The DNA form of a retroviroid-like element characterized in cultivated carnation species

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Carnation small viroid-like RNA (CarSV RNA) is a small (275 nt), circular molecule which is unique among plant viroid-like RNAs in having a tandemly repeated homologous DNA. This DNA form was found fused to DNA sequences of carnation etched ring caulimovirus (CERV) in certain Spanish carnation plants. The observation of a growth abnormality consisting of extensive shoot proliferation in cultivated carnations in Hungary prompted the molecular analysis of these plants, in which both CarSV RNA and DNA forms were detected. Several CarSV DNA sequences were characterized in various Dianthus caryophyllus cultivars which were symptomless or showed different symptoms. CarSV DNA forms showing minor sequence heterogeneities and deletions occurred in the same plant. Unit-length CarSV DNA sequences were proven to accumulate in the plant cell nucleus. The plants studied here were not infected by any of the viruses (including CERV) or other cellular pathogens described previously in carnation.

The complete nucleotide sequence of carnation small viroid-like RNA (CarSV RNA), a molecule with self-cleaving hammerhead structures in its plus (+) and minus (−) strands, was first reported in 1992 (Hernández et al., 1992). The structure and sequence of the CarSV RNA (+) hammerhead show extensive similarities with the genomic satellite transcript of the newt Notophthalmus viridescens (Darós & Flores, 1995b) which, together with the schistosome satellite DNA transcript, are the only animal RNAs known to have a hammerhead structure (Epstein & Gall, 1987; Ferbeyre et al., 1998).

In addition to the predominant 275 nt CarSV RNA, several coexisting RNA forms with duplications and deletions were observed and have been divided into groups. Each group showed heterogeneities in the exact positions of deletions or duplications. In addition to major deletions and duplications, minor sequence heterogeneities were observed between the cDNA clones (Darós & Flores, 1995a).

Regarding the biological nature of CarSV RNA, initial transmission assays suggested that it could be a viroid (Boccardo et al., 1988), but further attempts in this direction did not confirm these results (Hernández et al., 1992). On the other hand, analysis of the distribution of trimer sequences indicated that CarSV RNA might be related to viroid-like satellite RNAs (Daroş & Flores, 1995b). This curious feature of CarSV RNA, together with the similarity of the CarSV RNA hammerhead structure to that of the newt RNA, which is the transcript of the tandemly repeated 330 bp satellite II DNA, suggested that a corresponding DNA form of CarSV RNA might also be present. The existence of these DNA forms was confirmed and the term retroviroid-like element was proposed for the CarSV RNA/DNA system, which so far is the only such system described (Darós & Flores, 1995b).

Characterization of CarSV DNA was performed by using total DNA of CarSV RNA-infected plants. The DNA was fractionated by gradient centrifugation and CarSV DNA was found in fractions containing low molecular mass DNA (Darós & Flores, 1995b). Several forms were detected consisting of a variable number of tandemly repeated CarSV DNA monomers joined to other sequences homologous to regions of the DNA of carnation etched ring caulimovirus (CERV), forming an extrachromosomal element (Darós & Flores, 1995b). These data support the idea that CarSV DNA might have emerged by the CarSV RNA making use of a reverse transcriptase, most likely that encoded by CERV. Plants containing the extrachromosomal element and negative for CERV by immunoassay also existed, so the element might replicate autonomously and be vertically transmissible (Darós & Flores, 1995b).

The presence of CarSV RNA was initially associated with a stunting syndrome in Italian carnations (Boccardo et al., 1988). However, the correlation was relatively low and was
Fig. 1. CarSV DNA and RNA sequences detected in different *D. caryophyllus* cultivars. (A) CarSV RNA (Hernández et al., 1992). (B) CarSV RNA sequence variation (Hernández et al., 1992). (C) cv. Praline, CarSV cDNA. (D) cv. Newton, CarSV cDNA. (E) cv. Tanga, CarSV cDNA. (F) cv. Tanga, CarSV DNA. (G) cv. Praline, CarSV cDNA. (H) cv. Praline, CarSV DNA. (I) cv. Praline, CarSV DNA. (J) cv. Praline, CarSV DNA. (K) cv. White Castellaro, CarSV DNA. (L) cv. White Castellaro, CarSV DNA. (M) cv. Domingo, CarSV DNA. (N) cv. Domingo (symptomless), CarSV DNA. Sequences (C)–(F) have been reported previously (Palkovics et al., 1997).
not substantiated in further analyses with Spanish material (Hernández et al., 1992).

