Infectivity of recombinant strawberry vein banding virus DNA

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Infectivity of the cloned DNA genome of strawberry vein banding virus (SVBV) was demonstrated by particle bombardment of 4-week-old strawberry (Fragaria vesca L. var. UC-5) plants with gold particles coated with the putative full-length 7–9 kb viral DNA. Vein banding symptoms developed on 15% of inoculated plants 6–7 weeks post-inoculation. An approximate 1–25-mer of the viral DNA was cloned into the binary vector pCGN1547. Particle bombardment of this construct into strawberry plants gave an infection rate of 75%. The construct was used for transformation of Agrobacterium tumefaciens, and infiltration of these cells into healthy strawberry leaves resulted in development of vein banding symptoms in 100% of inoculated plants. Gel electrophoresis, Southern blot hybridization with an SVBV probe and sequence analyses of PCR-amplified DNA fragments were used to confirm SVBV infection in symptomatic plants.

Strawberry vein banding virus (SVBV) has a double-stranded DNA genome (Stenger et al., 1988; Petrzik et al., 1998) of approximately 8 kbp encapsidated in icosahedral particles of approximately 45 nm diameter (Kitajima et al., 1973; Morris et al., 1980). SVBV is transmitted in a semi-persistent manner by several aphid species and infects only strawberry (Frazier, 1955). SVBV is classified as a member of Caulimoviridae (Kitajima et al., 1973; Morris et al., 1980; Petrzik et al., 1998). However, due to difficulties in virus purification and mechanical inoculation of strawberry plants, the capacity of this virus alone to induce the vein banding disease has not been unequivocally demonstrated. Purified particles of SVBV have never been shown to be infectious. Stenger et al. (1988) constructed a genomic clone (pSVBV-E3; Stenger et al., 1988) and a strawberry plant infected with SVBV (same isolate from which the clone was obtained) were kindly provided by T. J. Morris (Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA).

Virion DNA isolation, PCR and partial sequencing were done as reported previously (Mahmoudpour, 2000) to verify that the pSVBV-E3 clone was full-length. Mechanical inoculation and particle bombardment were used to inoculate indicator plants (UC-4 and UC-5 strawberries, Chenopodium spp. and Nicotiana spp.) with different inocula. Partially purified virions (prepared according to Stenger et al., 1988 and/or Hull et al., 1976), viral genomic DNA and/or cloned SVBV DNA released from pSVBV-E3 were used as inocula. To prevent browning of plant extracts β-mercaptotethanol (β-ME) was added to a final concentration of 1–0% (v/v). A helium particle gun (Biolistic Particle Delivery System, model PDS-1000, Dupont) was used to bombard 4-week-old UC-5 strawberry plants with gold particles coated with DNA according to Paplomatas et al. (1994). Partially purified virus DNA, cloned SVBV DNA released from pSVBV-E3, and the SVBV 1-25-mer in pCGN1547 were used as DNA sources and delivered at a pressure of 1550 p.s.i. Gold particles alone were used as negative control.

Inoculation experiments, except where noted, were repeated at least three times. Inoculated plants were maintained in a glasshouse and were observed periodically for symptom expression. All inoculated plants were also assayed for SVBV infection by PCR (Mahmoudpour, 2000). pCGN1547 (McBride & Summerfelt, 1990), was used to construct a multimeric copy of SVBV DNA. This vector, in

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the presence of a 200 kb disarmed Ti-plasmid in Agrobacterium tumefaciens LBA4404 (Invitrogen), is commonly used for gene transformation of plants and also has been used for agroinoculation of geminiviruses (Hou et al., 1998).

To engineer the 1:25 × construct, a 2-0 kb PstI–BamHI SVBV fragment (nucleotides 5899 to 33 of SVBV) was excised from pSVBV-E3 and inserted into the multiple cloning site (MCS) of pCGN1547 digested with PstI and BamHI, to give a pCGN1547/2-0-kb SVBV recombinant. The full-length SVBV DNA insert was released from pSVBV-E3 by EcoRI digestion, and then re-ligated to obtain circular or linear multimeric SVBV DNA. The re-ligated DNA was digested with PstI to generate a 7-9 kb PstI fragment, which was ligated into pCGN1547/2-0-kb SVBV digested with PstI giving rise to pCGN1547SVBV1-25 recombinant. Through restriction analysis, recombinant plasmids having a 7-9 kb BamHI fragment were considered to have the 1:25-mer in the correct orientation, the infectivity of which was demonstrated by bombarding 12 UC-5 plants (one replicate only).

