ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2007 Vol. 50, Št. 1: 41–51

Sprejeto (accepted): 2007-11-26

New molecular diagnostic methods for detection of *Chrysanthemum stem necrosis virus* (CSNV)

Nove molekularne diagnostične metode zaznavanja virusa nekroze stebel krizantem (CSNV)

Jana BOBEN[1], Nataša MEHLE[1], Manca PIRC[1], Irena MAVRIČ PLEŠKO[2], Maja RAVNIKAR[1]
[1]National Institute of Biology, Večna pot 111, Ljubljana, Slovenia; Tel: +386-1-4323388, Fax: +386-1-2573390; E-mail (*Corresponding author): jana.boben@nib.si
[2]Current address: Agricultural institute of Slovenia, Hacquetova 17, Ljubljana, Slovenia

Abstract. *Chrysanthemum stem necrosis virus* (CSNV), an RNA virus, belongs to the genus *Tospovirus* and family *Bunyaviridae*. The disease symptoms on host plants can not be distinguished from those caused by closely related viruses of the same genus, such as *Tomato spotted wilt virus* (TSWV). The disease symptoms may vary between host plants and can be quite severe. Diagnostics of tospoviruses is of great importance in prevention of greater economic and environmental damage. Viruses within the genus show serological similarity and therefore make the detection of the viruses by serological methods unreliable. To avoid false positive results other methods can be used for detection of RNA viruses such as RT-PCR or RT real-time PCR. CSNV was first found in Slovenia in 2001. Different methods were used to confirm the identity of the isolate. Development of disease symptoms was observed on 15 different test plants and was compared to the symptoms caused by closely related TSWV. Since serological cross-reactivity was observed, molecular tests were developed (RT-PCR and real-time PCR) that further confirmed the identity of the isolate and also increased sensitivity of the assays. CSNV can now be reliably detected and distinguished from related tospoviruses.

Key words: CSNV, diagnostics, distinguishing, ELISA, real-time PCR, RT-PCR, test plants, tospoviruses, TSWV.

Izvleček. Virus nekroze stebel krizantem (*Chrysanthemum stem necrosis virus* – CSNV) je RNA virus in spada v rod *Tospovirus* in družino *Bunyaviridae*. Na gostiteljskih rastlinah povzroča podobna bolezenska znamenja kot sorodni tospovirusi, na primer Virus pegavosti in uvelosti paradižnika (*Tomato spotted wilt virus* - TSWV). Prizadetost gostiteljskih rastlin zaradi pojava bolezenskih znamenj je lahko različna, pojavi se lahko zelo velika škoda na pridelku. zaradi preprečevanja večje ekonomske škode in vpliva na okolje je zelo pomembna diagnostika tospovirusov. Virusi rodu *Tospovirus* so si serološko dokaj podobni. Med njimi tako prihaja do navzkrižne reaktivnosti s protitelesi, ki se uporabljajo v diagnostičnih testih, ki so zato nezanesljivi. V izogib lažno pozitivnim rezultatom lahko uporabimo druge diagnostične metode za določanje prisotnosti RNA virusov: RT-PCR ali RT-PCR v realnem času. Leta 2001 je prišlo v Sloveniji do prve najdbe CSNV virusa. Uporabili smo različne metode za potrditev identitete virusnega izolata. Opazovali smo razvoj bolezenskih znamenj na 15 različnih testnih raslin in smo jih primerjali z znamenji, ki jih povzroča sorodni TSWV. Ker smo opazili pojav serološke navzkrižne reaktivnosti, smo razvili molekularn detekcijski metodi RT-PCR in RT-PCR v realnem času. Z njima smo potrdiliidentiteto izolata in povečali občutljivost diagnostičnega testa. CSNV lahko zato zanesljivo določimo in razlikujemo od sorodnih tospovirusov.

Ključne besede: CSNV, diagnostika, ELISA; PCR v realnem času, razlikovanje, RT-PCR, testne rastline, tospovirusi, TSWV.

Introduction

The genus *Tospovirus* belongs to the family *Bunyaviridae* and contains plant pathogenic viruses that are transmitted by thrips species in propagative manner. Tospoviruses have a wide host range and cause significant economic losses to vegetables and ornamental plants. The genus *Tospovirus* has 8 species and 6 tentative species (Nichol & al. 2005) with new viruses emerging. Species are defined on the basis of their vector, host range, serological relationships of the N (nucleocapsid) protein, and their N protein sequence that should show less than 90 % of amino acid identity with that of any other described tospovirus species. *Chrysanthemum stem necrosis virus* (CSNV) is a tentatitve species in the genus (Nichol & al. 2005) and was for the first time reported in Brasil (Duarte & al. 1995). Since then the finding of the virus was also reported in the Netherlands (Verhoeven & al. 1996), United Kingdom (Mumford & al. 2003), and Japan (Matsuura & al. 2007).

