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Nucleotide sequence and symptom modulating analysis of a Peanut stunt virus-associated satellite RNA from Poland: high level of sequence identity with the American PSV satellites

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Abstract

Two peanut stunt virus isolates from Poland (PSV-Ag and PSV-P) have been studied. The isolates produce similar systemic symptoms on *Nicotiana tabacum* plants but the symptoms on *N. benthamiana*, *Pisum sativum* and *Datura stramonium* plants are much stronger for the PSV-P isolate. Analysis of the RNA extracted from purified virions by gel electrophoresis and RT-PCR amplification allowed the detection of a satellite RNA in the PSV-P isolate. The nucleotide sequence of this European PSV satellite was determined and found to have a high degree of identity with the sequences of the four American PSV satellites previously studied, which were found to have either no effect or ameliorate the PSV symptoms in tobacco plants (Collmer et al., 1985; Naidu et al., 1991). The possible role of the European PSV satellite in the modulation of viral symptoms has been studied but no effect was observed when the purified satellite was used with the PSV-Ag isolate as helper virus on any of the three hosts cited above.

Abbreviations: CMV – Cucumber mosaic virus; cDNA – complementary DNA; dsRNA – double stranded RNA; min – minute; PSV – Peanut stunt virus; RT-PCR – reverse transcription polymerase chain reaction; sec – second.

Introduction

Peanut stunt virus (PSV) is a member of the genus *Cucumovirus* of the family *Bromoviridae* (Murphy et al., 1995). Like cucumber mosaic virus (CMV), the type species, virions of some PSV strains are able to encapsidate satellite RNA molecules which specifically depend on their corresponding helper virus for replication (Kaper and Tousignant, 1977; Kaper et al., 1978).

More than 25 sequence variants of CMV satellite RNAs have been studied all over the world (Roossinck et al., 1992). Most of them present viral disease-modulating properties (Collmer and Howell, 1992) which result in an attenuation of symptoms in most host plants (Waterworth et al., 1979). However, some exacerbate the viral symptoms in some hosts (Gonsalves et al., 1982; Kaper and Waterworth, 1977; Takanami, 1981). The symptom attenuating ability of CMV satellite RNAs has been successfully used to obtain transgenic protection against CMV (Harrison et al., 1987; Jacquemond et al., 1988; McGarvey et al., 1994; Peña et al., 1994; Yie et al., 1992). A similar satellite-mediated transgenic protection could be applied for PSV control, specially if all or most PSV satellite RNAs presently known would express symptom-attenuating activity.

To date only four PSV satellite RNAs have been sequenced; they all are from the USA and share a high level of nucleotide sequence identity (Collmer et al., 1985; Naidu et al., 1991). Two of them have no effect on symptoms but the other two ameliorate the symptoms produced on tobacco by a particular, satellite free, PSV isolate and this effect seems to be PSV strain-specific (Naidu et al., 1991; 1992).

In this report, two PSV isolates (PSV-Ag and PSV-P) from Poland were studied and one of them (PSV-P) was shown to produce stronger symptoms on several host plants than the other isolate. Moreover, this PSV-P isolate was found to have a satellite RNA. Since no increase in viral symptoms has been reported so far in association with any PSV satellite RNA, a fact that would represent a significant drawback to the transgenic protection strategy (Naidu et al., 1995; Roossinck et al., 1992), and since all the PSV satellites described by now came from the USA, experiments were designed to determine both the symptom-modulation property of this European PSV-P satellite and its nucleotide sequence identity with that of the American PSV satellites. The results are reported here.

Materials and methods

Virus sources, propagation, purification and RNA analysis

Polish isolates of PSV were gifts of A. Twardowicz-Jakusz and H. Pospieszny (Institute of Plant Protection, Poznań, Poland). PSV-Ag and PSV-P were originally isolated from naturally infected celery (*Apium graveolens*) and yellow lupin (*Lupinus luteus*) plants, respectively (Pospieszny and Frencel, 1983; Twardowicz-Jakusz et al., 1983). The strains were first maintained in *Pisum sativum* and later in *Nicotiana tabacum* L. *cv. Xanthi nc* plants. PSV-76-69, a satellite containing isolate from USA (Díaz-Ruíz and Kaper, 1983; Kaper et al., 1978) was maintained in *N. tabacum* L. *cv. Xanthi nc* plants and used as control.

Bentonite-dusted leaves were inoculated with plant sap or with 25 μl of 10 μg/ml of purified PSV in 0.02 M sodium phosphate buffer pH 7.2. The plants were grown in a growth chamber at 24 °C with a 16 h light period (12,000 lux).