Electro–competent A. tumefaciens LBA4404 cells (Invitrogen) were transformed with pCGN1547SVBV1-25 construct by electroporation according to the manufacturer’s instructions. Transformants were selected on 2 × YT agar plates (Sambrook et al., 1989) supplemented with 10 μg gentamicin ml⁻¹. Colonies were screened for the presence of the 24 kb plasmid or 7-9 kb PstI fragment by gel electrophoresis. PCR was used to identify the transformants using primers specific for the SVBV coat protein gene.

The A. tumefaciens strain carrying pCGN1547SVBV1-25 was propagated in liquid culture at 28 °C using 2 × YT medium supplemented with 100 μg streptomycin ml⁻¹ and 10 μg gentamicin ml⁻¹. Non-transformed LBA4404 cells (negative control) were grown under the same conditions except without gentamicin. Cells were recovered by spinning at 5000 g and suspended in 0-1 vols 100 mM phosphate buffer, pH 6-5-7-0. Bacterial suspensions were infiltrated into the intercellular spaces of 4-week-old strawberry leaves using a 5-0 ml syringe without any needle. Multiple sites were inoculated per leaf and three to five leaves were inoculated per plant. Inoculated plants were maintained in a glasshouse.

Viral DNA was prepared according Mahmoudpour (2000) and analysed in 1% agarose gels in Tris/acetate/EDTA (TAE) buffer followed by ethidium bromide staining. The electrophoresed DNA was transferred to nylon membranes and analysed by Southern blot hybridization. Colorimetric or chemoluminescent detection methods were used to detect DNA on membranes. The digoxigenin-11-dUTP-labelled pSVBV-E3 probe was prepared by random priming using Klenow fragment according to Miltenburg et al. (1995). Viral DNA obtained from infected plants was examined with or without restriction enzyme digestion (BamHI and BamHI/EcoRI) along with total genomic DNA from uninfected UC-5 strawberry plants as a control. pSVBV-E3 digested with EcoRI was used as a marker and a positive control in all gels and Southern blot hybridization analyses.

To confirm the integrity of the cloning site of the putative full-length clone in pSVBV-E3 (i.e. to be certain that a small EcoRI fragment was not released during cloning) a 1500 bp fragment of virion DNA, including the cloning site, was PCR-amplified and sequenced on both strands. Only a single EcoRI site was identified in this 1500 bp fragment and the sequence across the cloning site was identical with the sequence of the two ends of the insert DNA. These results are consistent with pSVBV-E3 having a full-length SVBV clone.

Repeated attempts to infect strawberry plants by mechanical inoculation with cloned SVBV DNA were unsuccessful, irrespective of using the linear or self-ligated monomer (i.e. circularized DNA). Table 1 summarizes the inoculation data obtained for the three replicates of biolistic inoculation and agroinoculation on UC-5 strawberries. Symptoms developed in 16% of plants inoculated with monomer-coated particles approximately 6 weeks post-inoculation. Control plants inoculated with gold particles alone or those bombarded with the gold particles coated with DNA from partially purified virus did not develop symptoms (Fig. 1). The plants bombarded with gold particles coated with pCGN1547SVBV1-25 developed symptoms even at a higher infection rate of 75%. Systemic infection with SVBV in symptomatic plants was verified by PCR analysis of the young terminal leaves.

Agroinoculation of both UC-4 and UC-5 strawberry plants with A. tumefaciens carrying the infectious pCGN1547SVBV1-25 construct resulted in 100% infection (Table 1). Vein banding symptoms similar to those of Fig. 1 developed in agroinoculated plants 3–4 weeks post-inoculation, compared with 6–7 weeks for particle bombardment.

As shown in Fig. 1 symptoms on SVBV-infected plants (vegetatively propagated California isolate) and on the plants infected after bombardment with cloned SVBV DNA, were indistinguishable (i.e. mild vein-banding symptoms and chlorosis along the veins). Infected plants were also stunted and their leaves were not expanded as fully as on non-infected plants. Vein banding symptoms in all infected plants eventually became attenuated, but reappeared on new growth after removal of older leaves.

