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Sequence comparison and transmission of *Blackcurrant reversion virus* isolates in black, red and white currants with black currant reversion disease and full blossom disease symptoms

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Abstract We collected samples from black, red and white currants showing symptoms of blackcurrant reversion disease (BRD) and full blossom disease (FBD), cultivated in the Czech Republic. *Blackcurrant reversion virus* (BRV) was detected in all symptomatic plants. After amplification, a substantial part of the 3′ non-translated region (3′-NTR) of RNA2 of 15 new isolates of BRV was sequenced and compared with sequences available in the literature and GenBank. We did not find significant sequence diversity among isolates associated with either FBD or BRD. BRV was graft-transmitted from FBD infected red currant to black currant where symptoms of BRD were observed. Further sequence analysis of BRV isolates resulted in a phylogenetic tree with four branches, each consisting of six to nine isolates. No correlation with geographic origin was visible on the tree as isolates from various countries occurred in all four branches. We also found no correlation between the host and the topology of the tree: most of black currant isolates occurred in branches 3 and 4, but also occurred in branches 1 and 2. Only one white currant and one red currant isolate occurred in branches 3 and 4, respectively. The sequence identity of the Czech isolates in this region ranged from 91.9 to 99.8%. The 17 plant species growing within and in the close vicinity of the BRD-infested plantation were tested negative for BRV by RT-PCR as natural hosts of BRV. BRV was successfully transmitted by mechanical inoculation from black currant to *Nicotiana occidentalis* and *N. tabacum* cv. Xanthi, the latter being a new host for BRV. The infection was confirmed by PCR and sequencing.

Keywords *Blackcurrant reversion virus* · *Ribes* · Transmission · RT-PCR

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Introduction

Blackcurrant reversion virus (BRV, genus *Nepovirus*, family *Comoviridae*) is a mite-, graft- and mechanically-transmitted virus with a positive-sense, bipartite RNA genome. BRV was originally isolated from black currants infected with blackcurrant reversion disease (BRD) by Lemmetty et al. (1997). Final proof of BRV as the causal agent of BRD was obtained when typical BRD symptoms were detected after back-inoculation of BRV to healthy black currant plants (Lemmetty and Lehto 1999). Two forms of the BRD have been described – the common European form (E) which often did not cause visible symptoms

on black currant, and the more severe R-form, with malformed flowers and/or malformed leaves, found in Finland, central Europe and countries of the former Soviet Union (Jones 2000). The occurrence of satellite RNA (satRNA) has recently, in 2003 been related to the severe form of BRD (Malinowski et al., unpublished), but the effect of the satRNA on symptoms is not known and no specific determinants on the BRV genome are known to be involved in the formation of the symptoms of BRD (Susi 2004).

BRD, the most important virus disease of *Ribes* spp., was first observed in Czechoslovakia by Blattný (1930). In 2002, we confirmed the presence of the *Blackcurrant reversion virus* in black currants in the Czech Republic by reverse transcription-polymerase chain reaction (RT-PCR) (Přibylová et al. 2002).

Full blossom disease (FBD) of red currant was first described by Rakús (1971) at Bojnice, Slovakia and found to be associated with phytoplasma infection (Rakús et al. 1974). In 2001, we proved the presence of BRV in red and white currants with symptoms of FBD (Přibylová et al. 2001). Recently we reported the occurrence of FBD in many currant cultivars and locations (Špak et al. 2006). The disease has been recorded in 2002 the Czech Republic and Poland (Malinowski et al., unpublished) only.

Until now, the detailed sequence comparison of BRV isolates relating to BRD and FBD has not been done. In the analysis by Lehto et al. (2004) eight of nine partially sequenced isolates originated in black currant and only one in red currant. One sequence of complete BRV genome is available and about forty partial BRV sequences originating in several isolates are scattered alongside the genome, which makes the comparison among isolates difficult. Moreover, due to lack of additional data it is impossible to relate isolates to their geographical origin, hosts, symptoms and other biological characteristics.

