Acta virologica **60**: 196 – 200, 2016 doi:10.4149/av_2016_02_196

Detection of herbaceous-plant pararetrovirus in lichen herbarium samples

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Received July 23, 2015; revised September 4, 2015; accepted April 19, 2016

Summary. – Cauliflower mosaic virus (CaMV) – a plant pararetrovirus that naturally causes diseases in *Brassicaceae* and *Solanaceae* plant hosts worldwide – has been detected by PCR for the first time in herbarium samples of *Usnea* sp. lichens. The virus's presence in these lichens did not result in any micro- or macromorphological changes, and the herbarium records were classified as representative for the distinct species. Sequence analyses classified all the detected viruses into one lineage of CaMV isolates. We have shown here that herbarium samples could be a good source for virus study, especially where a longer time span is involved.

Keywords: cauliflower mosaic virus; dsDNA; pararetrovirus; persistent virus

Introduction

Lichen thallus may be inhabited by a plethora of such animals as tardigrades, rotifers, nematodes (Sohlenius *et al.,* 2004), and microarthropods (Materna, 2000) along with protozoans (Bamforth, 2004), epiphytic fungi (Lawrey and Diederich, 2003; Suryanarayanan *et al.,* 2005), and a wide range of bacteria (Bates *et al.,* 2011; Muggia *et al.,* 2013). In addition to the lichen-forming associations between symbiotic fungi and a photosynthesizing organism, a number of endolichenic fungi grow in association with algal photobionts inside asymptomatic lichen thalli and resemble fungal endophytes of plants (Arnold, 2007; He and Zhang 2012). Despite the complexity of the lichen biome, until recently no virus has been described in lichen fungus, in its photobiont, or in any other accompanying organism while performing control or balancing functions. Nevertheless, more than 200 viruses have been described in fungi (89 complete genomes and about 120 partial genomes; GenBank, Mar. 2015), as have been numerous cyanophages in cyanobacteria, (14 complete genomes, about 400 partial genomes), and unique large DNA viruses in algae (40 complete genomes, about 200 partial genomes).

There are assumed to be no viruses of angiosperms that are able to infect primitive nonvascular plants (e.g. mosses) under natural conditions, and no plant virus has been isolated from a nonvascular plant growing in the wild. Nevertheless, tobacco mosaic virus and cucumber green mottle mosaic tobamovirus' antigens have been detected by ELISA in arctic *Barbilophozia* and *Polytrichum* mosses (Polischuk *et al.,* 2007). In a laboratory experiment, it was proven that *Physcomitrella patens* moss supported multiplication of Tomato spotted wilt virus (Hühns *et al.,* 2003). In addition, highly effective *Agrobacterium tumefaciens* transformation of the lichen fungus *Umbilicaria muehlenbergii* performed by mixing and cocultivation opens the possibility for how lichen could be transformed or infected in the wild (Park *et al.,* 2013). Recently, plant-infecting Apple mosaic virus (a segmented virus with positive ssRNA genome from the genus Ilarvirus) and putative cytorhabdovirus (with negative ssRNA genome) have been detected in *Usnea*, *Cladonia, Pseudevernia, Xanthoria*, and *Lasallia* sp. (Petrzik *et al.,* 2014). Furthermore, complete genome of plant CaMV (a pararetrovirus with a circular dsDNA genome) has been sequenced from lichen photobiont *Trebouxia* sp. and CaMV was detected in free-living fresh water algae from the *Chlorellales*, *Prasiolales*, *Microthamniales*, and *Oocystales* orders, thus indicating a wider range of hosts for this virus than had been previously assumed (Petrzik *et al.,* 2015).