In 1993, a new virus was found in Brasil on *Chrysanthemum morifolium* Hendt. and was at first named Chr1 (Duarte *et al*., 1995). The symptoms of Chr1 and *Tomato spotted wilt virus* (TSWV) could not be distinguished on affected cultivars (Alexandre & al. 1996). In spite of this, the symptoms of the two viruses were often variable: necrosis and yellow spots, only chlorotic spots, chlorotic spots with necrotic pin-points on leaves and stem necrosis (Alexandre & al. 1996). Serological studies demonstrated that the Chr 1 isolate differed from TSWV, *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV), *Watermelon silver mottle virus* (WSMoV), and *Zucchini lethal chlorosis virus* (ZLCV), but the host range, *in vitro* properties and particle morphology of the causal virus were typical of a *Tospovirus* (Duarte & al. 1995; Resende & al. 1996; Bezerra & al. 1999). The N-gene partial nucleic acid sequence of Chr 1 confirmed results obtained with serology assay (Resende & al. 1996). Based on the biological and molecular features, Chr 1 was proposed as new *Tospovirus* species, designated *Chrysanthemum stem necrosis virus* (CSNV) (Bezerra & al. 1999).

Further studies revealed that CSNV also naturally attacks tomato (*Lycopersicon esculentum*) (Nagata & al. 1998) and can be present in mixed infections on some ornamental plants (*Callistephus* sp., *Eustoma grandifolium*, and *Senecio* × *cruentus*) with other tospoviruses such as TSWV, ZCSV and/or GRSV (Alexandre & al. 1999). The results obtained by Nagata & de Avila (2000) showed that *Frankliniella occidentalis* and *F. schultzeri* are the major vectors of CSNV in Brazil, while *Thrips tabaci* did not transmit CSNV, although low amounts of the virus were detected in 75% of the tested population.

With closely related viruses, the serological detection methods might have a downside – serological cross-reactivity and thus unreliable positive results (Leschert & al. 2002, Matsuura & al. 2007). This problem was avoided in the case of tobamoviruses by development of RT-PCR method that differentiated between tobamoviruses in group 1 (Leschert & al. 2002). Molecular detection methods for tospoviruses developed after the discovery that the N gene sequence of TSWV proved to be highly conserved among different isolates and can present the target in diagnostic assays (Heinze & al. 2001). Eiras & al. (2001) published a PCR assay that was designed to match conserved regions of a tospovirus' genome and was universal for detection of tospoviruses. A set of degenerated primers for a RT-PCR assay was reported by Okuda & Hanada (2001) that could detect 5 different tospoviruses: INSV, TSWV, *Melon yellow spot virus* (MYSV), WSMoV, and IYSV, however no report was made about the specific CSNV detection. With the development of new molecular detection methods, such as real-time PCR, the sensitivity of the tests improved, since Roberts & al. (2000) report a 10-times better sensitivity of the TSWV specific real-time PCR over the previously used RT-PCR method.

In 2001 finding of three tospoviruses (TSWV, INSV, and IYSV) was reported in Slovenia (Mavrič & Ravnikar 2001). They infected ornamentals and vegetables, mostly in greenhouses. First suspicion of

CSNV infection occurred on chrysanthemum plants, showing TSWV-like disease symptoms and strong cross reactivity in ELISA test. Later, CSNV was also found on an isolated gerbera plant. To identify and characterize CSNV isolates from Slovenia, different tests were performed and developed. Here we report the results of these tests and their importance in tospovirus diagnostics as well as the advantages of new, highly sensitive molecular method real-time PCR for distinguishing CSNV from TSWV.

Materials and methods

Virus isolates

Chrysanthemum stem necrosis virus (CSNV) was initially isolated from chrysanthemum (*Dendranthema grandifolium*) and gerbera (*Gerbera spp.*) plants. *Impatiens necrotic spot virus* (INSV) was maintained on *Nicotiana benthamiana* Domin. as well, whereas isolates of *Tomato spotted wilt virus* (TSWV) were maintained on *Nicotiana rustica* L.. For ELISA test lyophilised positive controls of *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) (Loewe, Germany) were used.

Test plants and monitoring of disease symptoms

Several plant species (Tab. 1) were tested for determination of CSNV and TSWV host range. Plants were mechanically inoculated using extracts of CSNV infected *N. benthamiana* and TSWV infected *N. rustica*. Young plants were mechanically inoculated by rubbing the extract onto the carborundum-dusted leaves, rinsed with tap water and grown at 20 ± 2 °C during the light period (16 h) and to 18 ± 2 °C during the dark period (8 h) with relative humidity 75 ± 2 %. Test plants, inoculated only with buffer presented negative controls. Disease symptoms were monitored up to 4 weeks after the inoculation. Inoculated and non-inoculated leaves were separately tested for the presence of viruses using ELISA. The leaves of the chrysanthemum and *N. benthamiana* plants, infected with CSNV were stored at -80 °C. Infectivity of CSNV was checked at different times after storage by mechanically inoculating *N. benthamina* test plants.

