted again in TE-saturated phenol:chloroform (1:1) mixture. The total nucleic acids obtained were precipitated again with ethanol and the pellet was resuspended in 20 μ l distilled water.

Dot blot hybridization. HLVD and HSVd specific probes were prepared from HLVD-cDNA cloned in this experiments and pCP316 containing dimeric cDNA of HSVd-cucumber (HSVd-c)¹¹. The full-length cDNAs of HLVD and HSVd-c were excised from recombinant plasmid by digestion with *Bam*HI and *Xho*I, respectively, and purified by electrophoresis in an agarose gel, followed by elution from the gel using Suprec-01TM (Takara Shuzo Co., Ltd.). The eluted cDNAs were labelled with [α -³²P]dCTP (3,000 Ci/mM) by a random priming method using a random labelling kit (Takara Shuzo Co., Ltd.). Unincorporated radioactive nucleotides were removed and radioactive cDNA probe was purified by a Sephadex G-50 column. The total nucleic acids (2 μ l; 1/10 volume) were mixed in 6 μ l of denaturing solution (65.6% formamide, 21.3% formaldehyde, 1.54 M MOPS, 6.5 mM sodium acetate, pH 7.0 and 1.3 mM EDTA), incubated for 15 min at 65°C, then quickly chilled and added equal volume of 20 $\times$ standard saline citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The samples (10 μ l; 5 times of 2 μ l aliquots, 2 μ l and corresponding to 0.4 μ l) were spotted onto Hybond N+ membrane filter (Amersham International plc) with 5-fold dilution, and air-dried. The membrane filter was transferred onto a sheets of filter paper containing 50 mM NaOH, and after 5 min, the membrane filter was rinsed in 2 $\times$ SSC for 1 min with gentle shaking. Prehybridization was carried out in 50% formamide (E. Merck AG), 5 $\times$ SSC, 100 μ g/ml yeast tRNA (Boehringer Mannheim GmbH), 0.5% SDS, 5 $\times$ Denhardt's reagent

(1×Denhardt's reagent : 0.02% Ficoll, 0.02% PVP and 0.02% BSA) for 2 hr at 46°C.

Hybridization was carried out in the same solution containing 1×10^6 cpm/ml of ^{32}P -labelled heat-denatured cDNA probe for 16 hr at 46°C. After hybridization, membrane filter was washed in $2 \times \text{SSC}$ containing 0.1% SDS for 15 min at 46°C (3 times), followed by washing in $1 \times \text{SSC}$ containing 0.1% SDS for 30 min at 65°C, $0.1 \times \text{SSC}$ containing 0.1% SDS for 30 min at 65°C with gentle shaking. The washed membrane filter was air-dried and autoradiographed for 24 hr at room temperature with intensifying screen.

RESULTS AND DISCUSSION

Detection of a viroid-like RNA by PAGE

The low molecular weight RNAs extracted from VF—cv. Kirin II were analysed by 2D-PAGE followed by silver staining. Figure 1a clearly showed that a viroid-like RNA was contained in VF—cv. Kirin II sample. Both circular and linear forms of the viroid-like RNA migrated considerably faster than

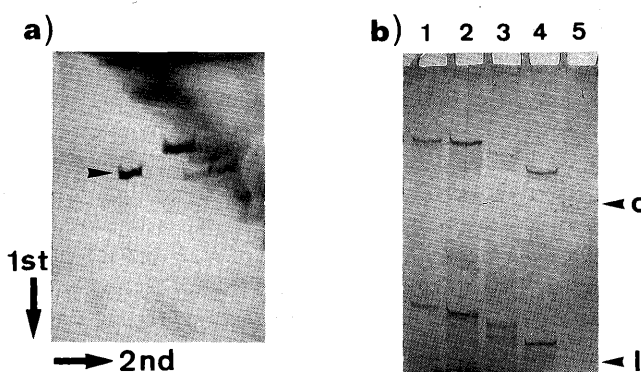


Fig. 1. a) Two-dimensional polyacrylamide gel electrophoresis of low molecular weight RNAs extracted from VF—cv. Kirin II. Arrows indicate the direction of electrophoresis. Arrowhead indicates the viroid-like RNA. b) Electrophoresis in a polyacrylamide gel (5%) under a denaturing condition. Lane 1 : CEVd (371 nucleotides), 2 : CSVd (354 nucleotides), 3 : ASSVd (330 nucleotides), 4 : HSVd-pear (297 nucleotides), 5 : the viroid-like RNA extracted from VF—cv. Kirin II. Arrowheads “c” and “l” indicate circular and linear form, respectively.

