Virus Infections in Gentiana Species

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Abstract

***Gentiana* species generally are of little importance to horticulture. This may explain that little attention has been given to virus infections in these ornamentals, so far. However, during the last couple of years the number of samples for virus diagnosis increased. The main symptoms recorded, were growth reduction, necrotic lesions and decline. The virus isolated most frequently was broad bean wilt virus 2, while the related broad bean wilt virus 1 was not detected. Impatiens necrotic spot virus was identified once and one natural infection by the beet ringspot strain of tomato black ring virus was found. In addition, several isolates of bean yellow mosaic virus were identified.**

INTRODUCTION

Species of *Gentiana* (gentian) originate in several temperate and arctic zones of North America, Asia and Europe, often in high mountain areas. Several of the over 300 *Gentiana* species are being grown as ornamental garden plants. Amongst these are *G. acaulis*, *G. asclepiadea*, *G. sino-ornata* and *G. verna*. Some, e.g. *G. triflora* var *japonica* 'Royal Blue', are also grown as cut flowers. Propagation is by seeds, as well as by cuttings or division for some species. Recently, in vitro propagated hybrid cultivars of *G. scabra* like 'Shusui' originating in Japan have been introduced as pot plants in Europe. However, the marketing of these vegetatively propagated plants increases the risk of spreading non-indigenous viruses.

During the last five years an increase was noticed in the number of symptomatic gentian plants that were submitted for diagnosis to the Plant Protection Service (PPS) of the Netherlands and to Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA) in Germany. Samples from cultivars of *G. scabra* were received most frequently. Most plants showed stunting and necrosis. Necrosis varied from necrotic lesions to necrosis of the whole plant. Occasionally also some leaf mottling was observed. Because of the nature of the symptoms several of these plants were initially tested for bacteria and fungi. However, as these tests were negative, tests for viruses were started. This paper reports on the identification of four different viruses in gentian plants.

MATERIALS AND METHODS

Samples of various species of *Gentiana* were obtained from growers in Germany, the Netherlands and the United Kingdom. The first step in the identification process was mechanical inoculation onto herbaceous test plants (Verhoeven et al, 1996). Depending on the symptomatology of the inoculated test plants additional methods were employed for further identification, i.e. (immuno-)electron microscopy (IEM/EM), enzyme linked immunosorbent assay (ELISA), reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis of cloned viral genome fragments.

For EM crude extracts were adsorbed to formvar carbon-coated copper or nickel

grids and negatively stained with 2% sodium phosphotungstate, pH 6.5, or with 1% aqueous uranyl acetate. Preparates were examined in a Philips CM 12 or a Zeiss EM 906 electron microscope.

For IEM particles were trapped from crude extracts of infected (test) plants on grids coated with antisera diluted 1:1000 prior to decoration tests at an antiserum dilution of 1:50. For assessing the relative intensity of decoration, titers were determined using a twofold dilution series in 0.1 M phosphate buffer pH 7 from 1:50 up to 1:12800. The highest dilution step yielding recognisable decoration is given as titer value. DAS-ELISA was performed according to Clark and Adams (1977) with some minor modifications. Antisera used in this study were from Bioreba (turnip mosaic virus, TuMV), DSMZ or the stock of BBA (bean yellow mosaic virus, BYMV; broad bean wilt virus 1, BBWV1; broad bean wilt virus 2, BBWV2; carnation vein mottle virus, CarVMV; clover yellow vein virus, ClYVV; tomato black ring virus, beet ringspot strain, TBRV/BRSV, and a potato isolate TBRV; watermelon mosaic virus, WMV), Plant Research International (BYMV), Sanofi (WMV) and Wageningen Agricultural University (impatiens necrotic spot virus, INSV).