Plant material for molecular analyses

Different plant material was tested for the presence of tospoviruses: 10 chrysathemum (*Dendranthema grandiflorum*), 3 pepper (*Capsicum annuum*), 2 gerbera (*Gerbera spp*.), 3 tomato (*Lycopersicum esculentum*) and 2 verbena (*Verbena spp*.) plants. Several infected and healthy controls of each species were also included in the testing. Mechanically inoculated test plant were also included in analyses: 1 *Nicotiana tabacum* cv. White Burley infected with extract of CSNV positive gerbera, 2 *Nicotiana benthamiana* plants, infected with CSNV and INSV, respectively, and 1 TSWV infected *Nicotiana rustica*.

Serological analyses

In order to detect the presence of viruses in different host and test plants, several specific antibodies, supplied by 4 different manufacturers (DSMZ, Loewe, Bioreba, Adgen) , were tested in different dilutions. The ELISA was performed according to the manufacturers' instructions, except the analysis for the presence of CSNV, where preabsorption of the conjugate antibodies was shown to give lower cross-reactivity with TSWV and lower OD values for healthy plant tissue. Preabsorption was performed by incubating the conjugate antibodies in 1/10 of total volume of conjugate buffer mixed with sap of healthy chrysanthemum leaves (diluted 1:20). After incubation at room temperature for 10-15 minutes the remainder of conjugate buffer was added and antibodies were used.

Plant sap was diluted 1:10 in PBS (pH 7,4) with 2 % PVP (MW 40,000) and 0,2 % BSA. 200 µl of extract (diluted 1:10) of each sample was tested in duplicate, along with appropriate controls. OD

values were measured 30 minutes, 1 hour, 2 hours, and more after loading the substrate (paranytrophenyl phosphate, 1 mg/ml) using the Dynatech MR 5000 (Dynex Technologies, USA) spectrophotometer. Data was processed using BioLinx software (Dynex Technologies, USA). The positive threshold was set to twice the value of corresponding average OD of negative controls.

RNA isolation, RT-PCR and sequence analysis

Total RNA was extracted from 300 mg of plant material using RNeasy Plant Mini Kit (Qiagen, the Netherlands) and diluted 1:10 and 1:100. 1 µl of total RNA or appropriate dilution was used in RT reaction: 1µl $MgCl_2$ (25 mM), 2 µl 10 x PCR buffer II, 4µl dNTPs (10 mM), 1 µl random hexameres (50 µM), 0,5 µl MuLV reverse transcriptase (50 U/µl), 0,25 µl RNase inhibitor (20 U/µl) (all chemicals by: Applied Biosystems, USA) and 0,25 µl H_2O. The RT step was performed at 42 °C for 1 hour. Entire contents of RT reaction were used in subsequent PCR reaction: 5 µl PCR buffer (100 mM Tris-HCl (pH 8.8), 15 mM $MgCl_2$, 500 mM KCl, 1 % Triton X-100), 32,5 µl H_2O, 0,5 µl Taq DNA Polymerase (5 U/µl, Applied Biosystems, USA), 1,1 µl of forward primer and 0,9 µl of reverse primer (both 10 pmol/µl). Two different sets of CSNV specific primers were designed for detection and identification of CSNV, CSNV UP1: 5′-AGCTGGTGAAGTTGAATTTGAG-3′, CSNV LO1: 5′-CATTCAAGCTAAGCCCGTATGC-3′ with specific product of 357 bp and CSNV UP2: 5′-GGCATACGGGCTTAGCTTGA-3′, CSNV LO2: 5′-GGAATAAGTTTAGATCCC-3′, with specific product of 384 bp. For detection of TSWV 0,9 µl of L1 TSWV primer and 1,1 µl of L2 TSWV primer were used (both 10 pmol/µl; Mumford & al., 1994). The PCR program used for amplification of CSNV was 94 °C 4 min; 30 cycles at: 94 °C 1 min, 48 °C 1 min, 72 °C 1 min, and end elongation at 72 °C for 9 min; for amplification of TSWV the annealing temperature was 55 °C.

CSNV from *Gerbera* was further amplified using primers BR060, BR066, CZ1 (Bezzera & al. 1999), and UHP (Cortez & al. 2001). The expected sizes of amplification products were 850 bp (BR060 – BR066) and 500 bp (CZ1 – UHP). RT step was performed as described. Entire contents of RT reaction was used in subsequent PCR reaction: 5 µl PCR buffer, 33,3 µl H_2O, 0,5 µl of Taq DNA Polymerase (5 U/µl, Applied Biosystems, USA) and 0,6 µl of each primer (50 µM). The PCR program used for amplification was 94 °C 5 min; 35 cycles at: 94 °C 1,5 min, 48 °C 2 min, 72 °C 2 min; and end elongation at 72 °C for 7 min. Both specific PCR products were cloned into pGEM-T Easy vector (Promega, USA), competent *E. coli* (DH5α) cells were transformed, and colonies containing plasmid with insert were selected by PCR. Products were sequenced on ABI PRISM 310 DNA Sequencer using BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA) according to manufacturer's protocol. All sequence analyses were conducted using tools available on the internet: http://searchlauncher.bcm.tmc.edu/ and http://www.ncbi.nlm.nih.gov/ .