In this study we report the results of sequence comparison of 15 new and all previously published sequences of 3′-NTR of RNA-2 of BRV in black, red and white currants showing symptoms of either BRD or FBD. Except for the genus *Ribes*, there are no known natural hosts of the virus. Therefore, we focused on natural host plants of BRV and conducted mechanical inoculation of several isolates of BRV onto herbaceous host plants.

Materials and methods

Plants

Selected plants (Table 1) with symptoms either of the R-form of BRD (Fig. 1) or typical symptoms of FBD were taken from the plantations in Lhenice and Chrastiny, South Bohemia, in 1998 and 1999, respectively, from a private garden in Rožmitál, South

Table 1 List of new *Blackurrant reversion virus* isolates from the Czech Republic

Currant	Cultivar	Location	Symptoms	AC number
Black	Karlštejnský dlouhohrozen A	Chrastiny	Reversion	DQ450984
Black	Karlštejnský dlouhohrozen D	Chrastiny	Reversion	DQ450985
Black	Karlštejnský dlouhohrozen E	Chrastiny	Reversion	DQ450986
Black	Black Smith 10[a]	Holovousy	Reversion	DQ450981
Black	Black Smith 11[a]	Holovousy	Reversion	DQ450982
Black	Black Smith 12[a]	Holovousy	Reversion	DQ450980
Black	Black Smith 14[a]	Holovousy	Reversion	DQ450983
Black	Viola 5 grafted[b]	Lhenice	Reversion	DQ450978
Black	Viola 100 grafted[b]	Lhenice	Reversion	DQ450979
Red	Vitan 2	Lhenice	Full blossom	DQ450977
Red	Vitan 3	Lhenice	Full blossom	DQ450976
Red	Vitan 8	Lhenice	Full blossom	DQ450975
Red	Red currant	Rožmitál	Full blossom	DQ450974
White	Blanka 1	Lhenice	Full blossom	DQ450972
White	Blanka 5	Lhenice	Full blossom	DQ450987

[a] Plants were imported from Slovakia.

[b] Scions of red currant ‘Vitan’ with symptoms of FBD were grafted in 1999.

