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Detection of high molecular weight dsRNA persisting in *Dianthus* species

Anita Szegő[1], Endre K Tóth[2], László Potyondi[3], Noémi Lukács[1]*

[1]Department of Plant Physiology and Plant Biochemistry, Faculty of Horticultural Science, Corvinus University of Budapest, Budapest, Hungary, [2]Óbuda Nursery Ltd, Budapest, Hungary, [3]Beta-Kutato Ltd, Sopronhorpács, Hungary

ABSTRACT Cryptic plant viruses are seed-borne dsRNA-viruses, which co-exist life-long with the host plant, without inducing any apparent symptoms. Since growth conditions and the host-virus combination (cultivar, strain, isolate, thermotherapy, etc.) are known to influence virus multiplication, we wanted to find out what effect long-term tissue culturing has on the survival of carnation cryptic virus (CarCV). 21 members of Hungarian *Dianthus* germplasm collection have been aseptically grown for 16 years. Total nucleic acids of these *Dianthus* species and of *Silene vulgaris* were separated by non-denaturing gel electrophoresis and the dsRNA-pattern was visualized by immunoblotting using dsRNA-specific monoclonal antibodies. Genomic dsRNAs of CarCV were detected in *D. caryophyllus*. In four additional species: *D. superbus*, *D. giganteus* *D. gratianapolitanus* and *Silene vulgaris* several dsRNA-species in the same size range as the genomic dsRNAs of CarCV were detected. We also show that three other cryptoviruses, the beet cryptic viruses BCV1, -2 and –3 can persist under *in vitro* conditions. Our results indicate that cryptic viruses are so well adapted to their hosts that they can persist after more than a decade of *in vitro* culturing despite the dramatic change of the environment.
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KEY WORDS

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Cryptic viruses belong to the *Cryptovirus* genus *(Alphacryptovirus* and *Betacryptovirus* subgenus) of the *Partitiviridae* family, and have a small, segmented double-stranded RNA (dsRNA) genome in the size range of 1-3 kbp. Cryptic viruses are widespread in several plant species and may be present in many others without being detected. The reason for this hidden existence is that their presence does not induce any apparent symptoms in the host and, as far as we know, does not lead to economic losses. The concentration of virus particles is usually low. Individual plants of the same species and cultivar may show large differences in virus content and in some cases more than one cryptic virus is present in the same host plant. Although cryptic viruses cannot be transferred horizontally by any of the usual virological methods, seed and pollen transfer them efficiently (Boccardo et al. 1987).

Our group is investigating the genomic organisation and the molecular features of selected cryptic viruses. One of our aims is to understand why the host does not / cannot eliminate parasitic cryptic viruses. Long-term cultivation of *in vitro* propagated plants is a dramatic change of environment compared to the natural life cycle of the plant. In this study we investigate the effects of *in vitro* culture conditions on the presence of cryptic viruses to find out whether seed-transmitted cryptic viruses can survive continuous tissue culturing. The most efficient way to detect cryptic viruses is dsRNA-immunoblotting (Lukács 1994). This method was used in this study to investigate the occurrence of carnation cryptic virus in various *Dianthus* species and to clarify whether cryptic viruses are able to persist after prolonged tissue culturing.

*Corresponding author. E-mail: noemi.lukacs@uni-corvinus.hu

Materials and Methods

Plant material

Eighteen different species of *in vitro* propagated *Dianthus*, two members of the *D. caryophyllus* variety "club" and *Silene vulgaris* from the Hungarian germplasm collection were used to search for the presence of carnation cryptic virus dsRNAs. The tissue cultures were initiated 16 years ago from sterilized seeds to eliminate single-stranded RNA-viruses and have been grown since then on hormone-free Murashige-Skoog (MS) basal medium in tissue culture tubes.

The *in vitro* cultured haploid and diploid *Beta vulgaris* lines were grown at Beta-Research Ltd., Sopronhorpács, Hungary. Diploid shoot cultures were initiated from sterilized seeds and have been aseptically grown for 6-7 years since then. Haploid plants were produced from *in vitro* cultures of ovules removed before anthesis from closed flowers of male sterile plants and have been grown aseptically for the last 5 years (Potyondi and Heszky 1992)

Total nucleic acid purification and dsRNA-immunoblotting

dsRNAs in crude nucleic acid extracts were detected on immunoblots as described by Lukács (1994). J2, a dsRNA-specific monoclonal antibody was used as primary antibody. This antibody specifically recognizes dsRNA independent

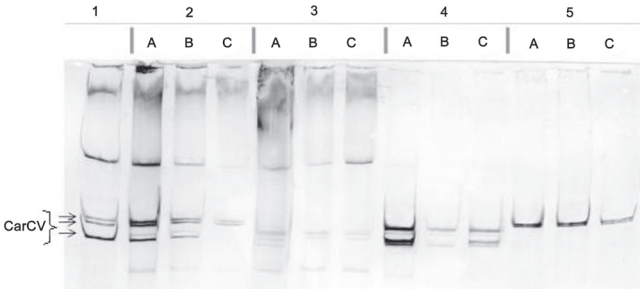

Figure 1. Detection of dsRNA species in *in vitro* plantlets regenerated from meristem tip culture after thermotherapy of carnation plants. Symbols: A, untreated control plants, B and C, shoots regenerated from treated and excised single meristems smaller than 0.8 mm (B) or larger than 0.8 mm (C). (1) *D. caryophyllus*, (2) *D. caryophyllus* 'Grenadin', (3) *D. caryophyllus* 'Chabaud', (4) *D. gratianopolitanus*, (5) *Silene vulgaris*.

of its nucleotide composition and sequence and does not cross-react with short double strand helices present in single-stranded RNAs (Schönborn et al. 1991). Fifty µg total nucleic acids were loaded onto each lane of the non-denaturing 5% PAA-TBE gel.