Virus purification from infected plants was according to Lot et al. (1972). Viral and satellite RNA was isolated from purified virion preparations and total nucleic acid was isolated from infected leaves both by conventional phenol/SDS extraction followed by several ethanol precipitation. Nucleic acids were analyzed by 2% agarose gel electrophoresis and staining with ethidium bromide.

Satellite detection by RT-PCR amplification

In order to detect possible satellite RNAs present in any of the two European PSV isolates, the reverse transcription polymerase chain reaction (RT-PCR) technique (Kawasaki, 1990) was used. Two oligonucleotides primers corresponding to nucleotides 1–18 and 379–393 of the published RNA sequence of the PSV-76-69 satellite from USA (Collmer et al., 1985) were designed. *Eco*RI or *Hind*III restriction enzyme recognition sites and two more nucleotides were added at the termini. The primer sequences were:

PS-5: 5′ ATATATGAATTGAATTCGTTTTGTTTTGTCGGGAG 3′
PS-3: 5′ AAATTAAAGGTTGGGTCGTGTAGGAGC 3′

About 0.5 μg of total RNA was used for cDNA synthesis. The samples, containing 1 unit of RNasin, were adjusted to 50 mM Tris HCl pH 8.3, 50 mM KCl, 7.5 mM $MgCl_2$, 1.25 mM dNTPs. Primer downstream (PS-3) was added to a final concentration of 2.5 μM and the reaction was started with the addition of 1 unit of AMV reverse transcriptase (Promega) in a final volume of 20 μl and incubated for 45 min at 42 °C. PCR mixtures contained, in a total volume of 100 μl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer (PS-3 and PS-5), and the 20 μl reverse transcriptase reaction in the buffer conditions supplied by Promega (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100). Reaction mixtures were heated at 95 °C for 10 min and cooled on ice before adding 2.5 U *Taq* DNA polymerase (Promega).

The conditions for the amplification reaction in an automatic thermal cycler were as follows: 10 cycles (30 sec denaturation at 92 °C; 30 sec annealing at 45 °C; 1 min extension at 72 °C), followed by 30 cycles (30 sec denaturation at 92 °C; 30 sec annealing at 70 °C; 1 min extension at 72 °C), and 10 min final extension at 72 °C. The amplified products were run on a 1.2% agarose gel and viewed by ethidium bromide staining.

Satellite sequencing

The RT-PCR satellite amplified band was cut from a low melting point 0.8% agarose gel and purified through a Promega Magic PCR column using the supplied protocol. 0.5 ng of the corresponding satellite DNA was sequenced as described on the fmol TM DNA Sequencing System from Promega. The same oligonucleotides described above were used as primers.

In order to read the sequences close to the 5′ and 3′ ends, two new oligonucleotides corresponding to nucleotides 123–141 and 309–326 of the cited PSV-76-69 satellite sequence (Collmer et al., 1985) were synthesized and the same sequencing protocol used.

Figure 1. Symptoms on *Nicotiana benthamiana* (a) and *Pisum sativum* (b) plants inoculated as follows: PSV-P (left), PSV-Ag (center), mock (right). More severe symptoms appear on the plants inoculated with PSV-P. The pictures were taken 13 (a) and 60 (b) days after inoculation.

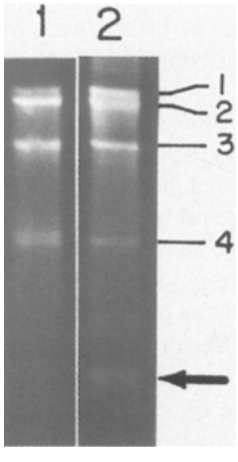

Figure 2. Electrophoretic analysis of viral RNA extracted from PSV virions purified from infected *Nicotiana benthamiana* plants. RNAs (1 μg) were analyzed on a 2% agarose gel and stained with ethidium bromide. Lane 1: PSV-Ag, lane 2: PSV-P. Viral RNAs 1–4 are indicated in the right margin. The arrow indicates the extra satellite band seen in the PSV-P isolate.

Satellite purification

RNA extracted from purified PSV-P preparations was used to purify the satellite RNA. In order to separate the satellite from the viral RNAs, the satellite band was cut with a sterile razor from a 0.8% low melting point agarose gel. The agarose was melted at 72 °C for 2 min and digested with the gelase TM enzyme preparation from Epicentre Technologies. After phenol extraction and ethanol precipitation, the RNA was resuspended in 20 μl of inoculation buffer (0.02 M sodium phosphate pH 7.2) and used for inoculation.