Real-time PCR analysis

Primer Express® software (Applied Biosystems, USA) was used to design CSNV specific primers and MGB probe. The nucleotide sequence for N gene of CSNV (AF067068), served as template andf resulted in the following primers and probe: CSNV-F 5′-TGAATTTGAGGAAGAACAGAACCA-3′ (starting at bp 215); CSNV-R 5′-CTGATCCAGGTTGTCATTGCA-3′ (starting at bp 284); CSNV-MGB 5′-TTGCATTCAACTTCC-3′ (starting at bp 241). The MGB probe was labelled with FAM (6-carboxyfluorescein) fluorescent dye at the 5′ end and a non-fluorescent quencher and a minor groove binding molecule at 3′ end. TSWV detection was performed using primers and probe by Boonham & al. (2002). The RNA extraction step was also checked by amplifying cDNA with host specific, broad spectrum COX (cytochrome oxidase) primers and probe (Weller & al. 2000 modified by Boonham, personal communication).

RT reaction was performed separately. 10 µl of isolated RNA was first denatured at 80 °C for 5 minutes. Then 12 µl of H_2O, 4 µl of 10 × PCR buffer, 8 µl of 10 mM dNTPs, 4 µl of random hexa-

meres (50 µM), 1 µl of RNase inhibitor (20 U/µl), and 1 µl of MuLV reverse transcriptase (50 U/µl) (all chemicals by Applied Biosystems, USA) were mixed. 30 µl of RT mix was added to the template. RT step was performed at 23 °C for 10 min and 42 °C for 60 min (GeneAmp® 9700 PCR System, Applied Biosystems, USA). Real-time PCR reactions were performed in 20µl reaction volume with 1 × TaqMan® Universal PCR Master MIX (Applied Biosystems, USA). 900 mM primers and 250 mM probe were used and 4 µl of cDNA was added to reaction. Real-time PCR reactions were run in triplicates on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems, USA) under universal cycling conditions. The threshold cycle (Ct) was determined after manual adjustment of the baseline and the fluorescence threshold using SDS 2.2 software (Applied Biosystems, USA). A zero template control (water) and a positive cotrol were also included in each run.

Results

Disease symptoms and maintenance of the virus isolate

CSNV was initially isolated from chrysanthemum and gerbera plants and maintained on *Nicotiana benthamiana* Domin. test plants. Infected chrysanthemum plants showed *Tomato spotted wilt virus* (TSWV)-like symptoms, i.e. necrotic lesions surrounded by yellow areas, and occasionally rings and line patterns on some leaves, followed by necrosis on stems, peduncles, and floral receptacles. Gerbera plant showed no such characteristic symptoms of tospoviral infection - only slight yellowing and necrosis on leaves. Using CSNV infected plant material, stored at -80 °C for different length of time it was discovered that the newly found virus isolate can keep its infectivity after at least one year of storage at -80 °C.

The disease symptoms expression was studied on many different test plants and confirmed identity of *Chrysanthemum stem necrosis virus* (CSNV) by ELISA (Tab. 1).

Table 1: Test plants after mechanical inoculation with TSWV and CSNV.
Tabela 1: Testne rastline po mehanski inokulaciji s TSWV in CSNV.

	CSNV		TSWV	
Test plants	Local	Systemic	Local	Systemic
Capsicum annum cv. Shorokshari	+,CNSp,ReSp	+,CSp,ReSp,NVVa	+,CNSp	+,C,Ld
Chenopidium amaranticolor	+,CSp,NSp	-,No		
C. murale	+,No	-,No		
C. quinoa	+,CNSp	-,No	+,NSp	-,No
Datura stramonium	+,CNSp	+,CSp,M,NSp	+,CSp	+,CSp,Y,Ld
Lycopersicon esculentum cv. Money maker	+,CNSp	-,No (+,CNSp,ReSp,Gr)*	+,CSp,ReSp	+, Gr,ReSp,C,YV
Nicotiana benthamiana	+,CNSp	+,Ld,CNSp,YNVVa,NS	+,CSp	+,Ld,CSp
N. clevelandii	+,NSp	-,No	+,NSp	+,NSp,NSpr
N. occidentalis P1	+,CNSprSp	+,NVVa,Ld,NSpr	+,N	+,Y,Gr,NV
N. rustica	+,CNSp	-,No (+,CNSp,NVVa)*!	+,CNSp	+,M,YVVa,CSp
N. tabacum cv. White Burley	+,CNSprSp	-,No	+NSp,NR	+,CSp,Ld,NVVa
Petunia hybrida cv. BW	+,CNSpr	-,No	+CNSpr	-,No
Physalis floridana	+,CNSp	-,No	+,CNSp	+,NV,Y,NS,W
Solanum melongena	+,CSp	-,No		
Solanum tuberosum cv. Desiree	+,NSp	-,No		