The problem of a growth abnormality of carnation plants, consisting of shoot proliferation, has also been observed in Hungary. Since none of the known pathogens proved to be responsible for the symptoms, these plants were analysed and both CarSV RNA and DNA forms were found, although they were also detected in symptomless carnation plants (Palkovics et al., 1997). We report here our attempts to define the nature of different sources of the CarSV DNA element to facilitate detection and further studies of this putative pathogen.

Carnation plants provided by the Óbuda Horticulture Co. were free of the usual pathogens in carnation (including CERV) and were maintained under normal greenhouse conditions. Total leaf DNA from different carnation cultivars was purified as previously described (Shure et al., 1983). Two pairs of CarSV-specific nested primers were used for PCR amplification resulting in sequences (H)–(O) (Fig. 1): PI (sense, nt 1–23), PII (antisense, nt 236–258), PIII and PIV (sense, nt 23–47; antisense, nt 216–240 respectively). Primer selection was based on previously described CarSV DNA and RNA sequences (Fig. 1A–F) where nt 1–72 and 216–275 were identical in all cases (Hernández et al., 1992; Palkovics et al., 1997). PCR amplifications were carried out with Taq DNA polymerase (Promega). The cycling profile of the first amplification with PI and PII was 5 min 95 °C, then 40 cycles of 30 s at 95 °C, 45 s at 55 °C and 60 s at 72 °C, followed by a final extension of 10 min at 72 °C. Conditions for the second PCR amplification with PIII and PIV were identical except that the annealing temperature was 57 °C. PCR products were examined in a 1:5% agarose gel and fragments of the expected sizes were excised from the gel, purified using a QIAEX II Gel Extraction Kit (Qiagen) and ligated in EcoRV-digested pBluescript II KS(+) plasmid (Stratagene) as previously described (Sambrook et al., 1989). Inserts of recombinant plasmids were sequenced with the fmol DNA Sequencing System (Promega) [except for sequence H (Fig. 1), which was obtained by automatic sequencing].

Total plant RNA was isolated from CarSV RNA-infected carnation leaves as described (Stiekema et al., 1988). cDNA synthesis was carried out using a Differential Display Kit (Display Systems Biotech) according to the manufacturer’s instructions. First strand cDNA synthesis was performed with primer PI and the resulting product was used for the synthesis of second-strand DNA using primer PII. Double-stranded cDNA was ligated into EcoRV-digested pBluescript and used to transform E. coli DH5α cells. Inserts were sequenced with an automated DNA sequencing system (ABI Prism; Perkin Elmer).

Plant nuclei, chloroplasts and mitochondria were isolated and the organellar DNAs purified as described [Jofuku & Goldberg (1988) and Pay & Smith (1988), respectively]. In order to PCR-amplify CarSV DNA from organellar DNAs the following primers were used: F1 (sense, nt 1–30) and F2 (antisense, nt 254–275) (Fig. 2). The cycling profile was 4 min 94 °C, then 40 cycles of 30 s at 95 °C, 30 s at 65 °C and 60 s at 72 °C, followed by a final extension of 10 min at 72 °C.

Nested PCR was used to establish whether multimeric copies of the CarSV DNA exist. To rule out the existence of a direct repeat the primers used were InPIV (sense, 216–240)/PII for the first PCR amplification and InPII (sense, nt 236–258)/PIV for the second reaction. To test for the alternative orientation the primers used were InPIV/PI and InPII/PIII. Conditions for PCR amplification were the same as mentioned previously except that the annealing temperature was 58 °C.

The comparison of our CarSV RNA and DNA sequences with those previously described from Spanish sources of
**Fig. 3.** Carnation plants showing different symptoms: (a) *Dianthus caryophyllus* L. cv. Praline plant showing proliferative symptoms; (b) *Dianthus caryophyllus* L. White Castellaro plant showing shoot proliferation and the same plant with enations on leaves (c, d).