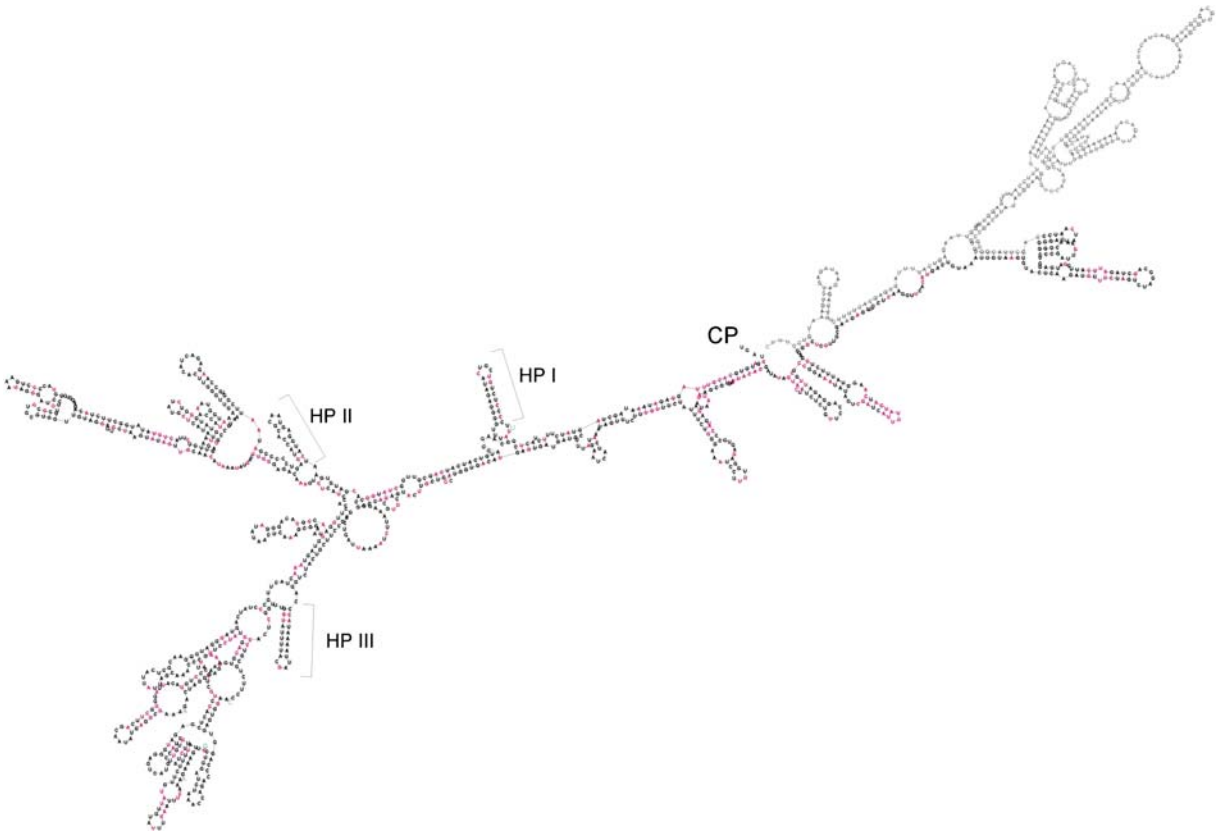

Fig. 1 Localisation of variable positions on one of possible foldings of the 3′-NTR of BRV. Analyzed positions of the Czech isolates are in *bold*, variable positions are *marked red*, inserted nucleotide positions are in *green*. HP I–III are conserved stem-loop structures identified by Karetnikov et al. (2004)

Bohemia, in 2001 and from the germplasm collection at The Research and Breeding Institute of Pomology, Holovousy, East Bohemia, in 1999. Plants of black currant 'Viola' purchased as commercial virus-tested seedlings, were used as test plants (Table 1). The plants marked as 'Viola 5' and 'Viola 100' were grafted in 1999 by scions from red currant 'Vitan' with symptoms of FBD (origin plantation Lhenice). The plants were kept in an insect-proof net-house at the Institute of Plant Molecular Biology (IPMB) in České Budějovice. All the plants were tested for BRV by PCR and for ApMV, ArMV, ToRSV, CLRV, AlMV, TSV, SLRSV, TBRV, RpRSV, RBDV and CMV by ELISA (Bioreba AG and Loewe Biochemica commercial kits).

Amplification of BRV nucleic acid and virus detection

RNA was isolated from about 100 mg of leaf tissue using RNeasy Plant Mini Protocol for isolation of total RNA from plant cells and tissues (Qiagen GmbH, Germany). The RNA extracts were used for standard RT-PCR protocol (AccessQuick™ RT-PCR System, Promega Corporation, USA). To amplify the 3′-NTR of RNA-2 of BRV, primers P5/P6 (PCR product of 481 nt), P16/BCP11 (PCR product of 786 nt) were used. The presence of BRV was detected by the RT-PCR method according to Lemmetty et al. (2001) using primers P1/P2 (PCR product of 215 nt; Table 2). Each 50 μl reverse transcription reaction contained 50 pmol of each primer, 1 μl of extracted RNA and 5U AMV reverse transcriptase. The cDNA was synthesized for 45 min at 45°C and reaction was stopped with 2 min denaturation at 95°C. The amplification consisted of 40 cycles with denaturation at 95°C for 30 s, annealing at 60°C (P5/P6), at 51°C (P16/BCP11), at 49°C (P1/P2) for 1 min, extension at 68°C for 2 min and the final step at 68°C for 7 min, and was conducted in a MJ Research thermocycler (Watertown, MA, USA).