Results and discussion

Characterization of the European PSV isolates: symptomatology and RNA analysis

Both *Nicotiana tabacum* cv. Xanthi nc, used as propagating host, and *N. benthamiana* plants inoculated

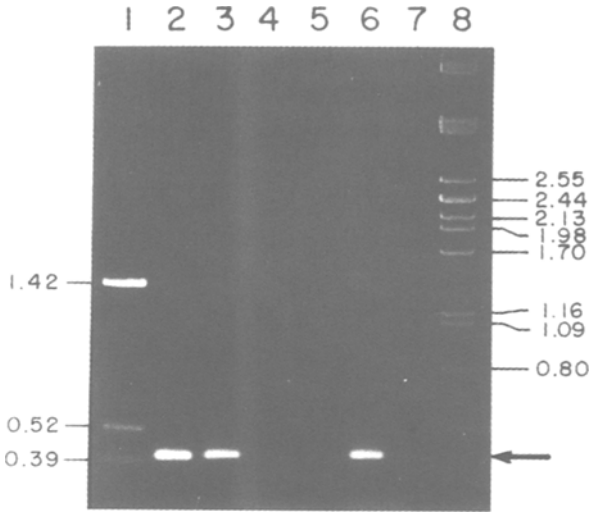

Figure 3. Analysis on 1.2% agarose gel electrophoresis of RT-PCR amplified PSV satellite from purified viral RNAs of PSV isolates. Lane 1: pUC13 digested with HinfI as molecular size markers, lane 2: PSV-P, lane 3: RNA from a PSV-P infected *Nicotiana benthamiana* plant, lane 5: PSV-Ag, lane 6: PSV-76-69, lane 7: H_2O, lane 8: λDNA digested with PstI as molecular size markers. The sizes of the markers (in Kb) are given in the margins. The arrow indicates the satellite band amplified from the PSV-P and the PSV-76-69 isolates.

with either purified virions or viral RNA from each of the two PSV isolates (Ag and P) showed systemic symptoms about 13 days after inoculation. However, while *N. tabacum* showed similar systemic symptoms with both PSV isolates (not shown), the symptoms on *N. benthamiana* plants inoculated with PSV-P were much stronger than in the case of PSV-Ag as shown in Figure 1a. The PSV-Ag infected plants were stunted and the leaves had a mosaic pattern but the PSV-P infected plants were smaller, the leaves were curled and presented blister deformations (Figure 1a). This severity of symptoms persisted one month after inoculation. PSV-P was again more aggressive in *Pisum sativum* plants (Figure 1b). One month after inoculation the plants were stunted, and presented chlorosis and necrosis in the lower leaves and tendrils; eventually the plants died. The symptoms in the PSV-Ag inoculated plants were milder and presented some chlorosis of leaves but they lived as long as the control plants (Figure 1b). A similar symptom severity situation associated with PSV-P infection was obtained with *Datura stramonium* plants (not shown).

Analysis of the RNA extracted from purified PSV-Ag and PSV-P virions by 2% agarose gel electrophoresis showed an extra low molecular weight band only associated with the PSV-P isolate (Figure 2). By running the products of the RT-PCR reactions performed with the purified viral RNAs or with the RNAs extracted from infected tissue on a 1.2% agarose gel, a band with the same molecular weight as the one corresponding to the USA satellite of the PSV-76-69 isolate used as control was detected in both PSV-P virion RNA and RNA from tissue infected with PSV-P but not PSV-Ag (Figure 3).

These results confirmed the satellite nature of the extra RNA band in PSV-P RNA and suggested that this European satellite could be responsible for the severity of the symptoms associated with PSV-P in these three hosts, an important event since none of the PSV satellites so far studied have been found to intensify the viral symptoms in any host plant.

PSV-Ag versus PSV-P as helper virus: effect on symptom modulation and satellite replication

The observation that the PSV-P isolate is clearly more aggressive in several host plants than the other PSV isolate that do not have a satellite, justified further study of this system. The following experiment was designed to check the possibility that the PSV-P satellite was responsible for the severity of the symptoms. The satellite RNA purified from a 0.8% low melting point agarose gel, as described in Methods, was inoculated on *N. benthamiana* plants together with the satellite-free, PSV-Ag isolate as helper virus. Although the PSV-Ag isolate supported the replication of the PSV-P satellite (not shown), no change in symptoms was seen in these plants over the plants inoculated with the PSV-Ag isolate alone (Figure 4a). Similarly,