RT-PCR was performed on total RNA isolated from 100 - 200 mg infected leaf material using the Quiagen RNAeasy plant Mini Kit according to the manufacturer's instructions. The eluted RNA was EtOH precipitated and after washing and drying dissolved in 20 µl H_2O. Prior to cDNA synthesis, 2.5 µl of total RNA was denatured by adding 4 µl of 5x First Strand Buffer (Life Technologies, Gaithersburg, U.S.A.), 1 µl of oligo-dT primer P9502 (100 ng/µl; Van der Vlugt et al., 1999) and 11.5 µl of H_2O, followed by incubation at 65°C for 5 min. Thereafter, cDNA was synthesized by adding 2 µl of 0.1M DTT (Life Technologies), 1 µl of dNTP mix (2.5 mM each), and 0.5 µl of M-MLV reverse transcriptase (Life Technologies; 200 U/µl). The reaction mixture was incubated at 37°C for at least 1 h.

The resulting cDNA was amplified by PCR using either a degenerate potyvirus-specific primer set P9502/CPUP (Van der Vlugt et al., 1999) or a newly designed BYMV-specific primer set (BYMV-UP: 5'- TTYCGCCARATAATGTG -3'; BYMV-DW: 5'- TTTTTTTTCTCGCTCTAC -3'). Three µl of cDNA was mixed with 21 µl of H_2O, 3 µl of 10x *Taq*-polymerase buffer (HT-Technologies Ltd., Cambridge UK,), 1 µl of each primer (100 ng/µl), 1 µl of dNTPs (2.5 mM each) and 1 µl of *Taq* polymerase (0.5 U/µl; HT-Technologies). Incubation was in a PTC 200 thermocycler (MJ Research Inc., Watertown, U.S.A.): 3 min at 94°C, followed by 35 cycles of 30 s at 92°C, 30 s at 52°C and 45 s at 72°C, and a final extension of amplification products for 10 min at 72°C. Amplification products were analysed for size and yield by electrophoresis on a 1% Tris-acetate EDTA agarose gel.

Cloning and sequencing of the amplification products was performed following the procedure as described by Van der Vlugt et al. (1999) with minor modifications in the selection of clones containing the expected insert. Sequence data were compiled and analysed with the UW-GCG (v.8) and the Lasergene software packages.

RESULTS

From the symptomatic plants of *Gentiana* four different viruses have been isolated, *i.e.*, BBW2, INSV, TBRV (beet ringspot strain) and BYMV.

The virus found most frequently was BBWV2. After mechanical inoculation, samples of systemically infected plants of *Chenopodium quinoa* or *Nicotiana benthamiana* were examined in EM. In this way angular isometric virus particles and empty shells were observed with a diameter of about 30 nm. The virus could be identified as BBWV2 by IEM using antisera to BBWV1 and BBWV2. Both viruses could be distinguished most clearly by cross tests using both antisera at a dilution of 1:400. Antisera diluted 1:50, as often used for decoration tests, did not always clearly differentiate between theseviruses. Thus, both viruses were cross-reacting to some extent, indicating a serological relationship. Later, isolates were also identified by testing systemically infected test plants in DAS-ELISA (tests performed at Naktuinbouw, the

Netherlands). Infections with BBWV2 were found in samples from both Germany and the Netherlands. In addition to recent samples from *G. scabra*, also a sample of *G. triflora* var *japonica* 'Royal Blue' from 1986 was shown to contain BBWV2 by IEM. The related BBWV1 was not detected in *Gentiana*, although it was often detected in various other cultivated or wild plants in Germany (D-E. Lesemann, unpublished).

INSV was identified once in plants from a greenhouse in the Netherlands. The identification was based on the reactions of mechanically inoculated plants of *C. quinoa*, *N. occidentalis*-P1, *N. tabacum* 'White Burley' and *Vicia faba* 'Witkiem Major' (Verhoeven et al., 1996) and DAS-ELISA. Using DAS-ELISA the virus was detected in symptomatic gentian plants and in mechanically inoculated plants of *N. occidentalis*-P1.
TBRV/BRSV was isolated from a naturally infected garden plant of *G. asclepiadea* with chlorotic mottling and stunting symptoms in Germany. After mechanical transmission the virus infected *N. benthamiana* and *C. quinoa* systemically. *N. benthamiana* showed systemic leaf mosaic and vein chlorosis, and *C. quinoa* necrotic local lesions followed by conspicuous apical necrosis. In IEM the isolate reacted strongly with two antisera to TBRV/BRSV but not or very weakly with an antiserum to a potato isolate of TBRV. The isolate did not react with antisera to 18 other members of the genus *Nepovirus*.

