Identification of tomato ringspot nepovirus by RT-PCR

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Tomato ringspot nepovirus (ToRSV) causing viral diseases and severe economic losses in vegetable, ornamental and fruit crops worldwide has been identified in Lithuania from a great variety of plant species on the basis of determination of host range, symptom expression on naturally infected plants and inoculated test-plants, morphological and serological properties of virus isolates. In this work, primers designed on the basis of published sequences were applied for amplification of ToRSV cDNA fragments in RT-PCR, using isolates from cucumber and iris. This confirmed ToRSV identification results obtained by investigating host range, virus morphological properties and DAS-ELISA. The RT-PCR method for virus detection and identification is less time-consuming and labour-intensive in comparison with the other methods.

Key words: tomato ringspot virus identification, RT-PCR

INTRODUCTION

Tomato ringspot virus (ToRSV) is a member of the *Nepovirus* group of plant viruses and causes economically important diseases in a range of crops. ToRSV is found in many perennial crops in North America, Europe, Asia, Australia and South America [1]. It affects 285 plant species in 159 genera of 55 botanical families [2]. ToRSV is readily transmissible by sap inoculation. It is transmitted by the nemathode of the genera *Xiphinema* and *Longidorus.* Seed transmission of ToRSV has been reported in several crops. The virus is also transmitted by vegetative propagation and pollen. It has a wide host range including various woody and herbaceous plants. The virions are icosahedral, about 28 nm in diameter, sedimenting as three components. The most characteristic type of foliar symptom induced by ToRSV is ring spotting. Cucumber and other cucurbits are infected by most nepoviruses and typically develop chlorotic lesions or no lesions on inoculated leaves and systemic mottle or mosaic with slight distortion on the first systemically infected leaves [3]. ToRSV was identified from many affected perennial ornamental species in Lithuania [4].

Employment of polymerase chain reaction (PCR) for virus detection has been very successful in the last decade. A prerequisite for the use of PCR for virus detection is the availability of sequence data on the viral genome. The genome of most plant viruses is made of RNA. Detection of RNA sequence by PCR requires some modifications. RNA has to be reverse-described into cDNA before amplification (RT-PCR) [5].

The aim of this work was to investigate the possibility to apply the RT-PCR method using primers designed on the basis of published sequences for ToRSV identification in cucumber and iris plants.

MATERIALS AND METHODS

The material for investigation was collected in different private gardens of vegetable crops, in vegetable and ornamental collections of Lithuanian Institute of Horticulture, Botanical Gardens of Vilnius University and Vytautas Magnus University, Experimental Station of Field Floriculture. The samples were collected from cucumber and iris plants showing visual virus symptoms on leaves. Virus has been identified by test-plant reaction and virus particle morphology, DAS-ELISA. DAS-ELISA was carried out at the laboratory of State Plant Protection Service according to standard protocol. Absorbtion was measured spectrophotometrically at 405 nm [6]. The inoculum for mechanical inoculation was prepared by homogenizing infected leaves with 0.1 M phosphate buffer, pH 7.0, containing as stabilizing agents 0.2% 2-mercapthoethanol or 0.01 M sodium diethyldithiocarbamate (DIECA). Virus particles were visualized in negatively stained dip preparations using

a JEOL JEM-100S electron microscope [7]. The following ToRSV isolates were used: isolates from *Cucumis sativus* L. cv. 'Ventura' (Kaunas region), cv. 'Polan' and cv. 'Restima' (Vilnius region), and the isolate from *Iris* L., found in Botanical Garden of VU. ToRSV isolates were purified by a modified method [1]. Frozen leaves of *Tetragonia expansa* and *Nicotiana rustica* showing systemic symptoms of ToRSV infection were ground in 0.2 M phosphate buffer pH 7.2 containing 0.1% 2-mercaptoethanol. The extract was clarified by 10% chloroform. Virus particles were precipitated with w/v 10% polyethylene glycol Mv 6000 and 0.2 M sodium chloride. Pellets were resuspended in 0.2 M phosphate buffer pH 7.2. Virus was purified by two cycles of differential centrifugation (10 min at 8000 rpm in K-24 centrifuge, and 3 h at 27000 rpm in VAC-601). Final purification was accomplished by sedimentation through a 20% sucrose cushion.

For detection of ToRSV isolates by RT-PCR, purified virus solutions were used. The tissue of healthy *N. rustica* plant was used as a control. Primers used in RT-PCR were designed from ToRSV viral sequence information [8]. The primers included U1, (5' to 3') GACGAAGTTATCA ATGGCAGC (nt 1.078 to 1,098) and D1, TCCGTCCAATCACGCGAAT (nt 1.506 to 1.527) of the putative viral polymerase gene. This putative enzyme sequence was selected as the amplification target assuming the likelihood of highly conserved sequences between isolates, as compared to the antigenically distinct and possibly more variable coat protein coding sequences [9].

