SHORT COMMUNICATION

INFECTIVITY OF *IN VITRO* TRANSCRIPTS FROM A FULL-LENGTH cDNA CLONE OF *PELARGONIUM FLOWER BREAK VIRUS* IN AN EXPERIMENTAL AND A NATURAL HOST

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SUMMARY

To carry out reverse genetics experiments with *Pelargonium flower break virus* (PFBV), one of the most important viruses affecting *Pelargonium* spp., cDNA clones were constructed from which RNA transcripts can be synthesized *in vitro*. Two populations of overlapping RT-PCR products encompassing the complete PFBV RNA were ligated into pUC18 with the T7 RNA polymerase promoter fused to the 5' extremity of the viral cDNA. The RNA transcripts derived from one of the resulting clones were infectious when mechanically inoculated to the experimental host *Chenopodium quinoa* and to the natural host *P. zonale* inducing local and systemic infections, respectively. The sequence of the infectious cDNA was almost 98% identical to that determined previously for a Spanish isolate of PFBV. This is the first description of a biologically active PFBV cDNA clone, an essential tool for detailed analyses of the viral genome.

Key words: PFBV, infectious transcripts, cDNA clone, *Pelargonium.*

Pelargonium flower break virus (PFBV) causes frequent infections in geraniums (*Pelargonium* spp.) throughout the world (Stone, 1980; Paludan and Begtrup, 1987; Adkins and Nameth, 1989; Bouwen and Maat, 1992; Franck and Loebenstein, 1994; Alonso and Borja, 2005). It may induce white flower streaking, chlorotic spotting of leaves and growth reduction (Stone, 1974), thus having detrimental effects on the quality and marketability of the affected plants. The rapid spread of the virus in recent years may have been facilitated by the practice of vegetative propagation and by the symptomless condition of many plants infected with PFBV isolates. Moreover, PFBV can be easily transmitted by the use of contaminated tools and, occasionally, via irrigation systems or by western flower thrips (*Frankiniella occidentalis*) (Krczal *et al.*, 1995).

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PFBV is a member of the genus *Carmovirus* (family *Tombusviridae*) and, like other carmoviruses, produces icosahedral virions that encapsidate a linear positive-sense single-stranded RNA (Lommel *et al.*, 2005). The complete nucleotide sequence of PFBV genomic RNA has been determined recently (Rico and Hernández, 2004). It comprises 3,923 nucleotides (nt) and contains five open reading frames (ORFs). The 5' proximal ORF encodes a 27 kDa protein (p27) and terminates with an amber codon which may be read-through into an in-frame p56 ORF to generate an 86 kDa protein (p86) that contains viral RNA-dependent RNA polymerase (RdRp) motifs. Two small ORFs, located in the central part of the virus genome, encode polypeptides of 7 (p7) and 12 kDa (p12), respectively, which are probably involved in virus movement. The 3' proximal ORF encodes a 37 kDa capsid protein (CP).

Due to the importance of PFBV in *Pelargonium* production and because of our interest in the dissection of viral genome elements involved in different steps of the infectious cycle, we generated a full-length viral cDNA clone from which biologically active transcripts could be synthesized *in vitro*.

Sap from a *Pelargonium zonale* (*Pelargonium x hortorum* Bailey) plant collected in Spain and naturally infected by isolate SP18 of PFBV, was mechanically inoculated to *Chenopodium quinoa*. Total RNA preparations from infected *C. quinoa* leaves were obtained by phenol extraction and lithium chloride precipitation (Verwoerd *et al.*, 1989) and used as templates for RT-PCR reactions. Different attempts to amplify the complete viral sequence in one step were unsuccessful. To avoid this problem, two different cDNAs were generated with Superscript II-RT (Invitrogen, San Diego, CA, USA): cDNA I, which was synthesized using primer CH44 (5'-CGAGTCGACAATTTATGTCCTTCATG-3'), complementary to nt 1,679-1,704 of the PFBV genomic RNA, and cDNA II, which was obtained with primer CH52 (5'-GGTCTAGAGGGCGGGTTAAGGTCTCCATC-3'), complementary to the 3' terminus of the viral sequence (nt 3,903-3,923) with an *Xba*I site (underlined) at the 5' end. Amplification of cDNA I was performed with the Expand High Fidelity PCR System (Roche) using primers CH44 and CH49 (5'-CCGCATG-