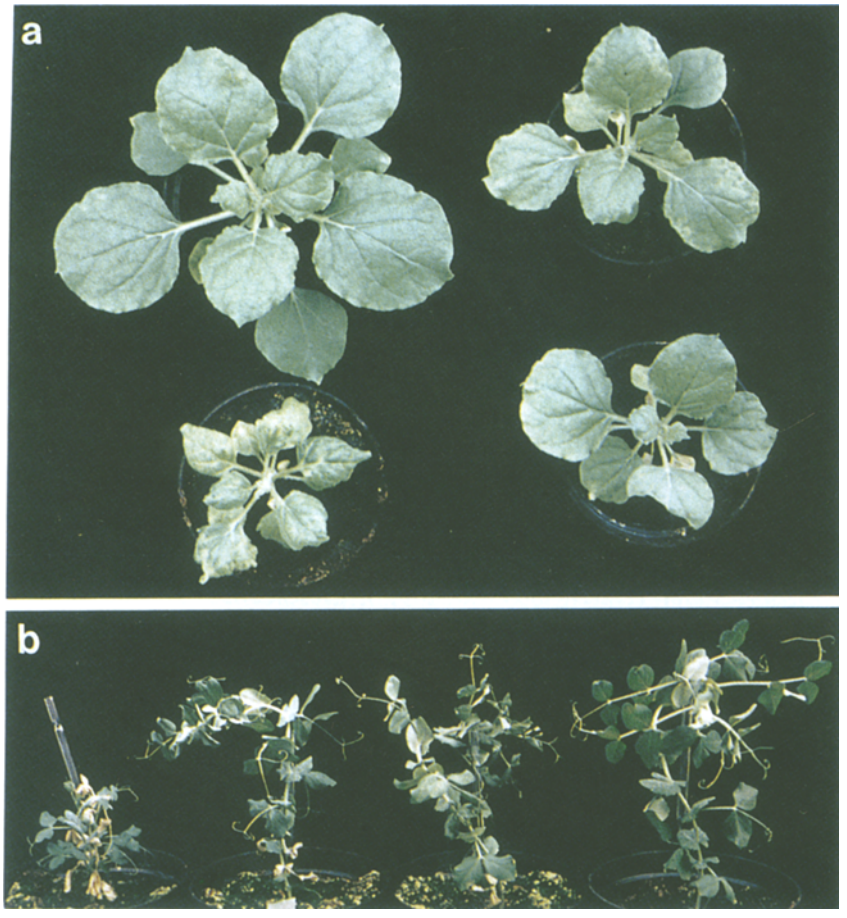

Figure 4. Symptoms on *Nicotiana benthamiana* (a) and *Pisum sativum* (b) plants inoculated as follows: (a) PSV-P (lower left), PSV-Ag (lower right), PSV-Ag + PSV-P satellite RNA (upper right), mock (upper left); (b) PSV-P (left), PSV-Ag (middle left), PSV-Ag + PSV-P satellite RNA (middle right), mock (right). Plants inoculated with the PSV-P satellite RNA plus PSV-Ag as helper virus show the same symptoms as the ones inoculated with PSV-Ag alone. The pictures were taken 20 (a) and 30 (b) days after inoculation.

no exacerbation of the PSV-Ag symptoms could be demonstrated either in *P. sativum* (Figure 4b) or in *D. stramonium* (not shown) plants when the satellite was added in the inoculum. These results indicated that the severity of symptoms in these hosts associated with the PSV-P isolate was not directly related to the presence of the satellite, but to either this particular PSV-P isolate or to the specific interaction between the PSV-P isolate and this satellite.

Analysis of total nucleic acids extracted from the inoculated *N. benthamiana* plants showed an extra band only from plants inoculated with either PSV-P or with the PSV-P satellite plus PSV-Ag as helper virus, with the same mobility that the satellite RNA from PSV-P virions (not shown). However, *N. benthamiana* plants inoculated with PSV-P consistently accumulated from two to five times more satellite RNA than *N. benthamiana* plants inoculated with PSV-Ag plus the PSV-P satellite (not shown). To confirm that the extra band found in plants inoculated with the PSV-Ag isolate plus the PSV-P satellite was indeed the satellite RNA, a RT-PCR reaction was performed on the extracted total nucleic acid samples, using the same primers as before. Only in samples from plants inoculated with the satellite, a band with the expected molecular weight was amplified (not shown).