Nucleic acids from purified ToRSV solution and investigated test-plants infected by ToRSV were extracted using the small-scale procedure proposed for extraction of nucleic acids from woody plants [10], with slight modifications.

Purified virus solution (about 100 µl) was transferred to 1.5 ml microfuge tubes. Tissue samples of infected test-plants were ground in liquid nitrogen and transferred to tubes, too. 600 µl 1 × STE buffer (0.1 M NaCl, 0.001 M Tris, 0.001 EDTA, pH 6.9), 80 µl of 10% SDS and 800 µl of 2 × STE-saturated phenol were added to the powdered tissues and purified virus solution. The mixture was vortexed thoroughly and centrifuged for 5 min at 16000 g. The aqueous phase was removed and transferred to a clean microfuge tube. Ethanol to a final concentration of 30% and then ~10 mg cellulose (whatman CF-11) were added and the tube was vortexed thoroughly. After centrifugation cellulose was pelleted and supernatant discarded. Cellulose CF-11 was washed by vortexing 3 times with 1 ml of 1 × STE/30% ethanol, collecting cellulose by centrifugation between washes and discarding supernatants.

RNA from cellulose CF-11 was eluted by adding 200 µl of 1 × STE buffer, vortexing to mix and centrifugation for 5 min. The supernatant was transferred to a clean tube. The total elution volume was ~400 µl. For precipitation of RNA, 40 µl of 3 M sodium acetate and 1 ml of ethanol were added. The tube was incubated at –20 °C for 2 h, centrifuged for 10 min at 16000 g, and the pellet was incubated with 80% of ethanol at –20 °C and air-dried. Pellets of RNA were resuspended in the mixture containing 1% of RNAse inhibitor, 0.4 µM of primer D1 and PCR water, then incubated at 70 °C for 5 min and cooled to 4 °C for 5 min. For the first strand cDNA synthesis in the mixture containing reaction buffer, 0.8% RNAse inhibitor, 200 mM dNTP mix and 10 units of M-MLV reverse transcriptase (MBI Fermentas) were added to the RNA samples. The first strand DNA synthesis was carried out at 37 °C for 60 min and at 70 °C for 10 min.

Amplification was performed using Eppendorf Mastercycler personal in 50 µl reaction mixtures containing each of the four deoxynucleoside triphosphates at a concentration of 200 µM, each primer at a concentration of 0.4 µM, and 0.25 U of recombinant *Tag* polymerase (MBI Fermentas). PCRs were carried out for 40 cycles using the following parameters: 1 min at 94 °C (4 min for the first cycle), 2 min at 55 °C, and primer extention for 2 min (10 min in final cycle) at 72 °C.

PCRs products were analyzed by electrophoresis through 5% polyacrylamide gel, stained with ethidium bromide and DNA bands visualized using a UV transilluminator.

RESULTS AND DISCUSSION

ToRSV was isolated and identified from naturally infected cucumber, cv. 'Ventura', 'Polan' and 'Restima', and iris, cv. 'Golden Flave' plants showing symptoms specific for this virus. Naturally infected cucumber plants showed chlorotic or necrotic spots, and iris had chlorotic streaks on leaves. Virus induced typical severe symptoms on the main diagnostic plant species: *Chenopodium quinoa*, *C. ambrosioides,* and *Celosia argentea* f. *cristata* (chlorotic or necrotic local lesions and systemic apical deformation); *Cucumis sativus* (local necrotic lesions and systemic mottle); *Gomphrena globosa* (local white ring spots); *Nicotiana rustica* (local ring flecks and systemic mottle); *Tetragonia expansa* (local chlorotic spots, systemic rings, stunting, chlorotic mottle and rugosity of leaves). ToRSV infected a wide experimental host range. Some differences between iris and cucumber ToRSV isolates were revealed in test-plants reaction. ToRSV isolates from iris did not induce reaction in *Cucumis sativus* and *Phaseolus vulgaris*

cv. 'Bataaf', isolate from cucumber – in *Datura stramonium, Nicotiana glutinosa, Nicandra physalodes* (Table).

Electron microscopy revealed isometric virus particles about 28 nm in diameter in preparations made from naturally infected cucumber, iris plants, and from various test-plants (Fig. 1).

