SHORT COMMUNICATION

IDENTIFICATION AND CHARACTERIZATION OF *POTATO SPINDLE TUBER VIROID* INFECTING *SOLANUM JASMINOIDES* AND *S. RANTONNETII* IN ITALY

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SUMMARY

Potato spindle tuber viroid (PSTVd), a quarantine pathogen included in the EPPO A2 list was identified in plants of *Solanum jasminoides* and *S. rantonnetii* in Italy. Molecular characterization of four *S. jasminoides* and one *S. rantonnetii* PSTVd isolates showed limited sequence variability. A tissue-printing hybridization method for detecting PSTVd in ornamental *Solanaceae* was successfully tested and its potential for large scale surveys is discussed. This appears to be the first report of PSTVd in Italy and the first report of PSTVd naturally infecting *S. rantonnetii.*

Key words: PSTVd, ornamental *Solanum*, sequencing, diagnosis, tissue printing hybridization.

Viroids are the smallest infectious agents known. With a genome of only 246-401 nt composed of circular, single-stranded RNA without coding capacity, they cause diseases in many kinds of plants (Flores *et al.*, 2005a). Based on biochemical, biological and structural properties, viroids have been grouped in two families: *Pospiviroidae* and *Avsunviroidae*, which comprise nuclear and chloroplast replicating viroid species, respectively (Flores *et al.*, 2005b). *Potato spindle tuber viroid* (PSTVd), the type species of *Pospiviroidae*, has a special historical importance because it was the first viroid to be identified and characterized when the aetiology of the potato spindle tuber disease was being investigated (Diener, 2001). In spite of its narrow range of natural hosts, which in addition to potato includes tomato (Puchta *et al.*, 1990; Elliot *et al.*, 2001; Verhoeven *et al.*, 2004), avocado (Querci *et al.*, 1995) and pepino (Shamloul *et al.*, 1997), PSTVd has been transmitted experimentally to a wide range of hosts, at least 156 species in 12 families (Diener, 1979). PSTVd is an EPPO A2 quarantine pest transmissible by seed and pollen (Fernow *et al.*, 1970; Singh, 1970; Sing *et al.*, 1992), as well as by contact mainly using contaminated tools.

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In 2006, symptomless plants of *Solanum jasminoides* infected by PSTVd were identified in several European countries, including the Netherlands (Anonymous, 2006a) and Germany (Anonymous, 2006b). More recently, PSTVd infections were recorded in the Netherlands in *Brugmansia* species (*B. suavolens*, *B. x candida*, *B. cordata* and *B. variegata*) and again in *S. jasminoides* (Anonymous, 2006c; Verhoeven *et al.*, 2007).

To test the occurrence of PSTVd in ornamental solanaceous plants grown in Italy, a limited survey was carried out at the beginning of 2007, the results of which are reported here together with an evaluation of the efficiency of a tissue-printing hybridization technique for the quick detection of this pathogen.

A total of 23 *S. jasminoides* plants were randomly collected in four different nurseries of central and southern Italy. Total nuclei acids (TNAs) were extracted from 100 mg of leaf tissue as described by Di Serio *et al.* (2002). TNA preparations were mixed with an equal volume of formamide and aliquots (5 µl) spotted onto positively charged nylon membranes (Roche Diagnostics GmbH, Germany) that were hybridized with a PSTVd-specific digoxigenin-labelled riboprobe in the DIG-Easy Hyb Granules solution (Roche Diagnostics GmbH, Germany) at 68°C. Membrane washing, incubation with the anti-digoxigenin antibody conjugated to alkaline phosphatase, and chemiluminescent detection with the substrate CPD-star were done as recommended by the supplier (Roche Diagnostics GmbH, Germany).