BYMV was the fourth virus identified. It was isolated from samples from Germany, the Netherlands and the United Kingdom. Based on the reactions of test plants (Table 1) the four isolates tested could be divided into two groups of two isolates each: one group (isolates PD99908417 and PD99913981) infected *N. hesperis*-67A, *N. miersii*, and *V. faba* systemically, while the other group (isolates PD99908965 and BBA99-710) did not. In contrast, only the two isolates not systemically infecting these species infected *C. quinoa* systemically. The following mechanically inoculated plants did not become systemically infected by any isolate: *Datura metel*, *D. stramonium*, *N. bigelovii*, *N. rustica*, *N. tabacum* 'White Burley' and *Trifolium repens* 'Barbian'. Potyvirus-like particles were observed by EM in all systemically-infected plants. In DAS-ELISA systemically infected test plants only showed reactions with an antiserum to BYMV, while no reactions were obtained with antisera to ClYVV, TuMV and WMV (Table 2). Also in IEM for all isolates the strongest reactions were obtained with an antiserum to BYMV. Heterologous reactivity of the gentian isolates as well as of two legume isolates of BYMV occurred with antisera to CarVMV, ClYVV and WMV. Heterologous reactivity was weakest with the antiserum to ClYVV although its homologous titer was very high (1:12,800). However, isolate BBA99-710 was decorated by antisera to ClYVV and WMV at just one dilution step below that of BYMV (Table 3). Nevertheless, the closest similarity of this isolate to BYMV was proven since the homologous titers of the antisera were 1:3,200, 1:12,800 (Table 3) and 6,400 (data not shown) for BYMV, ClYVV and WMV, respectively.

In RT-PCR all four isolates reacted positive with the BYMV-specific primer set. Isolates PD 99913981 and BBA99-710 were also subjected to RT-PCR using the degenerate potyvirus primer set (Van der Vlugt et al., 1999). With this primer set both isolates clearly generated a PCR product of approximately 650 bp. Cloning of these DNA fragments in pGEM-T easy vector followed by sequence analysis and alignment of these fragments revealed high levels of homology with BYMV sequences from the EMBL database (Table 4), both in the (partial) CP encoding region (> 94%) and in the 3' non translated region (3NTR; 81 – 100%). Alignments of the sequences of these isolates with sequences of the related ClYVV revealed lower levels of homology for the (partial) CP sequence (82 - 86%) and the 3NTR (50- 56%). An overview of homologies between the BYMV and ClYVV isolates is given in table 4. Sequences were submitted at the EMBL Nucleotide Sequence Database and assigned the accession numbers AJ289199 and AJ289200 for respectively PD99913981 and BBA99-710.

DISCUSSION

From symptomatic plants of *Gentiana* four viruses have been isolated: BBWV2, INSV, TBRV/BRSV and BYMV. However, discrimination between these viruses on the

basis of symptoms on gentian plants appeared difficult, as most of them were isolated from plants showing more or less similar symptoms. This means that specific tests are needed for the identification of viruses from gentian. In addition to the viruses detected in this study, the following viruses have been reported in gentian: ClYVV (Kaji et al., 1993a; Sasaya et al., 1997), cucumber mosaic virus (Schmidt, 1972; Nagao et al., 1978; Yamashita, 1990), tobacco rattle virus (Li et al., 1983), a member of the Genus *Carlavirus* referred to as *Gentiana* carlavirus (Koenig, 1985; Brunt et al., 1996) and a member of the family Rhabdoviridae referred to as *Gentiana* rhabdovirus (Thaler and Gailhofer, 1996).