Identification of ToRSV isolated from cucumber plants was confirmed by a positive reaction in DAS-ELISA test. The positive results in this test were obtained also with inoculated test-plants *N. rustica* (isolates N 9802 and 9807), *C. sativus* (isolate N 9804) and *G. globosa* (isolates N 9805 and 9807).

ToRSV cucumber isolates (N 9805 and 9802) and the iris isolate (N 9809) were purified. For purification ToRSV isolates were propagated in *T. expansa.* The purified virus preparations had A_{max} at 260 nm and A_{min} at 240 nm, $A_{260/280}$ ratio being 1.2. The yield of purified virus, taking into account ToRSV specific absorbance $A^{0.1\%}_{260nm}$ being 10.0 [1] was calculated to be 31 mg (cucumber isolate) and 40 mg (iris isolate) from 1 kg of infected plant tissue.

The purified ToRSV preparations were used in RT-PCR. RT-PCR for detection of ToRSV in cucumber and iris isolates was successfully used. The specific PCR product was obtained in all investigated isolates, but not with negative control. Specific bands were observed in gel analysis at the position corresponding to the expected size of the amplification product of 499 bp (Fig. 2). DNA from symptomatic samples along with a specific band yielded also smaller PCR products. Possibly it was connected with not completely optimized PCR conditions. This investigation was only a first attempt to use RT-PCR for ToRSV detection in our laboratory.

Table. **Reaction of test-plants to infection of ToRSV isolated from iris and cucumber plants**

Test plant	Symptoms induced by ToRSV from plants	
	iris	cucumber
Amaranthus caudatus L.	L:LL	–
Atriplex hortensis L.	0	–
Capsicum annuum L.	–	0
Celosia argentea f. cristata (L.) Kuntze	–	L:RLL; RSp,Ru
Chenopodium amaranticolor Coste et Reyn.	L:ClLL; S:VStu,TCrl, NT	L:WhLL; S:TR,Dis,Stu
Chenopodium ambrosioides L	–	L:SmNLL; S:TR,TDis
Chenopodium foetidum Schrad.	–'	0
Chenopodium murale L.	–	L:ClLL; S:VC,TDis
Chenopodium quinoa Willd.	L:LLCl; S:ClDot,LeDis	L:ClLL; S:TR,TDis,W
C. urbicum L.	L:LL; S:VStu	–
Cucumis sativus L.	0	L:NLL, S: ClSp
Cucurbita pepo L.	–	S:ClMo
Datura stramonium L.	L:SmLL	0
Gomphrena globosa L.	L:GSpN; S:Dis,LeM	L:WhLL or NRLL
Lycopersicon esculentum Mill.	S:M, Cl, VN	S:VN, Cl Mo
Nicandra physalodes (L.) Gaertn.	S:ClSp, NSp	0
Nicotiana debneyi L.	–	S: ClMo
Nicotiana glutinosa L.	L:SmNLL	0
Nicotiana rustica L.	L:SmNDot	L:RNLL; S:RSp,LeDis
Nicotiana tabacum L. 'Samsun'	L:NSp; S:NSp,Str	L:RLL
'White Burley'	L:NSp; S:NSp,Str	–
'Xanthi'	L:NSp; S:NSp,Str	–
Petunia hybrida Vilm.	L: GRSp; S: LeRu,ClSp	–
Phaseolus vulgaris L. 'Bataaf'	0	L:SmNLL
'Baltija'	S: RuNLe	–
'Prince'	S: ClDot, NT	–
Physalis floridana Rybd.	L; LLN, LeCrl	–
Pisum sativum L. 'Žalsviai'	–	0
Sinapis alba L. –	0	
Tetragonia expansa Murr.	L: ClDifSp; S:Dis,ClDot	L:ClLL; S:DifClSp,Stu
Verbesina encelioides Benth. et Hook.	–	L:SmBlLL or BrLL

Abbreviations: L – local reaction, LL – local lesions, LeCrl – leaf curling, M – mosaic, Cl – chlorosis, S – systemic reaction, R – red, Wh – white, NT – top necrosis, TR – top rugosity, TCrl – top curling, Dis – deformation, Str – streaking, Stu – stunting, Sm – small, VC – vein clearing, W – wilting, VN – vein necrosis, Dif – diffuse, Dot – dots, Bl – black, BrL – brown lesions, Sp – spotting, G – gray, 0 – no symptoms, – – not tested.