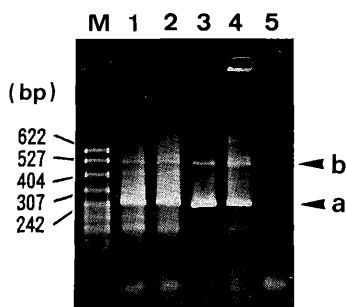


Fig. 2. Electrophoresis in an agarose gel (3%) of RT-PCR products ($10 \mu\text{l}$; 1/5 volume of reaction mixture) amplified from low molecular weight RNAs extracted from hops. Lane 1 : VF—cv. Kirin II, 2 : HLV infected cv. Kirin II, 3 : HMV infected cv. Brewers Gold, 4 : ApMV infected cv. Kirin II, 5 : negative control (distilled water was added RT reaction mixture instead of low molecular weight RNAs of hop sample). The size markers are *HapII*-digested-pBR322 (lane M). Arrowheads “a” and “b” indicate expected 250 bp fragment and 506 bp fragment corresponding to nearly two unit length of HLVd, respectively.

CEVd (371 nucleotides), CSVd (354 nucleotides), ASSVd (330 nucleotides) and HSVd-pear (297 nucleotides) in 5% PAGE under a denaturing condition (Fig. 1b). Its migration rate suggested that the viroid-like RNA was regarded as HLVD¹³⁾.

PCR-mediated cloning of full-length cDNA of HLVD

The viroid-like RNA suspected to be HLVD was confirmed by molecular cloning and nucleotide sequencing. The low molecular weight RNAs extracted from following four hop samples were examined by RT-PCR using HLVD specific primers: VF, HLV infected, ApMV infected cv. Kirin II and HMV infected cv. Breweres Gold. When PCR products were analysed by electrophoresis, the DNAs approximately 250 bp, expected from the nucleotide sequence information¹³⁾, was detected in all four hop samples (Fig. 2, arrow a). An arrow b in Fig. 2 apparently indicated that the bands of about 500 bp are detected in all four hop samples. In reverse transcription, cDNAs of longer than one unit length may be occasionally synthesized for single-stranded circular RNA in rolling circle. When nearly two unit length cDNAs of HLVD are synthesized in reverse transcription, a 506 bp fragment is expected to be produced after amplification in PCR.

The amplified HLVD-cDNA of nearly two unit length in PCR products was digested with *Bam*HI (position No. 52-53¹³⁾). The resulting full-length cDNA of HLVD, which did not contain PCR primers, were ligated into *Bam*HI site of pUC119, and cloned into *E. coli* JM 109 cells.

The described PCR-mediated cloning procedure can be generally applicable for the efficient full-length cDNA cloning of single-stranded circular RNA such as viroids and virusoids from the nucleotide sequence information.

Nucleotide sequence of HLVD

The four cDNA clones from VF—cv. Kirin II and one cDNA clone from ApMV infected cv. Kirin II were completely sequenced. Furthermore, two cDNA clones from ApMV infected cv. Kirin II and two cDNA clones from HLV infected cv. Kirin II were partially sequenced. The nucleotide sequences of the cDNAs from the eight clones were completely identical to that of HLVD described previously¹³⁾. In one clone from VF—cv. Kirin II, the nucleotide sequence changed at position No. 198 to C from U, which may be caused by misincorporation during PCR-mediated cloning.

Detection of HLVD and HSVd by dot blot hybridization

In order to investigate the extent of distribution of HLVD in commercial cultivars, the presence of