Figure 1 shows that 17 samples gave a strong hybridization signal, indicating infection in the corresponding plants. These results were confirmed by RT-PCR using the primers PSTVd-32 (5'-AAACCCTGTTTCGGCGGGAATTAC-3') and PSTVd-33 (5'-TCACCCTTCCTTTCTTCGGGTGTC-3'), complementary and identical, respectively, to positions 156-179 and 180-203 of the PSTVd reference variant (accession number: NC002030). The region covered by these adjacent primers is highly conserved in most known PSTVd variants, including that recently isolated from *S. jasminoides* in the Netherlands (accession number: EF192393; Verhoeven *et al.*, 2007). Using these primers, a cDNA of about 360 bp corresponding to the full-length PSTVd RNA, could be amplified from infected samples. RT-

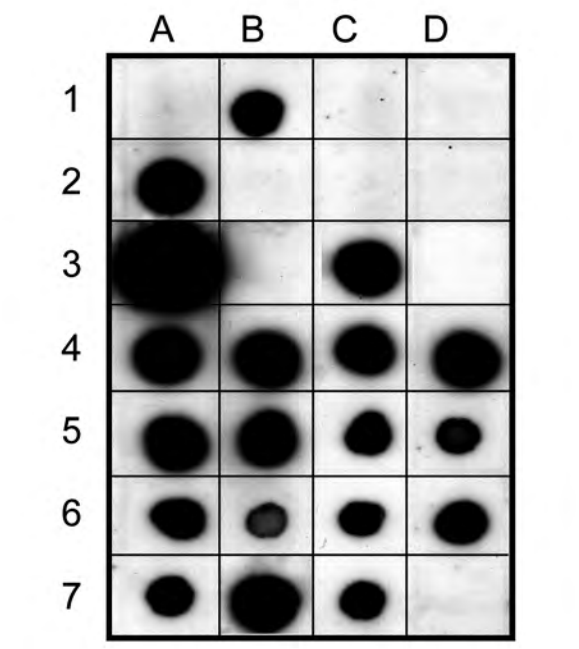

Fig. 1. Dot-blot hybridization assay with a digoxigenin-labeled PSTVd-cRNA probe. Aliquots (5 µl) of TNA preparations from each *S. jasminoides* plant were spotted on each square. Positive controls, consisting of a plasmid with a full-length PSTVd-cDNA, and of a TNA from a PSTVd-infected *N. benthamiana* plant, were spotted in A3 and C3, respectively. Positions B3, D3 and D7 are free spaces in the membrane.

PCR analyses showed that all samples testing positive by dot-blot hybridization, generated an RT-PCR amplicon of this size, thus strongly supporting the conclusion that they were actually infected by PSTVd.

Definitive identification of the pathogen was obtained by cloning and sequencing the amplified products generated from four different *S. jasminoides* isolates. The twelve sequenced clones (three for each *S. jasminoides* isolate) had a size of 357 nt, consistent with that of some PSTVd sequence variants characterized previously. Multiple alignments showed that nine clones were identical to the PSTVd variant previously reported from *S. jasminoides* in the Netherlands, which is taken as the reference variant in the alignment (Fig. 2). Interestingly, this variant was recovered from all *S. jasminoides* isolates characterized in the present study. However, three variants with point mutations [PS-Sj-5F2, PS-Sj-T3 and PS-Sj-T4 (accession numbers: EF459697, EF459699, EF459698, respectively)] were identified in two Italian isolates (Fig. 2). Altogether, these data show that PSTVd infects *S. jasminoides* plants grown in Italy; this apparently represents the first report of this viroid in this country.

S. rantonnetii has previously been reported as an experimental host of PSTVd (Sing, 1973). Therefore, dot-blot hybridization and RT-PCR amplification analyses were also extended to two plants of *S. rantonnetii* growing in one of the nurseries surveyed. One of the plants tested proved to be infected by PSTVd when analysed by RT-PCR (data not shown) and dot-blot hybridization (Fig. 3, lanes Sr1 and Sr2). The RT-PCR amplicon was subsequently cloned, and sequencing of five clones confirmed the identification of the pathogen: four variants had the same sequence (accession number: EF459700) as the reference PSTVd variant previously isolated from *S. jasminoides* (Fig. 2), whereas variant PS-Sr2 (accession number: EF459701) differed by one point mutation (Fig. 2).

This appears to be the first report of PSTVd naturally infecting *S. rantonnetii.* Whether the identity between the Dutch reference variant and the most frequently recovered variant from the Italian isolates reflects a common origin of the PSTVd infecting ornamental *Solanaceae* in these two countries is not known. Alternatively, a strong host pressure could have selected this specific variant in *S. jasminoides* and *S. rantonnetii* from different PSTVd-infected sources. Experimental testing of the latter hypothesis may prove interesting because rapid evolution of natural PSTVd variants was previously reported in other hosts (Góra-Sochacka *et al.*, 1997). It could be speculated that since both solanaceous plants are native from South America, they may represent original sources/reservoirs of PSTVd.