BBWV2 was the virus detected most frequently. It was mainly detected in vegetatively propagated plants of *G. scabra*. These in vitro propagated plants originate in Japan, where the virus was already isolated from gentian before (Nagao et al., 1978; Yamashita, 1990; Kobayashi, 1999). So far, in the Netherlands and in Germany BBWV2 was detected only in imported plants from *Petunia* originating in Japan and in *Gentiana* and *Verbena* (Roenhorst and Verhoeven, 1998 and 1999). New species and cultivars of these ornamentals have been introduced from Japan frequently. Therefore, it is supposed that many BBWV2 infections in Western Europe originate in Japan. Also tests on samples of spinach from China (Feng, L.-X., H.L.Paul and D.-E. Lesemann, unpublished) *Capsicum annuum* in China (S.K. Green and D.-E. Lesemann, unpublished) and *Megakepasma* from Singapore (S.M. Wong and D.-E. Lesemann, unpublished), and reports on BBWV2 in China (Xu et al, 1988) and Japan (Kobayashi et al., 1999) indicate that Asia is the continent of origin of this virus. On the other hand, the related BBWV1 is a common virus of wild plants and ornamentals in Germany, and was also recorded in *Eustoma russellianum* (syn. *Lisianthus*), another member of the family Gentianaceae (Lesemann, 1987 and unpublished).

The identification of INSV in gentian represents the first finding of a tospovirus infecting plants of the genus *Gentiana* (Roenhorst and Verhoeven, 1997). In the Netherlands infections by INSV mainly occur in greenhouses, as the conditions in the open air generally are unfavourable for its main vector (*Frankliniella occidentalis*). The infection of gentian is an example of bringing a virus and a plant species from different origins together at one location. In the Netherlands 50 different ornamental plants species have been found infected by INSV, so far. In ornamental crops the number of INSV infections gradually equals or even slightly surpasses the number of TSWV infections.

Also the infection of *Gentiana* by TBRV/BRSV has not been reported before. Although this virus is quite widespread in Europe, it generally occurs at low incidence. Since it was only found in one single plant, this virus does not seem of economic importance for gentian.

Concerning the identification of BYMV, a closely related virus was reported in gentian in Japan (Kaji et al., 1993b). Additional studies indicated that the isolated potyvirus most resembled ClYVV (Kaji et al., 1993a). Later ClYVV (Sasaya et al., 1997) was identified from gentian based on studies including nucleotide sequence comparison of the 3'-terminal region. In this study BYMV infections of gentian were identified on the basis of biological, serological and molecular data. Especially, the molecular analysis provided convincing data to distinguish BYMV from the closely related ClYVV, the only potyvirus previously reported in gentian. For the partial CP amino-acid sequence and the full length 3'-NTR sequences, levels of homology among BYMV isolates and among ClYVV isolates always was above 93%. Levels of sequence homology between BYMV and ClYVV isolates were nearly always below 86% (CP) and 56% (3'-NTR). This is in good agreement with the generally accepted levels of sequence homology within and between potyvirus species (Shukla et al., 1988; Frenkel et al., 1989).

Remarkably the published sequence of the BYMV-GDD isolate (D00490; Hammond and Hammond, 1989) generally showed significant lower levels of homology with the other BYMV isolates in the 3'-NTR (75 - 82%) while for the (partial) CP amino-acid sequence levels of homology are above 93% for all BYMV isolates. The sequence alignment revealed a total of five nucleotide deletions and five unique nucleotide changes

in the 3'-NTR of BYMV-GDD in comparison to the other BYMV isolates. Whether these differences represent true characteristics of this BYMV isolate or merely represent sequencing artefacts remains to be established. Together with the variability of the reactions observed on the mechanically inoculated test plants, these results indicate that there is significantbiological variation within BYMV. It also indicates that the infected plants represented different sources of infection.

In conclusion, the fact that four different viruses have been identified in gentian demonstrated that both new ways of propagation and introduction of plants into new areas will often be accompanied by spreading viruses. Since gentian is becoming more popular as an ornamental, more new virus infections can also be expected in this crop.

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Tables

Table 1. Local/systemic reactions of mechanically inoculated test plants for four potyvirus isolates from *Gentiana*.