Table 2 Primers used in the study

Primer	Sequence 5′ to 3′	Position	Orientation	References
P1	GTAATACGCTGGTGTCTC	6185–6202	Sense	Lemmetty et al. 2001
P2	GAAAGGACATTTCAGACTC	6382–6400	Antisense	Lemmetty et al. 2001
P5	AAACCAGACCCAGGTGAGTG	5655–5674	Sense	Lemmetty et al. 1998
P6	GGACACTTCCATATAAGTCGGC	6114–6135	Antisense	Lemmetty et al. 1998
BCP11	ATTTCGAGCTGTATGGTCG	4951–4969	Sense	Lemmetty et al. 2001
P6	CTCGGAAGCAGTAGACCT	5720–5737	Antisense	Lemmetty et al. 2001

Sequence analysis

PCR products of 215 nucleotides (nt) generated by amplification with primers P1/P2 (position 6185–6400), 481 nucleotides (nt) generated by amplification with primers P5/P6 (nt position 5655–6135, numbering according to accession number AF020051) and products of 786 nt generated with P16/BCP11 primers (nt position 4951–5737), respectively, were obtained from each sample and sequenced directly using a BigDye sequencing terminator kit (PE Biosystems, Warrington, UK). Sequencing was performed in an ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, USA), from both directions. The sequences of BRV isolates used in this study are listed in Table 3. For phylogenetic analysis, sequences were truncated to encompass nt 5156–6102 of the 3′-NTR. The sequences were aligned using Clustal W, phylogenetic analysis was done with DNADIST and NEIGHBOR programmes of the PHYLIP package (Felsenstein 1989). The secondary structure of the 3′-NTR end was computed with the RNA fold via the web service: http://www.tbi.univie.ac.at/~ivo/RNA/.

Searching for the natural host of BRV

Leaf samples from herbaceous plants (weeds) growing between the rows of shrubs and trees growing at the hedge margin of an old plantation of the black currant ‘Karlštejnský dlouhohrozen’ with symptoms of the R-form of BRD at location Chrastiny, South Bohemia were collected in May 2005. One mixed leaf sample of three plants of each species *Acetosa pratensis*, *Achillea millefolium*, *Alchemilla vulgaris*, *Cirsium arvense*, *Corylus avellana*, *Daucus carota*, *Geum urbanum*, *Leontodon autumnalis*, *Leontodon hispidus*, *Prunus domestica* subsp. *domestica*, *Quercus robus*, *Potentilla reptans*, *Rumex obtusifolius*,

Table 3 Published and unpublished accession numbers of *Blackcurrant reversion virus* used in this study

Currant	Cultivar	Origin	AC number	Symptoms	References
Black	line 1873–01E1	CAN	AF321568	E-form	Lehto et al. 2004
Black	P9/5/1	FIN	AF321566	R-form	Lehto et al. 2004
Black	unknown	FIN	AF321571	R-form	Lehto et al. 2004
Black	unknown	FIN	AF321572	R-form	Lehto et al. 2004
Red	wild	FIN	AF321573	galled plant	Lehto et al. 2004
Black	Silvergieter	NZ	AF321565	E-form	Lehto et al. 2004
Black	Bona	POL	AF321569	R-form	Lehto et al. 2004
Black	Bona	POL	AF321570	R-form	Lehto et al. 2004
White	Blanka	POL	B 18	not reported	Malinowski et al. (unpublished)
White	Blanka	POL	JOR 10	not reported	Malinowski et al. (unpublished)
White	Blanka	POL	JOR 11	Not reported	Malinowski et al. (unpublished)
Red	Jonkheer vT	POL	Jv 7	Not reported	Malinowski et al. (unpublished)
Black	unknown	POL	Kal 28	Not reported	Malinowski et al. (unpublished)
Black	Yubileinaya	RUS	AF321567	Reverted plant	Lehto et al. 2004
Black	Sunderbyn	SWE	AF321564	Reverted plant	Lehto et al. 2004

Sambucus nigra, *Taraxacum officinale*, *Urtica dioica*, *Veronica persica* was tested for the presence of BRV by RT-PCR using the primers P1/P2.