The data here reported additionally highlight the need of large-scale surveys to determine the spread of this viroid in Italy and other European countries, and to produce PSTVd-free ornamental *Solanaceae.* Since simple, fast and economic detection procedures will be necessary for this, a comparative test was conducted to evaluate the possibility of detecting PSTVd in these plants by tissue-printing hybridization (TPH), a method that does not require skilled technical expertise and is particularly suitable for massive surveys. Tissue prints were done by directly pressing freshly cut herbaceous stems of *S. jasminoides* and *S. rantonnetii* onto nylon membranes (Roche Diagnostics GmbH, Germany). TPH was extremely efficient for detecting PSTVd in both *S. jasminoides* and *S. rantonnetii,* generating strong signals that perfectly matched those obtained by dot-blot hybridizazion, while healthy controls were consistently negative (Fig. 3). These results were confirmed by extending the TPH assay to all *S. jasminoides* plants previously analysed (data not shown).

PSTVd shares high sequence similarity with several species in the genus *Pospiviroid.* Therefore, a PSTVd-specific cRNA probe may hybridize also other species in the genus, including *Chrysanthemum stunt viroid* (CSVd), another quarantine pest in the A2 list of EPPO, which was recently found in *S. jasminoides* in the Netherlands (Verhoeven *et al.,* 2006). According to EPPO diagnostic protocols for regulated pests (Anonymous, 2004), PSTVd diagnosis in potato using a dig-labelled probe must be confirmed by a different detection method, such as RT-PCR and sequencing of the generated amplicon. By analogy, it seems plausible that the same procedure could be validly used also for hosts other than potato, as it was done in the present instance.