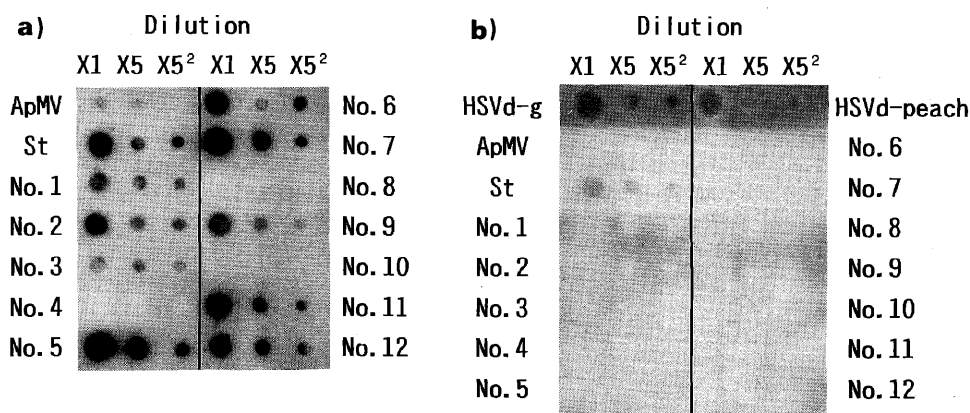


Fig. 3. Dot blot hybridization of HLVD (a) and HSVd (b) to total nucleic acids extracted from hop samples. ApMV: ApMV infected cv. Kirin II, St: cv. Kirin II showing stunt symptom. Sample No. 1: cv. Shinsyu Wase, No. 2: VF—cv. Shinsyu Wase, No. 3: cv. Furano Ace, No. 4: VF—cv. Furano Ace, No. 5: cv. Sorachi Ace, No. 6: VF—cv. Sorachi Ace, No. 7: cv. Saaz YG, No. 8: VF—cv. Saaz YG, No. 9: cv. Tett nang YG, No. 10: VF—cv. Tett nang YG, No. 11: cv. Saaz Oswald Clone 72, No. 12: VF—cv. Saaz Oswald Clone 72. HSV-g: low molecular weight RNAs containing HSVd-grapevine, extracted from cucumber, HSVd-peach: low molecular weight RNAs containing HSVd-peach (AF), extracted from cucumber.

the viroid in hop samples collected from the Sapporo Breweries Ltd. was examined by dot blot hybridization. The total nucleic acids extracted from hop samples No. 1-12 and cv. Kirin II showing stunt symptom, and low molecular weight RNAs extracted from ApMV infected cv. Kirin II were tested. Hybridization signal was detected in nine samples, but not in samples, No. 4, 8 and 10 (Fig. 3a).

These hop samples were examined to be free from HSVd by the following experiment. The HLVD-cDNA probes hybridized in the above experiment were removed from the membrane in boiling water. The nucleic acid samples on the membrane was re-hybridized with ^{32}P -labelled cDNA of HSVd-c. Low molecular weight RNAs containing HSVd-grapevine and HSVd-peach(AF)²¹⁾, which were extracted from cucumber, were spotted on another new membrane filter as positive control. Hybridization signal was detected only in cv. Kirin II showing stunt symptom, HSVd-grapevine and HSVd-peach(AF) samples, but none in samples No. 1-12 (Fig. 3b).

Detection of HLVD by RT-PCR

The nucleic acid samples used in dot blot hybridization were examined by RT-PCR using HLVD specific primers. Each sample was ten-fold diluted with distilled water, and 1/50 volume of the sample was applied to RT-PCR. In this experiment PCR was performed in reaction mixture containing 1% formamide which is reported to be effective for elimination of non-specific products and increase of the expected product¹⁷⁾. The result showed that the PCR products expected in size were detected in samples except for No. 4 and 10 (Fig. 4). In sample No. 2, the observed band was thin, though a strong signal was detected in dot blot hybridization analysis (Fig. 3a). This may occur when reverse transcription reaction and/or PCR was obstructed by insufficient clarification of hop sample. In sample No. 8, no signal was detected by dot-blot hybridization (Fig. 3a), but the expected band was obviously observed in RT-PCR. The result indicates that RT-PCR is potentially more sensitive than dot-blot hybridization using a radioactive probe.

HSVd was not detected in hop samples No. 1-12 cultivated in Hokkaido Prefecture (Fig. 3b). However, HLVD was detected in all six mother cultivars (Fig. 3a and Fig. 4). It is shown that HLVD is distributed in Hokkaido Prefecture of Japan. HLVD was also detected from hop cultivars cultivated in Yamagata and Iwate Prefectures of Japan (unpublished result). It seems that HLVD is widely distributed in hop cultivars cultivated in Japan. Barbara *et al.*²⁾ reported that HLVD decreased total cone yields, individual cone weights, and alpha-acid content in hops cv. Omega and Wye Northdown cultivated in UK.