Mechanical transmission of BRV to herbaceous hosts

Five plants of black currant 'Karlštejnský dlouhohrozen' (marked A–E) with symptoms of the R-form of BRD were collected in May 2003 at the location Chrastiny, dug out, potted and transferred to the IPMB. In spring 2004 symptomatic first leaves of each plant were homogenized in 1:10 ratio with 0.1 M phosphate buffer pH 7.5 containing 1% of nicotine. The extract was rubbed by a glass rod on carborundum-dusted first true leaves of herbaceous test plants, which were sensitized in a dark cold room for one day before inoculation. Fifteen plants of *Cucumis sativus*, *Lycopersicon esculentum*, *Datura stramonium*, *Chenopodium quinoa*, *Spinacea oleracea*, *Petunia hybrida*, *Calendula officinalis*, *Physalis floridana*, *Phaseolus vulgaris*, *Nicotiana glutinosa*, *Nicotiana. aucalis*, *N. occidentalis* 37B, *Nicotiana rustica*, *N. tabacum* cv. *Xanthi* were inoculated, maintained in a greenhouse at about 21°C and observed for symptoms for eight weeks. Samples were taken from indicator plants displaying symptoms, homogenized in the same inoculation buffer and inoculated onto *N. glutinosa*, *N. aucalis*, *N. occidentalis* 37B, *N. rustica*, *N. tabacum* cv. *Xanthi*, *P. floridana*, *C. quinoa* for the virus passage and maintaining of the isolates. The presence of BRV in symptomatic plants was verified by PCR, using primers P1/P2. The experiment was repeated in 2005.

Results

During monitoring of BRV at 21 locations in the Czech Republic only the R-form of the virus causing blackcurrant reversion disease was found in one plantation, two germplasm collections and one home garden. Isolates from two locations were involved in this study. BRV was routinely detected with RT-PCR and primers P1/ P2 in all symptomatic plants with BRD and FBD (Table 1). All the plants tested positive for BRV by PCR and negative for ApMV, ArMV, ToRSV, CLRV, AlMV, TSV, SLRSV, TBRV, RpRSV, RBDV and CMV by ELISA (Bioreba AG and Loewe Biochemica commercial kits). The plants marked as 'Viola 5' and 'Viola 100' grafted in 1999 by scions from red currant 'Vitan' with symptoms of FBD (origin plantation Lhenice) exhibited symptoms of the R-form of BRD, respectively. The plant marked 'Viola 5' exhibited typical flower malformation and leaf deformation in 2002 and the symptoms remained unchanged until 2006. The second plant marked 'Viola 100' revealed the first symptoms in 2005, although the virus was detected by PCR in 2004.

The comparison of isolates from red currant 'Vitan' and black currant 'Viola 100' showed more than average sequence identity (98.3%) of the sequences (AC: DQ450978, DQ450975) – only 36 of 1,063 nt were different. Results of this experiment suggest that BRV is associated with both diseases and at least some isolates from red and white currants could be transmitted to black currant.

The 3′-NTR of RNA2 genomic segment of BRV was exceptionally long (above 1,360 nt) in comparison to related comoviruses, and was efficient in forming highly stable secondary structures (Fig. 1). We found that most of the nucleotide changes in one position of the 3′-NTR were compensated with changes in another position to recover the complementary pairing in hairpins and stability of the structure.