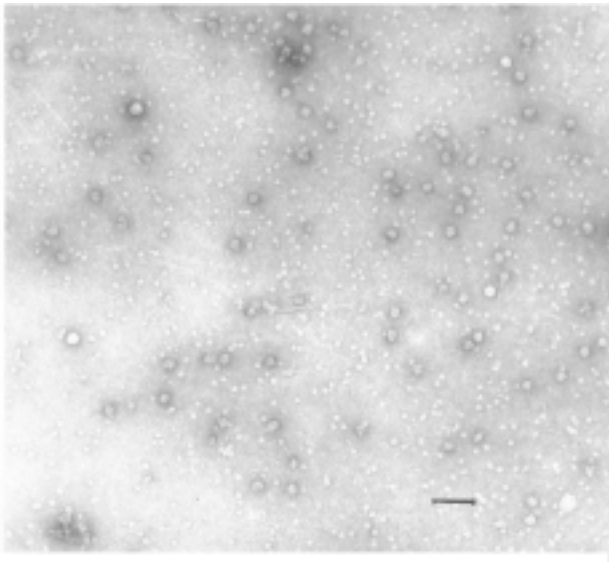

Fig. 1. Tomato ringspot virus particles in inoculated *Tetragonia expansa* plants. Bar represents 100 nm

So, the primers designed on bases of published sequences successfully amplified ToRSV cDNA templates in RT-PCR using virus isolates from cucumber and iris. This confirmed results of identification obtained by investigating host range, virus morphological properties and results of DAS-ELISA. The RT-PCR method for virus detection and identification is less time-consuming and labour-intensive in comparison with the other methods.

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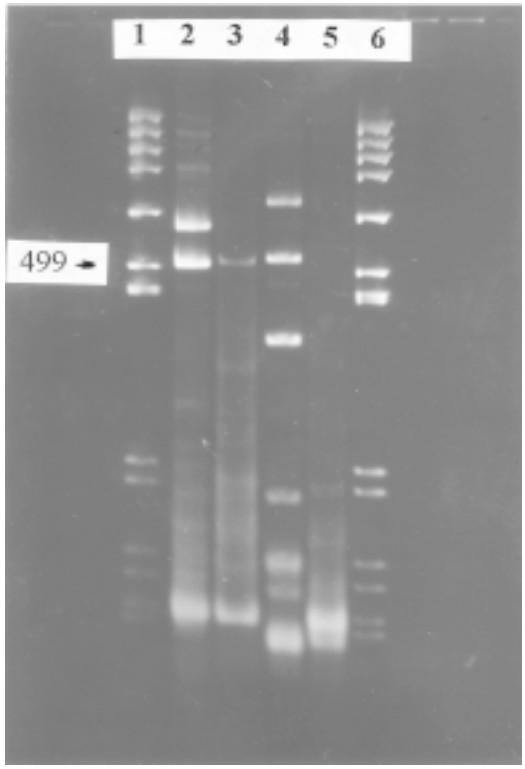

Fig. 2. 5% polyacrylamide gel electrophoresis of polymerase chain reaction products of amplified ToRSV samples and controls. Lane 1 and lane 6, DNA ladder, Lane 2, ToRSV isolated from cucumber and purified from *Tetragonia expansa* tissue; Lane 3, ToRSV from cucumber infected *Nicotiana rustica* tissue; Lane 4, ToRSV from iris; Lane 5, healthy tissue of *N. rustica*

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POMIDORŲ ŽIEDIŠKOSIOS DĖMĖTLIGĖS VIRUSO IDENTIFIKAVIMAS RT-PCR METODU

S a n t r a u k a

Pomidorų žiediškosios dėmėtligės virusas (*Tomato ringspot nepovirus*, ToRSV), plačiai paplitęs visame pasaulyje, pažeidžia daugelį daržovių, dekoratyvinių, vaisinių ir uoginių augalų rūšių padarydamas ekonominių nuostolių. Pagal natūraliai pažeistų augalų ir inokuliuotų augalų-indikatorių simptomus, eksperimentiškai pažeidžiamų augalų spektrą, morfologines ir serologines savybes identifikuotas ToRSV Lietuvoje yra aptiktas įvairiose augalų rūšyse. Šiame darbe pateikiami RT-PCR, atliktos su išgrynintais ToRSV izoliatais iš agurkų ir vilkdalgių, panaudojant publikuotų nukleotidinių sekų pagrindu parinktus pradmenis, rezultatai. Elektroforezės gelyje išryškėję DNR produktai ir jų lokalizacijos vieta patvirtino ToRSV identifikavimo rezultatus, gautus tiriant viruso augalų-šeimininkų ratą, morfologines savybes ir DAS-ELISA duomenis. RT-PCR yra labai tikslus ir patogus metodas fitovirusams nustatyti ir identifikuoti.