CAAGCTTG**TAATACGACTCACTATAGGG**ATACATTACACTCGGTATCTGG-3'), which contains a *Hind*III site (underlined) fused to a T7 RNA polymerase promoter sequence (in bold) followed by 22 nt of the 5' end of the virus sequence. PCR amplification of cDNA II was done using primers CH52 and CH29 (5'-ATGAAGGACATAAATTGTCGACTCG-3'), homologous to nt 1,680-1,699. RT-PCR products obtained with primers CH44 and CH49 were digested with *Hind*III and *Sal*I while RT-PCR products obtained with primers CH52 and CH29 were digested with *Sal*I and *Xba*I. The two digested RT-PCR products were ligated at the *Sal*I site (present in the PFBV sequence at nt 1,696-1,701 and embedded in primers CH44 and CH29) and cloned into *Hind*III and *Xba*I sites of pUC18. Recombinant clones were confirmed as such by restriction analysis. This approach allowed simultaneous generation of a large pool of independent clones and was similar to the population cloning strategy used previously to synthesize full-length clones of other RNA viruses (Yu and Wong, 1998). Since for each RT-PCR several point mutations may arise, this strategy maximizes the probability of obtaining infectious cDNA clones.

Ten clones were randomly selected and, after linearization with *Xba*I, transcripts were generated by using T7 RNA polymerase (Roche Diagnostic, Indianapolis, USA) following standard protocols (Sambrook and Russell, 2001). As a consequence of the design of the 3' end primer used for cDNA synthesis, the *in vitro* transcripts should contain five extra nucleotides at the 3' terminus compared with the wild type viral RNA. No cap analog was included in the *in vitro* transcription reactions as the cap structure is not required for infectivity of viral RNA of members of the family *Tombusviridae* (Rochon, 1999). Batches of four *C. quinoa* plants were mechanically inoculated (three leaves per plant) with the RNAs derived from each clone (approximately 0.7 µg per leaf) including one mock inoculated batch as a negative control. Seven days after inoculation, only the leaves inoculated with one of the clones (designated as pSP18-IC) developed chlorotic lesions that were identical to those produced by the wild type virus (Fig. 1A). The infectivity of pSP18-IC was confirmed in numerous independent experiments and plant infection was further verified by Northern analysis (Fig. 1B). In addition, viral double-stranded RNAs were isolated from the infected leaves (Morris and Dodds, 1979) and polyadenylated using yeast poly(A) polymerase (U.S. Biochemical Amersham, Little Chalfont, England) according to the manufacturer´s instructions. After phenol-chloroform extraction and ethanol precipitation, the polyadenylated RNAs were reverse transcribed using GeneRacer Oligo dT oligonucleotide (Invitrogen, San Diego, CA, USA), which has a 3' terminal 18 nt dT tail and contains the priming sites for the GeneRacer 3' and GeneRacer 3' Nested oligonucleotides (Invitrogen, San Diego, CA, USA) at 5' end. RT products were PCR amplified using either primer GeneRacer 3' or GeneRacer 3' Nested in combination with CH11 (5'-CCTTCTGCCAGAGGTTCCCG-3'), homologous to nt 3,704-3,723, to amplify the 3' terminal region. Sequencing of the PCR products showed that the progeny viral RNA had the precise 3' terminus indicating that the extra nucleotides of the original transcripts are removed during replication in plants.

The full-length cDNA inserted in pSP18-IC was completely sequenced (Accession number: DQ256073). Comparison of its nucleotide sequence with that reported previously (Rico and Hernández, 2004) for another Spanish PFBV isolate, showed an overall sequence identity of 97.7%. Only ten of the 94 nucleotide substitutions detected resulted in amino acid changes, five affected the RdRp (one of them non-conservative, Ser to Phe at position 511 of p86), one affected p7 (non-conservative, change Ser to Pro at position 19), two affected p12 (both of them non-conservative, changes Thr to Ile at position 12 and Val to Gly at position 14) and two affected the CP.

To get insights into the lack of infectivity of the other clones that were screened, the nucleotide sequence of one of them was also fully determined. One nucleotide insertion was detected at position 2,371 that led to a frameshift within the p7 and p12 genes. This mutation may have been introduced during reverse transcription and/or PCR because it seems unlikely that non-viable PFBV variant was present in the initial viral RNA. This observation supports the rationale of using a population cloning strategy.