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Abbreviations: CaMV = cauliflower mosaic virus

CaMV was the first plant virus to be discovered to contain DNA as genetic material and the first virus to be sequenced completely (Franck *et al.,* 1980). It is disseminated worldwide wherever its hosts are planted and is transmitted by several aphid species. Transmission by other vector types or by pollen has never been reported in nature, although CaMV can readily be transmitted by infected sap to a mechanically injured host plant (Blanc *et al.,* 2001). Members of the *Brassicaceae* (cabbage family) have been reported as systemic plant hosts, but B29, W260, Japan-S, and NY8153 CaMV isolates are able to infect also the *Solanaceae* (nightshade family) species *Nicotiana clevelandii* (wild Cleveland's tobacco) and *Datura stramonium* (Jimson weed) (Haas *et al.,* 2002). Sequence analysis of a large set of CaMV isolates from geographically distant locations recently showed that this virus probably spread from a single population in Turkey around 400–500 years ago and is known in four geographically distributed lineages (Yasaka *et al.,* 2014).

In this paper, we describe for the first time *Usnea* lichens as new hosts of CaMV and show herbarium records to be a suitable source for virus molecular analysis.

Table 1. Lichen samples used in this study

Lichen	SM OP catalogue No.	Year of collection	Country/place of origin	Sequence length (nt)	Localization on CaMV genome*	GenBank Acc. No.	Blastn E-value/ mostly related sequence %	Blastx E-value/ mostly related sequence %	CaMV presence
Usnea amaliae	10734	1961	Uruguay, Maldonado	–	–	–	–	–	–
Usnea barbata	134472	1848	North Wales	–	–	–	–	–	–
Usnea cavernosa	142754	1952	USA, New Mexico, Spirit Lake	–	–	–	–	–	+, NS
Usnea cladocarpa	164797	1978	Brasilia, Serra da Mantiqueira	349	908–1256	KP292822	0.0 KF550287 100%	1e-82 KF550287 100%	+
Usnea fasciata	117262	1946	Falkland Islands, Port Stanley	–	–	–	–	–	+, NS
Usnea gigas	162660	1937	Zaire, Yangambi	–	–	–	–	–	–
Usnea hawaiiensis	102211	1964	USA, Hawaii, Lanai	349 336	908–1256 7014–7349	KP292820 KP292824	3e-178 KF550287 99% 3e-174 KF550287 99%	2e-80 KF550287 98% 3e-70 KF550287 99%	+
Usnea longissima	170728	1986	Abkhazia, Sukhumi	748	3616–4363	KP292819	0.0 KF550287 99%	2e-180 KF550287 100%	+
Usnea marocana	125543	1970	France, Port-Cros	–		–	–	–	–
Usnea osseina	123586	1969	Tanzania, Uluguru Mts.	386	2318–2703	KP292823	0.0 KM502557 100%	5e-66 KM502557 100%	+
Usnea scabrata	100726	1959	Canada, Saskatchewan, Beaver Lake	349	908–1256	KP292821	3e-178 KF550287 99%	9e-81 KF550287 98%	+
Usnea simplicissima	150412	1976	Tanzania, Kilimanjaro	–	–	–	–	–	–
Usnea squarrosa	168692	1975	Indonesia, Java	–	–	–	–	–	+, NS
Usnea sulphurea	124272	1967	Iceland, Tungnafellsjokull	744	3617–4363	KP292818	0.0 KF498706 99%	8e-174 KF498706 99%	+
Usnea tanzanica	147838	1972	Tanzania, Rungwe	–	–	–	–	–	–
Usnea welwischiana	153490	1968	Kenya, Tsavo	–	–	–	–	–	–

*Numbering according to isolate 219-1d sequence, GenBank Acc. No. KF550287. KM502557 = CaMV isolate from *Graesiella vacuolata*, KF498706 = CaMV isolate CB1 from cauliflower, NS = not sequenced.

Material and Methods

Material. Lichen samples collected between 1848 and 1986 at different locations were obtained from Silesian Museum's lichen herbarium, Opava, Czech Republic (SM OP) (Table 1). The lichens had been stored in paper envelopes and maintained in a temperature- and humidity-controlled location. All the samples were analysed twice, using different DNA isolation methods, at different time.