The cv. Omega was severely affected, and the cv. Wye Northdown was less severely affected. As hop plants are propagated vegetatively, its elimination may be important for hop production. The two samples No. 4 (cv. Furano Ace) and 10 (cv. Tettnang YG) showing negative reaction in both dot blot hybridization and RT-PCR analysis were virus-free clones derived from samples No. 3 and 9 by

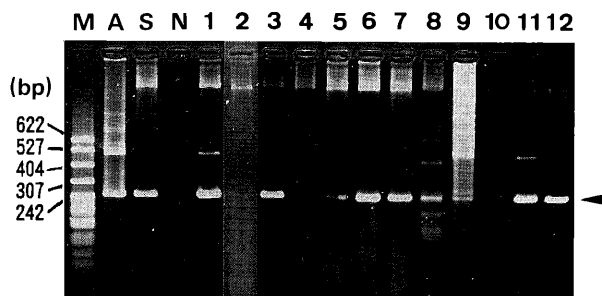


Fig. 4. Electrophoresis in an agarose gel (3%) of RT-PCR products (5 μl ; 1/10 volume of reaction mixture) amplified from total nucleic acids extracted from hop samples. Lane A: ApMV infected cv. Kirin II, S: cv. Kirin II showing stunt symptom, N: negative control (distilled water was added RT reaction mixture instead of total nucleic acids of hop sample). Samples in lane 1-12 were identical to sample No. 1-12 using in Fig. 3. The size markers are *Hap*II-digested-pBR322 (lane M). The arrowhead indicates expected 250 bp fragment.

meristem culture. The result shows that HLVD probably can be eliminated by meristem culture as indicated in several plant viruses and viroids. As we described here, the RT-PCR is potentially high sensitive method to detect HLVD, possibly other viroids, plant viruses, and other plant pathogens from small amount of plant tissue. Our results suggest that hop clones obtained by meristem culture to eliminate HLVD, can be diagnosed at an early cultured stage by RT-PCR method.

Literature cited

1. Barbara, D.J., Morton, A. and Adams, A.N. (1990). Assessment of UK hops for the occurrence of hop latent and hop stunt viroids. *Ann. appl. Biol.* 116: 265-272.
2. Barbara, D.J., Morton, A., Adams, A.N. and Green, C.P. (1990). Some effect of hop latent viroid on two cultivars of hop (*Humulus lupulus*) in the UK. *Ann. appl. Biol.* 117: 359-366.
3. Gross, H.J., Krupp, G., Domdey, H., Raba, M., Jank, P., Lossow, C., Alberty, H., Ramm, K. and Sanger, H.L. (1982). Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. *Eur. J. Biochem.* 121: 249-257.
4. Hadidi, A. and Yang, X. (1990). Detection of pome fruit viroids by enzymatic cDNA amplification. *J. virol. Methods* 30: 261-270.
5. Hashimoto, J. and Koganezawa, H. (1987). Nucleotide sequence and secondary structure of apple scar skin viroid. *Nucleic Acids Res.* 15: 7045-7052.
6. Korshineck, I., Himmler, G., Sagl, R., Steinkellner, H. and Katinger, H.W.D. (1991). A PCR membrane spot assay for the detection of plum pox virus RNA in bark of infected trees. *J. virol. Methods* 31: 139-146.
7. Kwok, S. and Higuchi, R. (1989). Avoiding false positive with PCR. *Nature* 339: 237-238.
8. Langeveld, S.A., Dore, J.-M., Memelink, J., Derks, A.F.L.M., van der Vlugt, C.I.M., Asjes, C.J. and Bol, J.F. (1991). Identification of potyviruses using the polymerase chain reaction with degenerate primers. *J. gen. Virol.* 72: 1531-1541.
9. Li, S., Hataya, T., Kudo, H., Ohmori, K. and Shikata, E. (1992). Molecular cloning and nucleotide sequence of chrysanthemum stunt viroid isolated from chrysanthemum cultivated in Japan. *Ann. Phytopath. Soc. Japan* 58: 146 (Abstr. in Japanese).
10. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-479.
11. Ohshima, K., Sano, T., Uyeda, I. and Shikata, E. (1988). Comparative studies on host range and the infectivity of hop stunt viroid—cucumber isolate (cucumber pale fruit viroid) native RNA and its cDNA. *Arch. Phytopathol. Pflanzenschutz.* 24: 475-484.
12. Pallas, V., Navarro, A. and Flores, R. (1987). Isolation of a viroid-like RNA from hop different from hop stunt viroid. *J. gen. Virol.* 68: 3201-3205.
13. Puchta, H., Ramm, K. and Sanger, H.L. (1988). The molecular structure of hop latent viroid (HLV), a new viroid occurring worldwide in hops. *Nucleic Acids Res.* 16: 4197-4216.
14. Robertson, N.L., French, R. and Gray, S.M. (1991). Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses. *J. gen. Virol.* 72: 1473-1477.
15. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
16. Sano, T., Hataya, T., Terai, Y. and Shikata, E. (1986). Association of a viroid-like RNA from plum dapple disease occurring in Japan. *Proc. Japan Acad.* 62B: 53-56.
17. Sarkar, G., Kapelner, S. and Sommer, S.S. (1990). Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Res.* 18: 7465.
18. Sasaki, M. and Shikata, E. (1977). On some properties of hop stunt disease agent, a viroid. *Proc. Japan Acad.* 53B: 109-112.
19. Schumacher, J., Randles, J.W. and Riesner, D. (1983). A two-dimensional electrophoresis technique for the detection of circular viroids and virusoids. *Anal. Biochem.* 135: 288-295.
20. Schumacher, J., Meyer, N., Riesner, D. and Weidemann, H.L. (1986). Diagnostic procedure for detection of viroids and viruses with circular RNAs by "return"-gel electrophoresis. *J. Phytopath.* 115: 332-343.
21. Shikata, E. (1990). New viroids from Japan. *Seminars in Virology* 1: 107-115.
22. Uyeda, I., Sano, T. and Shikata, E. (1984). Purification of cucumber pale fruit viroid. *Ann. Phytopath. Soc. Japan* 50: 331-338.
23. Vunsh, R., Rosner, A. and Stein, A. (1990). The use of the polymerase chain reaction (PCR) for the