Legende: + positive results; - negative results; C chlorotic, chlorosis; Gr growth reduction; Ld leaf deformation; M mosaic; N necrotic, necrosis; No no symptoms; P petioles; R ring; Re red; S stem; Sp spots; Spr round spots; Va along to vein; V vein; W wilting / total necrosis; Y yellowing; * rarely; ! recovery

Double infections with CSNV and TSWV were monitored and recorded on *Nicotiana tabacum* cv. White Burley, *Physalis floridana*, and *Datura stramonium*. *N. clevelandii*, *N. tabacum* cv. White Burley, and *P. floridana* were the test plants that successfully differentiated between CSNV and TSWV infections. While CSNV caused only local disease symptoms and the presence of the virus was not systemically confirmed, TSWV caused both, local and systemic damage. Two types of lesions were shown on *N. tabacum* cv. White Burley, infected with both viruses simultaneously, each characteristic for the specific virus (data not shown). This was not the case with *D. stramonium and P. floridana*, since only TSWV characteristic symptoms developed when the plants were infected with both viruses (data not shown). Some other test plants also showed difference in development of disease symptoms: systemic symptoms on *Capiscum annum* were more severe in TSWV infected plants, whereas on *N. occidentalis* P1 CSNV caused appearance of round, necrotic, local spots that later on became irregular and were not observed in TSWV infected leaves.

Using CSNV infected plant material, stored at -80 °C for different length of time it was discovered that the newly found virus isolate can keep its infectivity after at least one year of storage at -80 °C.

Comparison of different antisera in ELISA

Infected chrysanthemum and test plants were tested with antisera of four different manufacturers: one for CSNV, TCSV and GRSV, two for IYSV and tospoviruses, four for TSWV and INSV (data not shown). All antibodies gave reactions to the specific viruses, however in some cases cross-reactivity was observed. TSWV specific antibodies cross-reacted with CSNV viruses. Cross - reaction of CSNV with antibodies against *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), and in some cases with *Impatiens necrotic spot virus* (INSV) was also observed.

The cross-reactivity of TSWV antibodies with CSNV was lower if higher dilution of antibodies than recommended by the manufacturers was used. The binding of CSNV antibodies to TSWV was reduced when preabsorption of conjugate antibodies was performed in combination with the higher dilution of antibodies. Antibodies against INSV and *Iris yellow spot virus* (IYSV) did usually not cross-react with other tospoviruses. CSNV could be detected with the antibodies against mixed tospoviruses (Loewe, Bioreba).

RT-PCR

RT-PCR analyses of CSNV and TSWV, using specific primers, showed presence of amplification fragments of expected size.

Viral infection was confirmed in leaves, stems and roots of infected chrysanthemum plants in the case of TSWV, while CSNV was detected only in leaves and shoots (Tab. 2). The difference in band intensity was observed between different parts of the plants (data not shown). In CSNV infected plants, the intensity of the PCR bands was the highest in the leaves, whereas in case of TSWV infected plants, the intensity of the bands was the highest in the roots..

Comparison of serological (ELISA) and molecular analyses (RT-PCR)

Using ELISA and more sensitive RT-PCR both CSNV and TSWV were found in leaves, stems, and roots of infected chrysanthemum plants, sometimes also in mixed infections (Tab. 2). RT-PCR test was shown to be more sensitive.

Table 2: Combined results of ELISA and RT-PCR tests on infected chrysanthemum plants, where different tissues of plants were tested

Tabela 2: Združeni rezultati ELISA in RT-PCR testa na okuženih krizantemah, kjer smo analizirali različne dele rastlin

	LEAVES				STEMS				ROOTS			
	ELISA		PCR		ELISA		PCR		ELISA		PCR	
Sample	CSNV	TSWV	CSNV	TSWV	CSNV	TSWV	CSNV	TSWV	CSNV	TSWV	CSNV	TSWV
645/01	+?	_	+	+	?	–	+	+	–	+	–	+
1000/01	–?	+	–	+	–	?	–	+	–	–	–	–
808/01	?	+	–	+	–?	+	+	+	+?	–?	–	+

Legende: – : negative results; –?: higher values as negative control, but never near to the positive threshold; ?: OD values below positive threshold after 1 hour, but near to it after 2 hours of incubation in substrate; +?: OD values near to the positive threshold after 1 hour of incubation in substrate, but above it after 2 hours; +: OD values between two and four times the value of negative control after 1 hour of incubation in substrate

Sequencing

The nucleotide sequence of the N gene of the CSNV isolate from gerbera was determined. After the sequencing reaction a 637 bp long nucleotide sequence was obtained. BLAST analysis showed a 98 % homology with the previously published AF067068 N gene sequence of CSNV. The alignment of the 636 bp long sequence starts at the 203rd bp and ends at the 838th bp of the AF067068.