Plant total genomic DNA seen in ethidium bromide-stained gels (Fig. 2A) did not hybridize with the probe in Southern blot hybridization analyses (Fig. 2B). A 7-9 kb viral DNA band shown in lane 1 was detected only in linearized form. A linear 7-9 kb fragment shown in lane 2 was generated by digesting the circular form of viral genomic DNA with BamHI. The bands running at 3-9 kb are presumed to be the product of BamHI digestion (the BamHI site is 33 bases from the alpha nick) and a physical breakage at the single-stranded beta-nick position.
Table 1. Results with two different procedures for inoculation of strawberry (F. vesca L., var. UC-5) plants with cloned SVBV DNA

For particle bombardment, gold particles were coated with the 7.9 kb SVBV monomer excised from pSVBV-E3 with EcoRI. pCGN1547SVBV1-25 supercoiled DNA was similarly inoculated to confirm its infectivity. For agroinoculation, Agrobacterium tumefaciens LBA4404 cells transformed with pCGN1547SVBV1-25 were infiltrated into leaves. Virus infection was verified by symptom expression and PCR analysis. Negative control plants were bombarded with gold particles alone (biolistic experiments), or infiltrated with non-transformed LBA4404 cells. NA, Not assessed.

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<th>Agroinoculation</th>
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<tr>
<td></td>
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<td>pCGN1547/SVBV1-25</td>
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Fig. 1. Symptoms of SVBV in leaves of indicator UC-5 strawberry. (A) Plant vegetatively propagated from a known SVBV-infected plant. (B) Plant inoculated by particle bombardment with the monomeric insert DNA of pSVBV-E3. (C) Uninfected healthy control.
The primary goal of this study was to investigate whether the cloned SVBV DNA in pSVBV-E3 was infectious. According to Stenger et al. (1988), the lack of infectivity of the SVBV clone might indicate that the genome was not intact. The presence of an additional EcoRI site adjacent to the cloning site of SVBV DNA in pSVBV-E3 could have resulted in the loss of a small DNA fragment during cloning, thereby rendering the clone noninfectious. In this study, a PCR-amplified SVBV DNA fragment, containing the EcoRI cloning site, was sequenced and shown not to have an additional EcoRI site near the cloning site. Therefore, the clone in pSVBV-E3 was most likely a full-length clone.

Sap transmission of SVBV has been unsuccessful (Frazier, 1955; Morris et al., 1980; Stenger et al., 1988), and previous attempts failed to demonstrate the infectivity of pSVBV-E3 (Stenger et al., 1988). Demonstrating the infectivity of pSVBV-E3 was necessary to fulfill Koch’s postulates and prove that SVBV causes the disease symptoms from which it has derived its name.

When linear monomers of the SVBV (released from pSVBV-E3) were bombarded into leaves of strawberry plants, vein banding symptoms developed 6–7 weeks post-inoculation, where infection was confirmed by PCR and Southern blot hybridization analyses. These results establish that SVBV is the causal agent of strawberry vein banding disease and that the disease is not caused by mixed infection with another virus.

Transmission of viruses by agroinoculation has been demonstrated previously for caulimoviruses (Gal et al., 1992), geminiviruses (Hou et al., 1998) and potexviruses (Lamprecht & Jelkmann, 1997). All three viruses are transmissible either mechanically or by a vector. This method gave an infection rate of 100% in strawberry plants with SVBV (Mahmoudpour, 2000). The high rate of SVBV transmission by agroinoculation established that this method could be applied to viruses which have no means of manual inoculation.

Agarose gel and Southern blot hybridization (Fig. 2) of SVBV DNA isolated from partially purified virion preparations from vegetatively propagated UC-5 strawberries or from particle gun or agroinoculated strawberries confirmed SVBV infection in plants showing the vein banding symptoms.

Digesting the DNA with two restriction enzymes with unique sites in SVBV DNA (BamHI and EcoRI) along with pSVBV-E3 (Stenger et al., 1988) as the control and hybridizing the bands with a pSVBV-E3-derived DNA probe provided further evidence for identity of the viral DNA from these preparations. As seen in Fig. 2(A), these DNA fragments co-migrated in the gel and similar restriction sites were present in both DNAs. The single-stranded nicks of SVBV DNA found in this study verified the work of Stenger et al. (1988).
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REFERENCES