Comparison of the European PSV-P satellite sequence with the published PSV satellite sequences from the USA

The PSV-P satellite nucleotide sequence was obtained using its RT-PCR amplified DNA fragment as described in Methods. The primers used for the satellite PCR amplification corresponding to the 5′ and 3′

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76-69                                                                GC
V                                                                    GC
P    GTTTTGTTTTGTCGGGAGTCACCGCGTAAAAACCCACTGTAATGGTGATGCGACAGCAGG    60
G                                                                    CG
WC                                                                   CG

76-69
V
P    TGAAGAAATGCCTGTATGTGCGTGGCGCTACGCCTTTGCCCGGGGGGATATACTGGGGGG   120
G
WC

76-69                                                          G
V                                                              T
P    GGGCGTATGCACGTAGGTGACCGATGAGAGAGGACGCCTCCGGGGGCGGAGGTAAGTCGG  180
G                                                              G
WC                                                             G

76-69                                                          C
V                                                              C
P    GGAGGGCGGCGTTCGTAGTGAAACGACCTCCTATCAGGACTGCTGTACCGTCGGGGGGAA  240
G                                                              T
WC                                                             T

76-69          T
V              C
P    ACCCCCCTGGGTCGAGCCCGATCCCTCTTCTTGTAAAACCGGTGGAACTTAACCTCATTT  300
G              C
WC             C                 Δ

76-69
V
P    CTGGAGAAGTCCGGACAACTGTGATGGAAGAAAGCAGTGTGGTGCCCCAGCGGCGACCAC  360
G
WC

76-69    A                   C
V        A                   C
P    GCAAGGCCACGGCCTTTAGCTCCTACACGACCC    393
G        C   Δ               C
WC       C   Δ               T
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Figure 5. Nucleotide sequence of the European PSV-P satellite RNA. The complete nucleotide sequence is shown aligned with all the published PSV satellite RNA sequences from USA. Sequences from the American PSV satellites showing either no effect on symptoms or attenuation of viral symptoms are above and below the European PSV-P satellite sequence, respectively. Only the nucleotides that are different to the PSV-P satellite are specified. Deletions are indicated by Δ. The numbering of the sequences is as in Collmer et al. (1985).

ends were used in two separate reactions to sequence the satellite from both ends. Since the nucleotides close to the primers could not be read, two new primers (see Methods) had to be designed. The complete sequence of the European PSV-P satellite is shown in Figure 5, aligned with all other sequences of the American PSV satellites known so far.

Surprisingly, the sequence of the satellite from the geographically distant PSV-P isolate is 99% identical to the published sequences of the PSV satellites from USA isolates. The position of the nucleotides that differ from each of the previously published sequences (Figure 5) are the ones already found to be different between them. Therefore, it seems that, like with CMV

satellites (Fraile and García-Arenal, 1991), nucleotide sequence variations only occur among PSV satellite at specific positions.

Studies with infectious *in vitro* transcripts from full length cDNA clones and chimeric or mutant cDNA molecules of the American PSV satellites, reduced the differences between satellites with and without attenuating properties in tobacco plants to two nucleotide changes in the satellite sequence (Naidu et al., 1992). These two nucleotides (a T and a C at positions 226 and 362, respectively) are also found in the sequence of the European PSV-P satellite. Therefore, although the nucleotide sequence would suggest an attenuating effect for the PSV-P satellite, our biological data (Figure 4) show that this European satellite does not have any effect on viral symptom expression in either *N. benthamiana*, *P. sativum* or *D. stramonium* plants when PSV-Ag is used as helper virus, nor in *N. tabacum* plants when it is inoculated with its natural PSV-P helper virus. These results, and the fact that the two PSV-G and PSV-WC satellites from USA found to be symptom attenuating in tobacco plants when inoculated with PSV-ER as helper virus are not attenuating when PSV-74 was the helper virus (Naidu et al., 1992), indicate that, like with CMV satellites (Kaper and Collmer, 1988; Wu et al., 1993), the viral symptom modulation property of PSV satellites is the result of a complex interaction between each specific combination of satellite, host plant and helper virus.

Conclusions

Two European PSV isolates from Poland, PSV-Ag and PSV-P, have been comparatively studied. Both isolates produced similar symptoms on *Nicotiana tabacum* plants but PSV-P induced stronger symptoms on *N. benthamiana*, *Pisum sativum* and *Datura stramonium* plants than PSV-Ag (Figure 1).

An extra RNA that has been detected associated with the PSV-P isolate (Figure 2), has been characterized as a satellite RNA by RT-PCR using the known sequence of the PSV-76-69 satellite as control (Figure 3). The determined nucleotide sequence of this European PSV-P satellite has been found to be 99% homologous to the four published American PSV satellite sequences, the only PSV satellite sequences known so far (Figure 5).

The possibility of having found an exacerbating PSV satellite for the first time, which could represent a drawback for the PSV control by the satellite-mediated transgenic protection strategy, justified further study of this European satellite. However, the purified PSV-P-associated satellite RNA, when inoculated with PSV-Ag as helper virus, does not change the symptoms of the helper virus inoculated alone, on any of the three host plant studied (Figure 4).

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