*Dianthus caryophyllus* (Hernández et al., 1992) revealed a close relationship between them (Fig. 1), although there might be other related molecules that have not yet been detected because they were incompatible with the primers used. Natural sequence variants coexisted in the same carnation cultivar both in material from different plants showing identical symptoms (Fig. 1K, L) and in the same plant (Fig. 1H, I, J). In the latter case, three different CarSV DNA forms were detected in cv. Praline, two of which were full-length sequence variants and one which contained a deletion between nt 59 and 138, which is nearly identical to a form observed in a group of previously described CarSV RNA species (Darós & Flores, 1995a). CarSV DNA could also be detected in symptomless plants (Fig. 1N, O). The level of CarSV DNA in most of the examined cultivars was relatively low and two rounds of PCR amplification were usually necessary in order to produce a quantity suitable for cloning. In the analysed plants that contained CarSV DNA (cvs Tanga, Praline, White Castellaro, Domingo and ‘1285’ hybrid; Fig. 1F, H–O) the corresponding CarSV RNA form was also present as revealed by Northern analysis (data not shown), except in a symptomless plant of cv. Domingo (Fig. 1N) that died in the course of the experiments and could not be analysed. Plants negative for CarSV RNA (*D. monspessulanus, D. plumarius* ssp. *praecox, D. caryophyllus* cv. Improved White Sim) were also negative for its corresponding DNA form. The coexistence of CarSV DNA and RNA suggests some functional link between them, but the role of the DNA form in replication or symptom generation is still unknown. In two cases, cDNAs were also sequenced (cv. Tanga and cv. Praline; Fig. 1E, G, respectively) and proved to be homologous to CarSV DNA sequences. Analysis of our CarSV DNA and cDNA sequences revealed that mutations mostly occurred outside the predicted region of the plus and minus self-cleavage domains of CarSV RNA (Hernández et al., 1992). Variations in DNA sequences showed no correlation with cultivar or with presence or absence of symptoms.
CarSV DNA sequences were initially obtained from total leaf DNA of carnation plants, as was also the case for the previously detected CarSV DNA molecules (Daròs & Flores, 1995b). In order to gain insight into the subcellular localization of this form, cell organelles were purified and the corresponding DNA was then isolated and PCR-amplified using CarSV-specific primers. CarSV DNA was only present in the nuclear DNA fraction, with no signal detected in DNA fractions originating from chloroplasts and mitochondria (Fig. 2).

In contrast to previous results indicating that CarSV DNA was organized as a series of head-to-tail multimers in certain Spanish carnation cultivars (Daròs & Flores, 1995b), our results indicate that longer-than-unit forms of CarSV DNA did not occur in our samples following PCR amplification using primers Fl, FII and then Southern blot hybridization (Fig. 2). This was substantiated in further analysis when the total DNA of a symptomatic cv. Praline plant was assayed by PCR amplification in order to rule out the existence of multimeric CarSV DNA forms. Using the above mentioned primer pairs, no signals indicating the existence of these forms were detected (data not shown).

CarSV RNA was first identified in the USA and then in Italy and was named CarSAV (carnation stunt associated viroid) because its presence was apparently correlated with a stunting symptom in carnation (Lommel & Morris, 1983; Boccardo et al., 1988), but further observations questioned the role of this RNA molecule as a causal agent of this symptom (Hernández et al., 1992; Daròs & Flores, 1995b). Our CarSV RNA-infected plants did not exhibit stunting, although other symptoms were detected, including extensive shoot proliferation, which was characteristic for the vegetative stage of certain D. caryophyllus cultivars (e.g. cv. Praline, cv. Domingo) and enations on leaves (e.g. cv. White Castellaro) (Fig. 3). Typical symptoms were detected on certain carnation varieties, although some symptomless plants also contained the CarSV RNA and DNA sequences. Several other D. caryophyllus L. cultivars and different Dianthus species were examined for the presence of CarSV RNA or DNA regardless of the presence of symptoms. Experiments revealed that in wild carnation species and in non-extensively propagated species (D. superbus, D. barbatus, D. chinensis, D. anatolicus, D. knappi, D. carthaianorum, D. monspessulanus, D. plumarius ssp. praecox) CarSV RNA was not found.

In conclusion, the presence of CarSV RNA and DNA might correlate with the genetic background of carnation species and cultivars, although the role of the environment or other unknown factors could also be important in leading to the expression of visible symptoms. In addition to investigating the possible origin of cultivated carnation species in order to reveal from which breeding line or lines this element could have originated, further analyses are also in progress to characterize the CarSV DNA form present within the nucleus. As already indicated, our plants were free of the known carnation pathogens, including CERV, although the presence of certain fragments of the CERV genomic DNA joined to CarSV DNA sequences to form an extrachromosomal element (Daròs & Flores, 1995b) cannot yet be excluded.

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References


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