Plant species	**Isolate**			
	PD99908417	**PD99908965**	**PD99913981**	**BBA99-710**
Chenopodium amaranticolor	cl,nl/-*[1]	cl,nl/s	nl/cl,nl	cl,nl/cl
C. quinoa	cl,nl/-	cl,nl/cl,(nl)[2]	cl.nl/-	cl,nl/cl,(nl)[2]
Nicotiana benthamiana	-/vc	-/-*	-/c,l,vc	-/(m),(s)
N. hesperis-67A	cl,nl/cl	-/-*	cr,nr/cl	-/-*
N. miersii	-/cl,cr	-/-*	cl/cr	-/-*
N. occidentalis-P1	cl,nl,nr/(l),(nl)	-/-*	cr,nl,nr/(cl),(l)	cr/(cl)
Phaseolus vulgaris 'Dubbele Witte zonder draad'	-/-*	-/-*	-/-*	-/(vn)
Pisum sativus 'Kelvedon Wonder'	-/-*	-/-*	-/(m)	-/-*
Trifolium pratense 'Roosendaalse'	-/-*	-/-*	-/(m)	-/-*
Vicia faba 'Witkiem'	-/m	-/-*	(nr),(n)/m	-/-*

[1] c = chlorosis, cl = chlorotic lesions, cr = chlorotic rings, d = dwarfing, l = leaf deformation, m = mosaic or mottle, n = necrosis, nl = necrotic lesions, nr = necrotic rings, vc = veinal chlorosis, vn = mild veinal necrosis, - = no symptoms, -* = no infection, s = symptomless infection, () = symptoms occasionally observed

[2] systemic symptoms are restricted to older leaves and often are close to the veins

Table 2. Extinctions values of four potyvirus isolates from *Gentiana* in DAS-ELISA, measured 2 h after substrate incubation.

Isolate	Antisera			
	BYMV (PRI)	**ClYVV** (DSZM)	**TuMV** (Bioreba)	**WMV** (Sanofi)
Gentiana PD99908417	0.96	0.02	0.00	0.01
Gentiana PD99908965	1.24	0.01	0.00	0.01
Gentiana PD99913981	1.53	0.02	0.00	0.01
Gentiana BBA99-710	1.34	0.01	0.00	0.01

Table 3. Reciprocal decoration titers of four potyvirus isolates from *Gentiana*.

Isolate	Antisera			
	BYMV 401/8	**CarVMV** 201/4	**ClYVV** 275/5	**WMV** 1025/5
Gentiana PD99908417	1600	400	< 50	200
Gentiana PD99908965	3200	400	< 50	400
Gentiana PD99913981	1600	100	100	200
Gentiana BBA99-710	1600	200	800	800
BYMV SP 7-88 Lebanon	3200	200	200	1600
BYMV PV0469 DSMZ D	800	100	< 50	400
ClYVV Vetten D	100	< 50	12,800	50

Table 4. Levels of homology between isolates PD99913981 and BBA99-710 and different BYMV and ClYVV isolates. Codes refer to EMBL accession numbers. Above diagonal levels of CP amino acid sequence homology, below diagonal levels of 3NTR nucleotide sequence homology.

Isolate	BYMV						ClYVV		
	PD9991 3981	BBA99 -710	D00490	D83749	D00604	X81124	D00605	S77521	AB003 308
PD99913981	-	98.0	93.2	95.9	95.9	93.9	84.4	85.0	85.0
BBA99-710	95.4	-	94.6	96.6	96.6	94.6	84.6	85.2	85.2
D00490	77.5	81.7	-	97.3	95.3	96.6	81.9	82.6	82.6
D83749	95.4	100.0	81.7	-	96.0	98.0	83.9	84.6	84.6
D00604	90.2	93.7	75.1	93.1	-	95.3	85.2	85.9	85.9
X81124	94.8	99.4	81.1	98.9	92.6	-	81.9	82.6	82.6
D00605	50.6	55.2	50.3	54.9	53.1	53.7	-	99.3	99.3
S77521	52.9	55.7	52.1	55.4	52.6	54.3	97.8	-	100.0
AB003308	51.1	55.7	50.9	55.4	53.7	54.3	99.4	98.3	-