Real-time PCR

For detection of CSNV a specific real-time PCR based method was developed that amplified a 70 bp long region of N (nucleocapsid) viral gene. The specificity of the developed test was checked by analyzing tobacco test plants, infected with closely related tospoviruses TSWV and INSV. In case of CSNV infected *N. benthamiana*, CSNV and TSWV specific assays gave positive and negative result, respectively, whereas in TSWV infected *N. rustica* the result was negative for the CSNV and positive for TSWV assay. The result for INSV infected *N. bethamiana* was negative for both CSNV and TSWV assays. Along with the different test plants, the specificity of the developed primers and MGB probe was also tested on several different types of plant samples that cross-reacted in previously performed ELISA assays (data not shown). The RNA extraction procedure was checked by performing RT real-time PCR for the COX (cytochrome oxidase) plant tissue specific gene and was considered successful when Ct values ranged from 23 to 29. All analysed samples (10 chrysathemum (*Dendranthema grandiflorum*), 3 pepper (*Capsicum annuum*), 2 gerbera (*Gerbera spp*.), 3 tomato (*Lycopersicum esculentum*) and 2 verbena (*Verbena spp*.) plants) as well as test plants (1 *Nicotiana tabacum* cv. White Burley infected with extract of CSNV positive gerbera, 2 *Nicotiana benthamiana* plants, infected with CSNV and INSV, respectively, and 1 TSWV infected *Nicotiana rustica* plant) fitted within the set interval. Specific analyses revealed that CSNV was present in 3 chrysanthemums (2 samples showed its presence in leaves and stems) and 1 gerbera. TSWV was present in 7 chrysanthemums (2 samples showed its presence in leaves, stems, and roots) and 2 tomatoes.

Discussion

Identification of CSNV isolate

Using different methods we were able to confirm the identity of the CSNV isolate found in Slovenia. A new virus, isolated from chrysanthemum and gerbera plants was maintained on *Nicotiana*

bethamiana Domin. test plants. That material was used to mechanically inoculate a set of other test plants and monitoring of disease symptoms. Key characteristic that was looked for was the ability of test plants to distinguish between CSNV and closely related TSWV by developing different disease symptoms. To find appropriate test plants, 15 different plants were chosen from the previously described broad spectrum of host plants for the CSNV and other tospoviruses (Bezerra & al. 1999, Nagata & al. 1998). It was discovered that *Nicotiana clevelandii*, *Nicotiana tabacum* cv. White Burley, and *Physalis floridana* successfully differentiate between TSWV and CSNV. The latter was also described by Verhoeven & al. (1996) along with *Datura stramonium* as plant, suitable for discriminating the CSNV from other tospoviruses. The symptoms were in the case of CSNV strictly local, whereas with TSWV they were both, local and systemic. Two types of lesions were present on *N. tabacum* cv. White Burley when the plant was inoculated with the mixture of two viruses. With assays on test plants it was shown that the newly found virus presents a novelty in Slovenia, showing characteristics other than closely related tospovirus TSWV and, considering the developed disease symptoms, presents a new isolate of CSNV. Using ELISA the identity of the isolate was further confirmed. The cross-reactivity with TSWV specific antibodies was observed, which was also reported by Matsuura & al. (2007). The common antigenic determinants on the nucleocapsid of the virus particles of the same serogroup contribute to this effect (Williams & al. 2001). We were able to optimize the procedure of ELISA by modifying the protocol and preabsorb the conjugate antibodies with the plant sap of healthy plants. Cross-reactivity of the antibodies was lower, however still present. Another difficulty presented low virus concentration in infected samples resulting in OD values which often did not reach or pass the set threshold. Therefore in the next phase molecular analyses were performed. For detection of CSNV with RT-PCR a two sets of primers were designed, producing 357 bp and 384 bp long products, respectively. The method was tested on different CSNV infected samples (Tab. 2) and produced no cross-reactivity with TSWV infected samples, again confirming the identity of newly found virus isolates as CSNV. The similar confirmation of the isolate with molecular method was also performed by Matsuura & al. (2007) that used a method for multiplex RT-PCR of TSWV and INSV (Uga & Tsuda 2005). To further confirm the identity of the CSNV isolate from gerbera plant, a nucleotide sequence of the part of the viral N gene was determined by the procedure, described by Bezerra & al. (1999). They used the described sets of primers to sequence a virus isolate from the chrysanthemum plants that is published under the accession number AF067068. We obtained a 637 bp long fragment of the N gene. The homology with the already published sequence (AF067068) was 98 %, which is the same percentage as was described by Pappu & al. (2000), who compared homology between nucleotide sequences of intergenic regions of S and M RNA of different tospoviruses. The S RNA being also the one where the N gene is located (De Haan & al. 1990). In Slovenia altogether 26 plants (25 chrysanthemums and one gerbera) were found infected with CSNV from 2001 until now.

