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Infectious cDNA clone used to identify strawberry mild yellow edge-associated potexvirus as causal agent of the disease

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A full-length *in vivo* infectious cDNA clone of strawberry mild yellow edge-associated potexvirus (SMYEaV) was constructed and used to inoculate *Fragaria vesca* 'Alpine' seedlings and *Rubus rosifolius*. Both host plants could be infected using particle bombardment or agroinoculation, but not by mechanical inoculation. A method that used potted strawberry plants for particle bombardment resulted in high survival and infection rates. The plants developed systemic infection and virus particles were detected by ELISA and immunoelectron microscopy. Mechanical inoculation of *Chenopodium quinoa* and *C. foetidum* with the 35S construct resulted in localized infections. *F. vesca* 'Alpine' indicator plants produced symptoms that were indistinguishable from control plants inoculated with a naturally occurring isolate of strawberry mild yellow edge by graft or aphid transmission. These results suggest that SMYE potexvirus is the causal agent of strawberry mild yellow edge disease. As this virus is capable of causing the disease, we propose the name strawberry mild yellow edge potexvirus, with the acronym SMYEPV, to replace the name strawberry mild yellow edge-associated potexvirus.

Introduction

Strawberry mild yellow edge is one of the most common virus diseases of strawberry and was long believed to be caused by a luteovirus (Converse *et al.*, 1987). The association of the pathogen with the luteovirus group was based on electron microscopy studies (Yoshikawa *et al.*, 1984; Martin & Converse, 1985), symptomatology, and its transmission in a persistent manner by the strawberry aphid *Chaetosiphon fragaefolii* (Krczal, 1980; Converse *et al.*, 1987). Recently, a second virus associated with the disease, strawberry mild yellow edge-associated potexvirus (SMYEaV), was described morphologically and by its nucleotide sequence and genome organization (Jelkmann *et al.*, 1990, 1992). This potexvirus has been found consistently with all sources of strawberry mild yellow edge disease investigated (Jelkmann *et al.*, 1990; Hepp & Martin, 1992; Kaden-Kreuziger *et al.*, 1995; Quail *et al.*, 1995), but the role of the virus in the aetiology of the disease remains to be determined. The natural host range of SMYEaV is limited to strawberry, but it has been graft transmitted to *Rubus rosifolius* causing systemic infection. Mechanical transmission to *Chenopodium quinoa* results in a local infection. It can be detected reliably by immunosorbent electron microscopy (ISEM); immunocapture RT–PCR (IC-RT–PCR) and TAS-ELISA, using a polyclonal coating antibody from fusion proteins expressed in *Escherichia coli* and a monoclonal antibody as second antibody (Kaden-Kreuziger *et al.*, 1995; Quail *et al.*, 1995); or indirect DAS-ELISA using a polyclonal and a monoclonal antibody (Jawee & Adams, 1995). The genome organization of SMYEaV is typical of potexviruses, consisting of five open reading frames (ORFs) with the 5′ ORF being the putative viral RNA-dependent RNA polymerase followed by the 'triple gene block' and the capsid protein. The only observable irregularity is the occurrence of a proposed AUU initiation of translation for ORF2 instead of an AUG. Remarkably for a potexvirus, SMYEaV is transmitted in a persistent manner by the strawberry aphid *C. fragaefolii*. Heterologous encapsidation by an associated luteovirus has been proposed as a possible mechanism of aphid transmission (Jelkmann *et al.*, 1990, 1992).

Full-length genomic cDNA clones have been produced for a number of plant viruses. They were used to synthesize infectious transcripts *in vitro* by use of a bacteriophage RNA polymerase (T7, T3, SP6) or were linked to the cauliflower mosaic virus (CaMV) 35S promoter to generate *in vivo* transcripts (Boyer & Haenni, 1994; Baulcombe *et al.*, 1995;

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Gal-On *et al*., 1995; Fakhfakh *et al*., 1996). The latter technique omits *in vitro* transcription, is independent of RNA degradation and does not require capping of transcripts (Boyer & Haenni, 1994). Another advantage of *in vivo* transcripts is the possibility of agroinfection, which has been used for viruses that cannot be mechanically transmitted (Grimsley *et al*., 1987; Leiser *et al*., 1992). Particle bombardment has also been used to infect plants with cDNA clones under the control of the 35S promoter, resulting in increased infectivity compared to mechanical inoculation of the constructs (Gal-On *et al*., 1995; Fakhfakh *et al*., 1996). Infectious *in vitro* transcripts have been produced to date for the potexviruses white clover mosaic (Beck *et al*., 1990), potato X (Hemenway *et al*., 1990), papaya mosaic (Sit & AbouHaidar, 1993) and clover yellow mosaic (Holy & AbouHaidar, 1993).