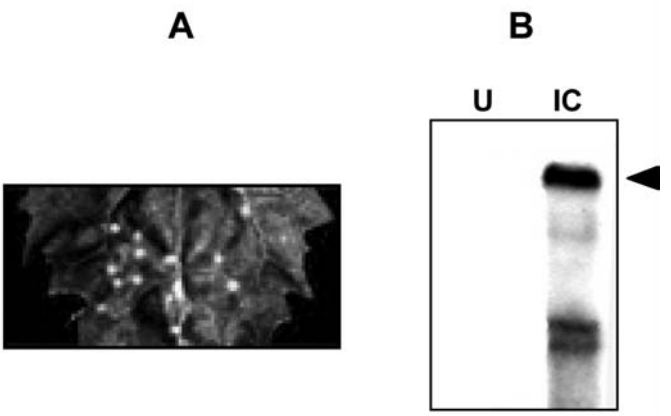

Fig. 1. A) Symptoms induced by *in vitro* transcripts derived from pSP18-IC in *C. quinoa* leaves. The picture was taken seven days post-inoculation. **B**) Northern blot hybridization of total RNA preparations from mock inoculated leaves (U) and from leaves inoculated with *in vitro* transcripts derived from pSP18-IC (IC). Two micrograms of total RNA samples were denatured by glyoxal-dimethyl sulfoxide treatment, electrophoresed in 1% agarose gels, blotted to nylon membranes, and hybridized with a ^{32}P-labeled DNA probe derived from the 3' terminus of the PFBV genome. The arrowhead points to the genomic viral RNA; lower bands correspond to subgenomic RNAs.

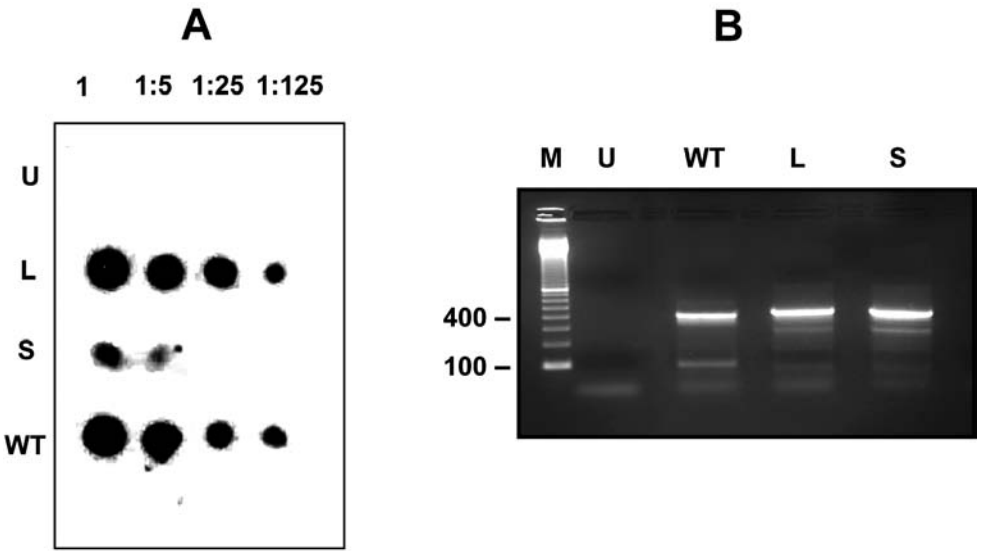

Fig. 2. Detection of PFBV in *P. zonale* plants inoculated with *in vitro* transcripts derived from pSP18-IC. **A**) Dot-blot hybridization of total RNA extracted from inoculated (L) and systemic (S) leaves (seven and thirty days after inoculation, respectively). Undiluted and five-fold dilutions (numbers on the top) of the RNA samples were applied onto nylon membranes and hybridized with a ^{32}P-labeled DNA probe derived from the 3' terminus of the PFBV genome. Total RNA from plants naturally infected by PFBV (WT) and from mock inoculated plants (U) was included as positive and negative control, respectively. **B**) RT-PCR analysis of the same RNA preparations using a pair of PFBV specific primers, CH3 and CH4. A 100 bp DNA ladder (M) was included as size marker (the bands of 100 and 400 bp are indicated on the left).