Comparison of different detection methods

In our study different detection methods were used that had different sensitivity. By comparing the results of serological method ELISA and molecular method RT-PCR (Tab. 2) the CSNV was more reliably determined by RT-PCR. Questionable results in case of ELISA method (OD values being at the threshold value) were analyzed as positive with RT-PCR. Our experiments show undoubted presence of CSNV in leaves and stems, whereas TSWV can be found in all tested parts of the plants, including roots (Tab. 2,).

By developing a CSNV specific real-time PCR method we were able to perform a comparative analysis of the infected samples – testing them for the presence of both, CSNV and TSWV. In case of sample 1001 (Tab. 2), we analysed different parts of the plant. The corresponding Ct values from the real-time PCR analysis (data not shown) were in concordance with the findings of Salmon & al. (2002), where Ct values corresponded to the band intensity of RT-PCR. CSNV specific real-time PCR gave lowest Ct values in leaves of the analyzed sample (data not shown), which corresponds to the

higher concentration of the virus and which in RT-PCR gave higher band intensity (data not shown). The same was observed in the case of TSWV specific test.

Development of a real-time PCR method

Due to the serological cross-reactivity of tospoviruses that was previously reported (Williams & al. 2001) and also observed in our experiments and due to the already reported real-time PCR assays for the presence of TSWV (Roberts & al. 2000, Boonham & al. 2002) we designed a CSNV specific real-time PCR assay. Boonham & al. (2002) have, unlike Roberts & al. (2000), tested the designed primers and probe against closely related viruses as was also done in our experiments. We tested the designed primers and probe on test plants infected with related TSWV and INSV and detected no signal. Since a broad spectrum of host for tospoviruses was previously reported (Nagata & al. 1998, Bezerra & al. 1999) different host plants were tested and revealed presence of CSNV in different chrysanthemum, gerbera and test plants.

Proposed protocol for analysis for the presence of CSNV

Due to the economic importance of tospoviruses that can cause great crop loss in the field (Nagata & al. 1998), there is a high need for sensitive and specific detection methods. In case of CSNV that serologically cross-reacts with TSWV, which was observed during our experiments as well as by others (Williams & al. 2001, Matsuura & al. 2007) the results of the serological analyses can not be reliable in order to distinguish between the two related viruses, but can, however be used in the first phase of analyses for screening of the plants for the presence of tospoviruses. In the next step the sensitive, specific real-time PCR for TSWV and CSNV can be used as a confirmation method. The success of RNA isolation is paralelly checked in the real-time PCR reaction, where an internal control gene (cox) is amplified. When Ct values of the internal control cox correspond to the values within the interval from 23 to 29 the extraction is considered successful. When the results of the controls of PCR reactions are suitable and the results for the samples are positive the infection of the samples with CSNV can be reliably determined.

Conclusions

Since the first finding of CSNV in Slovenia, 25 chrysanthemum plants and one gerbera plant tested postive for CSNV until now. The identity of the initial isolate was confirmed using different methods: ELISA, test plants, RT-PCR, sequencing and also RT real-time PCR. Three different test plants were selected that show differences in developed disease symptoms between CSNV and TSWV. Molecular detection methods improved detection of CSNV and increased sensitivity of the diagnostic assay, making serological methods more suitable for screening analyses of plant samples.

Acknowledgements

This work was co-financed by the Slovenian Research Agency. The authors would like to thank Silvestra Darja Gačnik for the monitoring of test plants and help in ELISA, dr. Nataša Petrovič and dr. Neil Boonham (CSL, York) for the help with the development and comparisons of diagnostic methods and the phytosanitary inspectors for providing us with the samples.

References

Alexandre M. A. V., E. B.Rivas, L. M Duarte. & M. H. Okuyama 1996: Serological survey of tospovirus on *Chrysanthemum* sp. Crops in São Paulo state, Brazil. Fitopatol. Bras. **21** (1): 80–84.

Alexandre M. A. V., L. M. L. Duarte, E. B. Rivas & C. M. Chagas 1999: Mixed infection by Tospovirus species in ornamental crops in Sao Paulo state, Brazil. Summa phytopathol. **25**: 353–356.

Bezerra I. C., R. de O. Resende, L. Pozzer, T. Nagata, R. Kormelink & A. C. de Ávila 1999: Increase of tospoviral diversity in Brazil with the identification of two new tospovirus species, one from chrysanthemum and one from zucchini. Phytopathology **89** (9): 823–830.

Boonham N., P. Smith, K. Walsh, J. Tame, J. Morris, N. Spence, J. Bennison & I. Barker 2002: The detection of *Tomato spotted wilt virus* (TSWV) in individual thrips using real time fluorescent RT-PCR (TaqMan). J. Virol. Methods **101**: 37–48.

Cortez I., J. Saaijer, K. S. Wongjkaew, A.-M. Pereira, R. Goldbach, D. Peters & R. Kormelink 2001: Identification of a novel tospovirus species using a new RT-PCR approach. Arch. Virol. **146**: 265–278.

Duarte L. M. L., E. B. Rivas, M. A. V. Alexandre, A. C. de Ávila, T. Nagata & C. M. Chagas 1995: Chrysanthemum stem necrosis caused by a possible novel Tospovirus. J. Phytopathol. **143**: 569–571.