The average sequence identity between BRV isolates was about 93.8%. In general, about 25.4% of positions could vary. The substitutions were evenly distributed along the NTRs and the longest stretch of 100% sequence identity in all isolates was therefore only 26 nt long. We found a close sequence relatedness among the Czech isolates A, D, E, from 'Karlštejnský dlouhohrozen' (98.5–99.3% identity) and also the isolates 11, 12, 14 from 'Black Smith' imported from Slovakia to the germplasm collection in Holovousy and grown in one location (96.5–97.5% identity). The exception was 'Black Smith' isolate 10, originating from the same locality as isolates 11 and 12, but with only 92.8 and 93.7% identity. This suggests that isolate 10 may have originated from a different source of infection.

Although the isolates originated from plants infected with FBD or BRD, longer motives or single nucleotides specific either for FBD or for BRD have not been identified. The number of variable positions of 18 BRV–R isolates was 208, the number of variable positions of 12 BRV in FBD plants was 177 on identical sequence length and the distribution of variable positions was highly similar.

Four branches, each consisting of six to nine isolates, occurred on the phylogenetic tree created

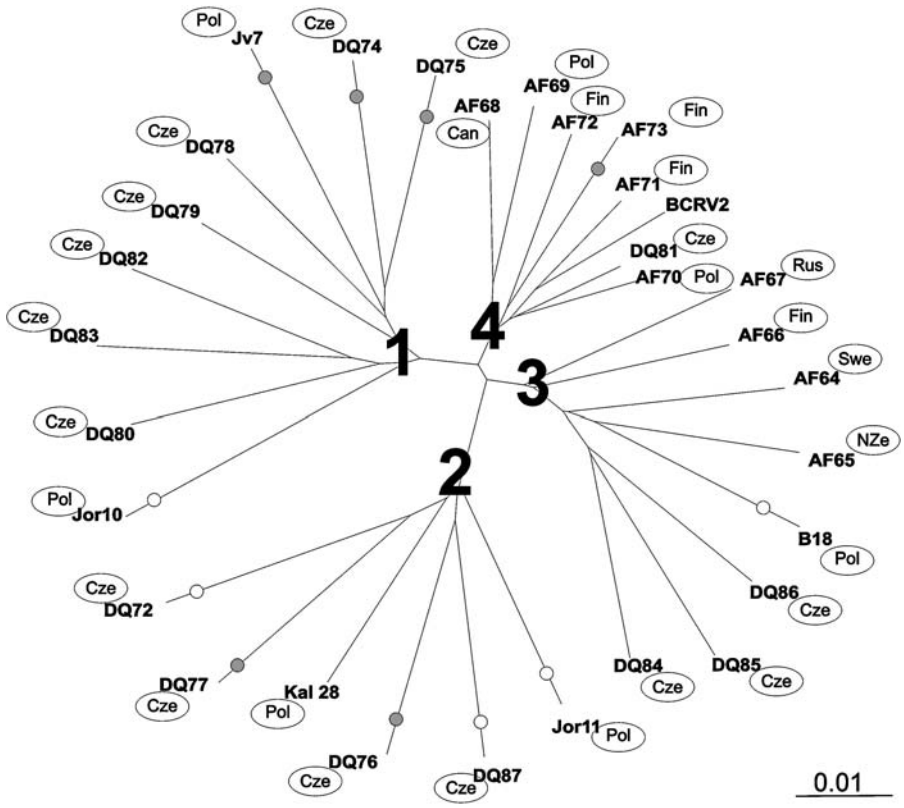

Fig. 2 Phylogenetic analysis of the nucleotide sequences of 31 *Blackcurrant reversion virus* isolates using DNADIST and Neighbor. The *bar* shows the number of substitutions per base. The last two digits of AC numbers are shown for clarity. Isolates from *red* and *white* currants are marked with *filled* and *open circles* on the branch, respectively.

after analysis of all available BRV sequences (Fig. 2). No correlations with the geographic origin is visible on the tree – the Czech and Polish isolates which are the most frequent in the comparison, occurred in all four branches and isolates from Finland occurred in two branches. The isolate AF321568 from Canada was closely related to isolate AF321569 from Poland with 96.6% sequence identity. The isolate AF321565 from New Zealand was closely related to the isolate from Poland B18 with 99.2% sequence identity. We also found no correlation between the host and the topology of the tree: Most of black currant isolates occurred in branches 3 and 4, but also occurred in branches 1 and 2. Only one white currant isolate B18 and one red currant isolate AF321573 occurred in branches 3 and 4, respectively.