In vitro transcripts derived from pSP18-IC were also inoculated to *P. zonale*, a natural host of PFBV. Except for one case in which chlorotic spots were observed on one leaf, the inoculated plants did not develop obvious symptoms under greenhouse conditions but the virus was detectable by dot-blot hybridization in both inoculated and systemic leaves (Fig. 2A). The infection was further confirmed by RT-PCR analysis with primers CH3 (5'-CGATATCTCAAGAAATTCGAAC-3'), homologous to nt 1,281-1,302 of the PFBV genomic RNA, and CH4 (5'-TCATGAGGTGCCTCGTTATG-3'), complementary to nt 1,664-1,683, which yielded a DNA of the expected size (~400 nt) from extracts of pSP18-IC inoculated plants but not from healthy controls (Fig. 2B). The usual lack of symptoms in the pSP18-IC infected plants parallels the behaviour of the original isolate, which only induced symptoms under certain, not well defined, conditions. This is in agreement with the results of a recent survey showing that the vast majority of naturally PFBV-infected plants are symptomless but some of them may develop chlorotic mottling or petal colour breaking at high temperatures or under water stress (Alonso and Borja, 2005), thus illustrating the strong influence of environmental parameters on symptom elicitation.

To conclude, we have produced a full-length clone of PFBV from which infectious transcripts can be obtained. The availability of this biologically active PFBV cDNA clone is currently allowing us to perform a genetic analysis of the virus.

ACKNOWLEDGEMENTS

We are grateful to Dr. M. Borja (Fundación Promiva, Madrid) for providing isolate PFBV-SP18 and for valuable comments in the course of this work. This research was supported by grants AGL2000-0942 and AGL2003-04249 from the Ministerio de Ciencia y Tecnología de España and by grant Grupos 03/064 from the Generalitat Valenciana (to C.H.). P.R. was the recipient of predoctoral fellowships from the Generalitat Valenciana and from CSIC (I3P program).

REFERENCES

Adkins S.T., Nameth, S.T., 1989. Detection of viruses in florist geraniums using a simplified method of dsRNA analysis. *Phytopathology* **79**: 1197.

Alonso M., Borja M., 2005. High incidence of *Pelargonium line pattern virus* infecting asymptomatic *Pelargonium* spp. in Spain. *European Journal of Plant Pathology* **112**: 95-100.

Bouwen I., Maat D.Z., 1992. *Pelargonium flower-break* and Pelargonium line pattern viruses in the Netherlands; purification, antiserum preparation, serological identification, and detection in pelargonium by ELISA. *Netherlands Journal of Plant Pathology* **98**: 141-156.

Franck A., Loebenstein G., 1994. Virus and virus-like diseases of pelargonium in Israel. *Acta Horticulturae* **377**: 31-39.

Krczal G., Albouy J., Damy I., Kusiac C., Deogratias J., Moreau J., Berkelman B., Wohanka W., 1995. Transmission of *Pelargonium flower break virus* by irrigation systems and by thrips. *Plant Disease* **79**: 163-166.

Lommel S.A., Martelli G.P., Rubino L., Russo M., 2005. Family *Tombusviridae*. In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (eds.). Eighth Report of the International Committee on Taxonomy of Viruses, pp. 907-936. Academic Press, San Diego, USA.

Morris T.J., Dodds J.A., 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology* **69**: 854-858.

Paludan N., Begtrup J., 1987. *Pelargonium flower break virus* and *Tomato ringspot virus*: infection trials, symptomatology and diagnosis. *Danish Journal of Plant and Soil Science* **91**: 183-194.

Rico P., Hernández C., 2004. Complete nucleotide sequence and genome organization of *Pelargonium flower break virus*. *Archives of Virology* **149**: 641-651.

Rochon D.M., 1999. Tombusviruses. In: Granoff A., Webster R.G. (eds.). Encyclopedia of Virology, 2nd ed., pp. 1789-1798. Academic Press, San Diego, USA.

Sambrook J., Russell D.W., 2001. Molecular cloning - A laboratory manual. 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Stone O., 1974. *Pelargonium flower break virus*. *CMI/AAB Description of Plant Viruses*, No. 130.

Stone O.M., 1980. Nine viruses isolated from pelargonium in the United Kingdom. *Acta Horticulturae* **110**: 177-182.

Verwoerd T.C., Dekker B.M., Hoekema A., 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research* **17**: 2362.

Yu H.H., Wong S.M., 1998. Synthesis of biologically active cDNA clones of cymbidium mosaic potexvirus using a population cloning strategy. *Archives of Virology* **143**: 1617-1620.

Received 22 November 2005
Accepted 31 January 2006