- detection of bean yellow mosaic virus in gladiolus. *Ann. appl. Biol.* 117: 561-569.
24. Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T. and Itakura, K. (1979). Hybridization of synthetic oligodeoxyribonucleotides to $\phi\chi$ 174 DNA: The effect of single base pair mismatch. *Nucleic Acids Res.* 6: 3543-3656.
 25. Wetzell, T., Candresse, T., Ravelonandro, M. and Dunez, J. (1991). A polymerase chain reaction assay adapted to plum pox potyvirus detection. *J. virol. Methods* 33: 355-365.

和 文 摘 要

畑谷達児・日影勝幸・須田成志・永田龍哉・李世訪・糸賀 裕・谷越時夫・四方英四郎：逆転写-polymerase chain reaction (RT-PCR) を用いたホップ潜在ウィロイド (HLVd) の検出

ウイルスフリーホップより、低分子 RNA を抽出し、二次元ポリアクリルアミドゲル電気泳動 (PAGE) で、ウィロイド様 RNA を検出した。この RNA は、8 M 尿素変性条件下での PAGE において、ホップ矮化ウィロイド (HSVd) よりかなり速く泳動した。この低分子 RNA に対し、既報のホップ潜在ウィロイド (HLVd) の塩基配列より設計した特異的プライマーを用い、逆転写-polymerase chain reaction (RT-PCR) を行ったら、HLVd の塩基配列から予想される長さの cDNA が増幅された。この RT-PCR 産物より完全長 HLVd・cDNA をクローニングし、その塩基配列を決定したところ、既報の HLVd と同一であった。また、ホップより全核酸を簡易抽出し、ドット・プロット・ハイブリダイゼーションおよび RT-PCR による遺伝子診断を行った。その結果、ある検体においては、ドット・プロット・ハイブリダイゼーションでは、HLVd が検出されなかったものの、RT-PCR 法で検出され、RT-PCR 法は、HLVd の検出に有効で、ドット・プロット・ハイブリダイゼーションより高感度である可能性が示された。また、検定した栽培ホップ全品種の元株から HLVd が検出されたが、それらの元株から茎頂培養によりウイルスフリー化された株のうち、2 検体では、RT-PCR 法でも、HLVd が検出されず、茎頂培養により、HLVd のフリー化が可能であることが示唆された。