Nucleic acid isolation and transcription. Before extraction, 100 mg of lichen thallus was pulverized in liquid nitrogen. First, a DNA plant kit (Macherey Nagel, Germany) was used according to the manufacturer's recommendation. This approach included degradation of contaminating RNA with 15 min of RNase treatment. DNA was ethanol precipitated, then dissolved in 20 μl of sterile water. Second, the DNA was isolated using a Wizard Magnetic DNA plant system kit (Promega Corporation, USA) and dissolved in 20 μl of sterile water. Total RNA was isolated from the same amount of sample using Plant RNA mini spin kit (Macherey Nagel, Germany). Contaminating DNA was on-column degraded with DNase digestion for 15 min. The RNA was ethanol precipitated and dissolved in 10 μl of sterile water. First strand cDNA was synthesized from the total amount of isolated RNA using the SuperScript III First-strand synthesis system (Life Technologies Corp.) according to the manufacturer's protocol.

CaMV screening. Virus screening was performed using five pairs of CaMV-specific primers (Table 2). The primers hybridize to gene I (Ca750 and Ca751), gene IV (Ca355 and Ca356), gene V (Ca470 and Ca476, Ca465 and Ca478), and a 35S promoter (Ca390 and Ca335) (Table 2). For PCR amplification, 2.5 U of DreamTaq DNA polymerase (Thermo Scientific), 2 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 200 nmol/l of each primer, and 1 μl of isolated DNA or 2 μl of cDNA were mixed in a 20 μl reaction and 35 cycles were performed. Amplified products were agarose gel separated, stained with SybrGreen and then visualized under UV light. Bands of expected size were excised, gel extracted using a NucleoSpin Extract II kit (Macherey Nagel, Germany), cloned and sequenced using the BigDye Terminator v3.1 Cycle sequencing kit (Life Technologies, USA). Nucleotide sequences and their *in silico* transcribed amino acid sequences were compared using blastn and blastp with GenBank data.

Results and Discussion

More than 50 herbarium samples 20–100 years old were tested for CaMV presence over the past 3 years in our laboratory. Not all primer pairs used for CaMV screening resulted in a visible amount of amplicons of expected size, however, even though they work with nucleic acid from infected plant host (Fig. 1). Primer pair Ca750/Ca751 producing the shortest amplicon from gene I and pair Ca390/Ca335 for 35S promotor sequence were the most effective, amplifying three and two templates, respectively. On the other hand, Ca355/Ca356 and Ca390/Ca335 pairs amplified one template each and Ca465/Ca478 produced no visible product with any sample. To date, CaMV was detected with at least one pair of primers in 9 of 16 *Usnea* samples (Table 1). The oldest CaMV-positive sample has been collected 62 years ago. Blastn and blastx comparison of those nucleotide sequences obtained with GenBank identified the Cauliflower mosaic virus nature of these sequences with the best expected value of all alignments from that database sequences (E-value close to zero) (Table 1). The CaMV 219-1d isolate from *Trebouxia* sp. lichen photobiont was recognized as the most closely

Table 2. CaMV detection primers

Primer	Sequence	Localization on CaMV genome*	Annealing	Expected product
Ca750	5'-CAGCCAAAGGTAATCTCGCA-3'	864–1256 gene I	55°C	393 bp
Ca751	5'-CATTGTTTCCTATTTGAAGACTATTACC-3'			
Ca355	5'-ACCAAATTATTGATCTAACC-3'	2318–2739 gene IV (capsid protein)	45°C	422 bp
Ca356	5'-AAGATAGTCTTCTCTATTGG-3'			
Ca470	5'-TTTAACCTTGATAGCTTTGCTTG-3'	3581–4363 gene V	50°C	783 bp
Ca476	5'-TAGAATACAAAGAAGAGGAAGAAGA-3'			
Ca465	5'-AGCAATGACAAAGAGACACTGG-'3	5387–6053 gene V-VI	55°C	667 bp
Ca478	5'-TAGCCTTGTCCCAGTCTGAAC-'3			
Ca390	5'-AGGACCTAACAGAACTCGCCG-3	7012–7392 promotor	50°C	381 bp
Ca335	5'-TAGAGGAAGGGTCTTGCGAAGG-3'			