Searching for the natural host of BRV

Our search for the possible natural host of BRV was not successful. All plants growing inside or closely to the severely BRV infected plantation of black currant in Chrastiny tested negative for BRV.

Mechanical transmission of BRV to herbaceous hosts

BRV was successfully transmitted from black currant cv. 'Karlštejnský dlouhohrozen' plants C, D (Fig. 3) and E. We observed local necrotic lesions on *N. occidentalis* 37 B (Fig. 4), *N. tabacum* cv. Xanthi and systemic mosaic on *N. occidentalis* 37 B (Fig. 5) and

Fig. 3 Flower malformation symptoms of R-form of reversion in *black* currant cv. Karlštejnský dlouhohrozen, plant D

Fig. 4 Local necrotic lesions on the leaf of *Nicotiana occidentalis* 37 B after transmission of BRV from black currant cv. Karlštejnský dlouhohrozen, plant C

N. tabacum cv. Xanthi. By PCR and sequencing the PCR product we confirmed BRV in local necrotic lesions on *N. occidentalis* 37 B, systemically infected leaves of *N. occidentalis* 37 B and *N. tabacum* cv. Xanthi. BRV sequences in herbaceous hosts were obtained by primers P1/P2. The latter species is a new host for BRV. Repetition of the experiment the next year was not successful, as passage of the virus in test plants, resulted in loss of infectivity of the virus after the second passage from tobacco plants.

Discussion

BRV RT-PCR detection in this study confirmed our previous findings of the presence of BRV in FBD-affected bushes of red and white currant (Přibylová et al. 2001). The rare occurrence of the R-form of BRD in the Czech Republic found only at five locations from 21 is in sharp contrast with the frequent occurrence of full blossom disease in red and white currants, which we reported recently (Špak et al. 2006). This may be a result of long-term use of certified black currant propagation material, controlled for the R- form of BRD by visual inspections and immediate removal of symptomatic plants in the field by growers, whereas FBD was neglected in red and white currants. Despite searching for the E- form symptoms described as "decrease the hairness of the sepals, so that flower buds are almost glabrous and appear brightly coloured, compared with the grey, downy appearance of normal buds" (Adams and Thresh 1987), we did not find black currant plants with similar symptoms in the Czech Republic.

Using specific BRV primers, we amplified practically the whole BRV RNA2 3′-NTR in 15 black, red and white currant plants showing symptoms either of BRD or FBD. BRV was also detected in two black currant plants of 'Viola' grafted in 1999 by scions taken from 'Vitan' with FBD symptoms and exhibiting the R-form of black currant reversion disease symptoms. This experiment proved the transmissibility of BRV isolates from red currant to black currant. It supports our hypothesis that BRV isolates associated with the two diseases do not differ substantially and that BRV may also be associated with FBD.