Thermotherapy and meristem isolation

Cultures were maintained at 30°C in a growth room for 5 days, followed by 5 weeks at 36°C. After excising the meristem (0.2-2 mm), meristems were transferred to MS medium containing kinetin and α-napthyl-acetic acid and shoots were regenerated for 6 weeks (Murashige and Skoog 1962).

Results and Discussion

Carnation cryptic virus (CarCV) can be detected in *in vitro* cultured *Dianthus species*

In virions purified from CarCV-containing plants three major dsRNA (MW 1.04×106, 0.95×106 and 0.84×106 Da, respectively) and a minor species (0.88×106 Da) have been described (Lisa et al. 1981a). The minor dsRNA was not present in all preparations (Lisa et al. 1981b). To find out whether CarCV is able to survive under the selection conditions of *in vitro* culturing we investigated different carnation species (Table 1) after 16 years of continuous *in vitro* cultivation. We found that dsRNAs of the same molecular mass as in CarCV are present in *D. caryophyllus* 'Chabaud' and 'Grenadin', but not every sample contained all four CarCV bands. The most prominent difference was seen in *D. caryophyllus* "Chabaud" where only the two smallest (0.95×106 and 0.84×106 Da) dsRNA fragments were detected (Figure 1). Although the presence of the larger fragments at concentrations below the detection limit cannot be excluded, we conclude that in this case the relative concentration of genomic fragments was altered after prolonged *in vitro* cultivation. Further experiments should clarify the effect of such alteration on the virus cycle. In addition to *D. caryophyllus,* dsRNAs were found in at least six other species. Four of them: *D. superbus*, *D. giganteus* *D. gratianapolitanus* and *Silene vulgaris* contained dsRNAs in the same size range as CarCV dsRNAs, but whether these dsRNAs are related to CarCV has not vet been proved. Two further species, *D. plumarius* and *D. chinensis* also exhibit prominent dsRNA bands but whose molecular mass is totally dissimilar to that of CarCV. The origin of these bands ranging from 1100 to 3600 bp is not known (part of the results are shown in Fig. 1, lanes A).

Influence of thermotherapy and meristem excising in *in vitro* cultured *Dianthus* species

Heat treatment or thermotherapy is a frequently used method for efficiently eliminating viruses from plants. However, it does not appear to work when applied to cryptovirus containing plants: After the treatment genomic dsRNAs of CarCV were still detected in *D. caryophyllus* and the uncharacterized dsRNA-species in *D. gratianopolitanus* and *Silene vulgaris* were also not eliminated (Fig. 1).

Beet cryptic virus (BCV1, -2 and -3) dsRNA persist in *Beta vulgaris* ssp. *vulgaris* after 6-7 years of *in vitro* culturing

To find out whether the same behavior is exhibited by other cryptic viruses we investigated tissue cultures of *B. vulgaris* ssp. *vulgaris*. In beet plants three kinds of probably unrelated cryptic viruses may occur and even coexist, *Beet Cryptic Vi-*

Table 1. Occurence of dsRNA-species in *in vitro* propagated carnation plants.

Species	dsRNA	Species	dsRNA
D. caryophyllus	+	*D. sylvaticus*	-
D. caryophyllus 'Grenadin'	+	*D. armeria*	-
D. caryophyllus 'Chabaud'	+	*D. giganteus*	+
D. chinensis	+	*D. deltoides*	-
D. giganteiformis	-	*D. knapii*	-
D. anatolicus	-	*D. carthusianorum*	-
D. superbus	+	*D. gratianopolitanus*	+
D. serotinus ssp. *regis-stephani*	-	*D. plumarius ssp. praecox*	+
D. fischeri	-	*D. gallicus*	-
D. pontederae	-	*D. monspessulanus*	-
Silene vulgaris	+		

rus 1, -2 and -3. All three known BCVs could be detected in the cultures after 6-7 years of culturing (results not shown). It was, however, also evident that many individual cultures were free of BCV dsRNA, although we know from many experiments that most individual sugar beet plants harbour at least one BCV.

Taken together our results indicate that cryptic viruses are so well adapted to their hosts that they can persist for 16 years even under the artificial conditions of tissue culturing and that they can even survive thermotherapy. Therefore, when generating virus-free plants by micropropagation it should be taken into account that cryptic viruses may survive and be present even in aseptically grown cultures. In addition, the undetected presence of cryptic viruses may invalidate tests to confirm that plants are virus-free , if these are based upon the isolation of high molecular weight dsRNA or of virus particles.

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