*Numbering as in Table 1

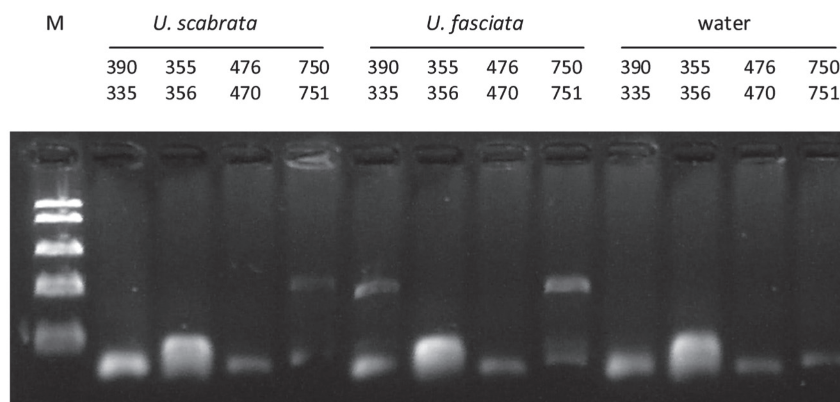

Fig. 1

CaMV detection in *Usnea scabrata* 100726 and *U. fasciata* 117262 with primers Ca390/Ca335 (381 bp product expected), Ca355/Ca356 (422 bp), Ca476/Ca470 (783 bp), and Ca750/Ca751 (393 bp)
M – FastRuller Middle Range DNA ladder of 5 kbp, 2 kbp, 850 bp, 400 bp, 100bp (LifeTechnologies).

related (more than 98% nt or amino acid identity) to *Usnea cladocarpa*, *U. hawaiiensis*, *U. longissima*, and *U. scabrata* samples, sequence from *U. osseina* was identical with CaMV from *Graesiella vacuolata* algae. Sequence from *U. sulphurea* was 99% identical to that of CaMV isolate CB1 from cauliflower. The sequences were deposited in GenBank under AC numbers KP29218-24. We performed an infectivity test and mechanically inoculated Chinese cabbage with homogenate of *U. fasciata*, *U. longissima*, and *U. sulphurea*. No disease symptoms developed and CaMV was not detected in the plants. We concluded that the viral genome had most probably been damaged during lichen storage and that the lichens no longer contained the virus in infectious form.

It has been reported that samples up to 35 years old have been used routinely for successful DNA extraction (Grube *et al.*, 1995) and that rDNA was amplified from herbarium samples of lichens more than 150 years old (Hawksworth, 2013). To obtain viral DNA of the highest possible quality from lichens, two different protocols were used: the Wizard system using paramagnetic particles (which is recommended for isolation of DNA from leaves and seeds) and the NucleoSpin system based on the established CTAB/SDS lysis method (which is recommended for isolation from plants and fungi). Both methods resulted in amplifiable DNA from the *Usnea* samples we had, but DNA from the Wizard system could be amplified easily with more primers.

In the case of the *Usnea* herbarium samples, we did not detect CaMV from transcribed RNA of any CaMV-positive sample. We could assume that a) the viral RNA transcript was more prone to degradation than was the genomic DNA; b) not all photobionts are identically suitable for hosting CaMV, thus resulting in notable differences in virus fitness and replication; c) such environmental conditions as low or high temperature, water content, and others could influence the cohabitation of lichen with the virus; and d) nothing is known about virus acquisition by the lichens, duration of infection, or virus distribution and movement (if any) in lichen thallus.

We may hypothesize that lichens could be accidental hosts of this plant virus. On the other hand, *Usnea* spp. are long-lived lichens and, in this case, they could be regular hosts of CaMV. In accordance with previous findings (Petrzik *et al.*, 2014, 2015), we suggest that lichens could serve as reservoirs for plant viruses, despite the fact that the mechanism of transmission among lichens themselves or among different organisms is not clear, and that herbarium samples constitute a good source for molecular study of viruses.

Acknowledgements. This work was conducted with support from Czech Science Foundation project no. P501/12/1747 and institutional support RVO60077344 from the Biology Centre. The authors thank O. Kubešová for her excellent technical assistance.

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