Malinowski et al. (unpublished) in 2002 found BRV in red currant cvs 'Jonkheer van Tets', 'Tatran' and 'Maraton' and white currant 'Blanka'. Severe symptoms were observed resembling FBD on some but not all BRV infected plants of 'Blanka' and 'Johkheer van Tets'. In addition, BRV was detected in nearly all 'Blanka' plants apart from the presence or lack of the

Fig. 5 Systemic infection on the leaf of *Nicotiana occidentalis* 37 B after transmission of BRV from black currant cv. Karlštejnský dlouhohrozen, plant E

full blossom symptoms. It was therefore suggested that BRV is not associated with 'full blossom of red currant' and that synergistic effects of BRV infection together with unknown factors could not be excluded as a cause of 'full blossom' disease. We confirm these observations by Malinowski et al. as typical FBD symptoms. Erratic distribution and low concentration of BRV in shrubs (Latvala et al. 1997) make the proof of correlation difficult between the presence of the virus and typical BRD or FBD symptoms. In our long term observations and experiments we found a reasonable variation of FBD and also BRD symptoms in individual years and cultivars (Špak et al. 2006). For example, the severe flower malformation, typical for the R-form was visible in cv. Black Smith only in some years. Indeed, sampling of different parts of symptomatic plants in spring and sometimes repeated PCR tests were necessary to detect the virus in our experiments. Therefore we suggest that our results and those of Malinowski et al. (unpublished) are correlated, but our different interpretation results from a more detailed study, including repeated testing of plants for the presence of BRV by PCR and other viruses by ELISA. Moreover, it should be taken into consideration that the E-form of BRV could occur in both black and red currants, without causing any symptoms of BRD or FBD, or could cause symptoms in one, but not in the other species. Therefore more efforts should be concentrated in the future on the comparison of BRV sequences causing the E- and R-form of reversion at the molecular level.

Recently, Karetnikov et al. (2006) presented data on the occurrence of the translational enhancer on the 3′-NTR of BRV RNA2; the stem-loop forming motif is located closely downstream from the capsid protein termination codon and interacts with the complementary motif on the 5′-NTR of RNA2. Surprisingly, seven of the Czech isolates presented here (two from red currant plants, five from black currant plants) posses substitutions abolishing correct pairing with the complementary motif. As a non-perfect pairing decreases the translational efficiency (Karetnikov et al. 2006) we could speculate that the translation of the virus could be changed, or a mutation restoring correct pairing could occur in the complementary position.

Results from the sequence analysis related to the host and geographic origin are consistent with those by Lehto et al. (2004). The long 3′-NTRs of BRV RNAs are highly conserved between virus isolates representing different phenotypes and geographic origins. However, data in databases are sometimes misleading. For example, the Canadian isolate AF321568 mentioned in the GenBank originates from Scotland, and occurs in Canada only as BRD-reference material in quarantine. We substantially extended the analysis by adding 15 new BRV sequences, including rare sequences from red and white currants, originating from our experiments and those of Malinowski et al. The absence of sequences from red and white currants in the literature and similarly like information on the transmissibility of reversion from black to red currants and vice versa, is probably due to the major importance of cultivation of black currants for processing in countries with significant *Ribes* production.

BRD was originally described by Ritzema Bos (1904). Attempts with mechanical transmission of the causal agent of BRD onto a herbaceous host were unsuccessful for decades. Lemmetty et al. (1997) reported the first successful transmission of BRV by mechanical inoculation onto numerous herbaceous host plants; despite many attempts the virus was transmitted from black currant cuttings infected with the R-form of reversion to *Chenopodium quinoa* only once. From *C. quinoa* the virus was sap-transmissible to many herbaceous test plants; however infection was proven by back-inoculation to *C. quinoa* only, as no other detection method was available at that time. In our experiments we are the first to confirm the presence of BRV in lesions and systemic infection of *N. occidentalis* 37 B by PCR. Also we found *N. tabacum* cv. *Xanthi* as a new experimental host of the virus. When comparing our results with those obtained by Lemmetty et al. (1997) we could speculate that the transfer of shrubs from the field to pots in our experiments, similar to the rooting of young cuttings by Lemmetty et al. (1997), induced a higher concentration of the virus in plants, which resulted in successful transmission of the virus. Nevertheless our experiments confirmed the difficulty in transmission of the virus from *Ribes* reported by many authors (Jones 2000).

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