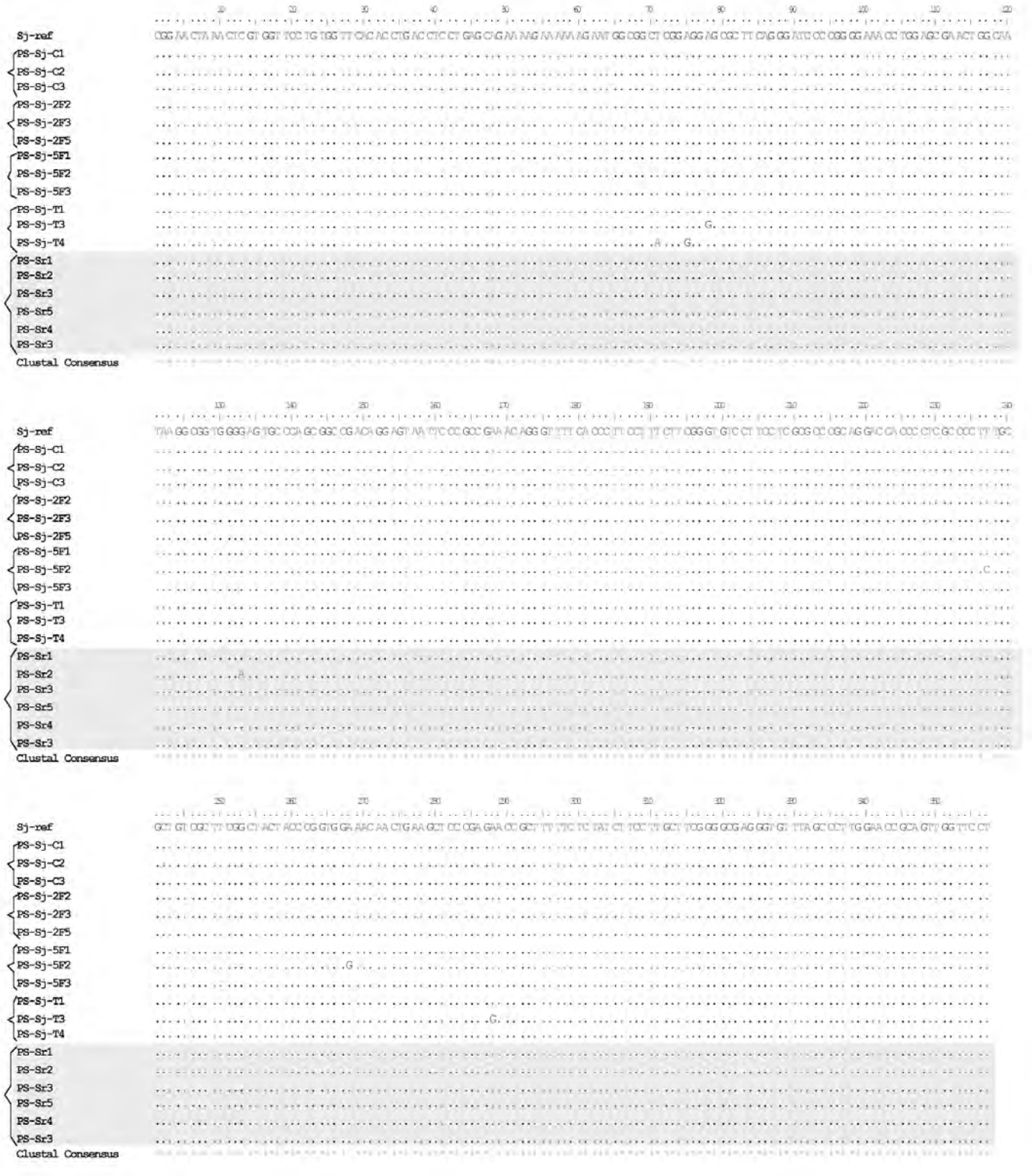

Fig. 2. Multiple sequence alignment of PSTVd-cDNA variants from isolates of *Solanum jasminoides* (white background) and *S. rantonnetii* (grey background). The reference sequence Sj-ref at the top of the alignment corresponds to the PSTVd variant from *S. jasminoid*es reported previously in the Netherlands (accession number: EF192393). Nucleotide identity with respect to the Sj-ref variant is indicated by dots. Branches comprise variants derived from the same isolate. Variants PS-Sj-5F2, PS-Sj-T3, PS-Sj-T4 and PS-Sr2, showing point mutations with respect to the reference variant, have been deposited in GenBank database with the accession numbers EF459697, EF459799, EF459698, EF459701, respectively. Asterisks denote positions of conserved nucleotides in all sequences aligned. Numbers on the top above indicate nucleotide positions in the multiple alignment.

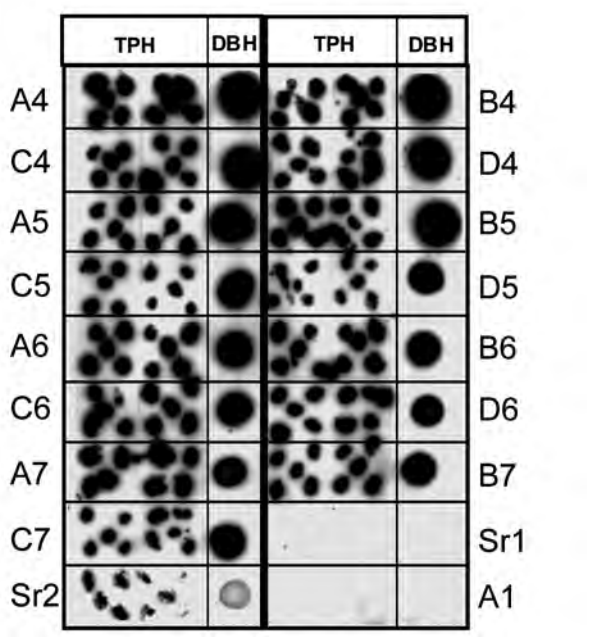

Fig. 3. Comparative detection of PSTVd on ornamental *Solanaceae* by tissue-printing (TPH) and dot-blot (DBH) hybridization. The analyses were performed using tissues and TNA preparations from most of the plants previously tested by dot-blot (Fig. 1) and RT-PCR (data not shown). On the left and the right are identified the specific samples, the same as in the membrane of Figure 1 with the exception of Sr1 and Sr2 that correspond to the two *S. rantonnetii* plants included in the preliminary survey.

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