Eiras M., R. O. Resende, A. A. Missiaggia & A. C. de Ávila 2001: RT-PCR and dot blot hybridization methods for a universal detection of tospoviruses. Fitopatol. Bras. **26** (2): 170–175.

Nichol S. T., B. J. Beaty, R. M. Elliot, R. Goldbach, A. Plyusnin, C. S. Schmaljohn & R. B. Tesh 2005: Genus *Tospovirus*. In: Faquet C. M., M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball (eds.): Virus Taxonomy. Classification and Nomenclature of Viruses, Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, Amsterdam, pp. 712–716.

de Haan P., L. Wagemakers, D. Peters & R. Goldbach 1990: The S RNA segment of tomato spotted wilt virus has an ambisense character. J. Gen. Virol. **71**: 1001–1007.

Heinze C., B. Letschert, D. Hristova, M. Yankulova, O. Kauadjouor, P. Willingmann, A. Atanassov & G. Adam 2001: Variability of the N-protein and the intergenic region of the S RNA of Tomato spotted wilt tospovirus (TSWV). Microbiologica **24**: 175–187.

Letschert B., G. Adam, D.-E. Lesseman, P. Willingmann & C. Heinze 2002: Detection and differentiation of serologically cross-reacting tobamoviruses of economical importance by RT-PCR and RT-PCR-RFLP. J. Virol. Methods **106**: 1-10.

Matsuura S., K. Kubota & M. Okuda 2007: First Report of *Chrysanthemum stem necrosis virus* on Chrysanthemums in Japan. Plant Dis. **91** (4): 468.

Mavrič I. & M. Ravnikar 2001: First report of Tomato spotted wilt virus and Impatiens necrotic spot virus in Slovenia. Plant Dis. **85** (12): 1288.

Mumford R. A., I. Barker & K. R. Wood 1994: The detection of tomato spotted wilt virus using the polymerase chain reaction. J. Virol. Methods **46**: 303–311.

Mumford R. A., B. Jarvis, J. Morris & A. Blockleey 2003: The first report of Chrysanthemum stem necrosis virus in United Kingdom. New Disease Reports, 7:feb.2003–jul.2003, http://www.bspp.org.uk/ndr/july2003/2003-06.asp (dec. 2005).

Nagata T., R. de O. Resende, E. W. Kitajima, H. Costa, A. K. Inoue-Nagata & A. C. de Ávila 1998: First report of natural occurence of Zucchini lethal chlorosis tospovirus on cucumber and *Chrysanthemum stem necrosis tospovirus* on tomato in Brazil. Plant Dis. **82**: 1403.

Nagata T. & A. C. de Ávila 2000: Transmission of Chrysanthemum Stem Necrosis Virus, a Recently Discovered Tospovirus, by Two Thrips Species. J. Phytopathol. **148** (2): 123–125.

Okuda M. & K.Hanada 2001: RT-PCR for detecting five distinct Tospovirus species using degenerate primers and dsRNA template. J. Virol. Methods **96**: 149–156.

Pappu S. S., A. I. Bhat, H. R. Pappu, C. M. Deom & A. K. Culbreath 2000: Phylogenetic studies of tospoviruses (Family: *Bunyaviridae*) based on intergenic region sequences of small and medium genomic RNAs. Arch. Virol. **145**: 1035–1045.

Resende R. de O., L. Pozzer, T. Nagata, I. C. Bezerra, M. I. Lima, L. B. Giordano, E. W. Kitajima & A. C. de Ávila 1996: New tospoviruses found in Brazil. Acta Horticulturae **431**: 78–81.

Roberts C. A., R. G. Dietzgen, L. A. Heelan & D. J. Maclean 2000: Real-time RT-PCR fluorescent detection of tomato spotted wilt virus. J. Virol. Methods **88**: 1–-8.

Salmon M. A., M. Vendrame, J. Kummert & P. Lepoivre 2002: Detection of *apple chlorotic leaf spot* virus using a 5' nuclease assay with a fluorescent 3' minor groove binder-DNA probe. J. Virol. Methods **104**: 99–106.

Uga H. & S. Tsuda 2005: A one-step reverse transcription-polymerase chain reaction system for the simultaneous detection and identifiaction of multiple tospovirus infections. Phytopathology **95**: 166–171.

Verhoeven J. Th. J., J. W. Roenhorst, I. Cortes & D. Peters 1996: Detection of a novel tospovirus in Chrysanthemum. Acta Horticulturae **432**: 44–51.

Weller S. A., J. G. Elphinstone, N. C. Smith, N. Boonham & D. E. Stead 2000: Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl. Environ. Microb. **66** (7): 2853–2858.

Williams L. V., P. M. Lopez Lambertini, K. Shohara & E. B. Biderpost 2001: Occurence and geographical distribution of tospocirus species infecting tomato crops in Argentina. Plant Dis. **85**: 1227–1229.