In this paper, we report the 5′ end sequence of SMYEaV (Jelkmann *et al*., 1992) and the construction of an *in vivo* infectious cDNA clone linked to the CaMV 35S promoter at the 5′ end and the corresponding termination signal at the 3′ end. The study was undertaken to determine the role of the potexvirus in yellow edge disease and to investigate whether the virus is aphid transmissible without an assistor virus. As mechanical inoculation of SMYEaV to *C. quinoa* is possible (Jelkmann *et al*., 1990), but failed to infect strawberries and *R. rosifolius* (unpublished observations), biolistic inoculation and agroinfection were investigated as infection techniques.

Methods

■ **Virus sources.** The SMYEaV isolates used in this study were MY-18, which had been grafted into *Rubus rosifolius* (Martin *et al*., 1989), and D-74 from *Fragaria vesca* (Jelkmann *et al*., 1990). All plants were grown in an insect-free containment greenhouse at 22 °C with a 14 h photoperiod.

■ **5′ end determination of SMYEaV.** The 5′ end nucleotide sequence of SMYEaV RNA was determined using single-stranded RNA (ssRNA) from *R. rosifolius* infected with the MY-18 isolate. RNA was prepared by immunocapture PCR as described by Kaden-Kreuziger *et al*. (1995). The sequence was obtained by applying the 5′-AmpliFINDER RACE Kit (Clontech) according to the manufacturer's instructions. Briefly, the ssRNA was reverse transcribed with oligonucleotide 1 (5′ CAGCACCCCGGGCATCAACC 3′, nt 222–203: accession no. D01227). After ligation of the AmpliFINDER anchor, a nested PCR was performed using oligonucleotide 2 (5′ TTATGCGCTTGTATGCCTCA 3′, nt 162–143) and the AmpliFINDER anchor primer. The PCR product was subcloned and its sequence determined.

■ **Mutagenesis and construction of full-length cDNA.** The cloned PCR product of the virus 5′ end (p7RACE-BS) was subcloned in consecutive steps into pG35S1 (Commandeur *et al*., 1991), kindly supplied by U. Commandeur, to yield pG35S3 (Fig. 1*a*). The pG35S1 vector contains the *Hin*dIII cassette of pRT103 (Töpfer *et al*., 1987), including the 35S promoter and the polyadenylation signal of CaMV which had been ligated into the multiple cloning site of pGEM-3Zf+. Fifteen non-viral nucleotides between the 35S promoter initiation site of transcription and the first nucleotide of SMYEaV were removed by site-directed mutagenesis as described by Kunkel *et al*. (1987) to give pG35S4 (Fig. 1*a*). Subsequently, fragments of five SMYEaV cDNA clones (Jelkmann *et al*., 1992) were cloned into a Bluescript vector using the restriction sites *Xba*I, *Sac*II, *Hpa*I, *Bln*I and *Bst*EII (Fig. 1*b*). Finally, the insert was excised from the Bluescript vector and ligated to pG35S4 yielding the full-length cDNA clone pG35S-SMYEaV (Fig. 1*c*). For inoculation experiments, pG35S-SMYEaV was prepared on a large scale using the Plasmid Mega Kit (Qiagen) according to the manufacturer's instructions.

For agroinfection the *Nhe*I fragment of pG35S-SMYEaV, containing the 35S promoter, the full-length SMYEaV sequence and the poly-adenylation signal, was ligated into pBIN19 (Bevan, 1984) digested with *Xba*I to yield pBIN-SMYEaV. The construct was used to transform *Agrobacterium tumefaciens* strain LBA4404.

■ **Infectivity assays.** The full-length clone pG35S-SMYEaV was used for mechanical inoculation of 33-day-old *C. quinoa*, *C. murale*, *C. foetidum* and *C. foliosum*, 54-day-old *F. vesca* 'Alpine' seedlings, and 7-month-old *R. rosifolius* seedlings. All plants were kept in the dark for 12 h prior to inoculation. Young leaves were lightly dusted with carborundum and the entire surface of one or two leaves of each plant was rubbed with 10 µl of inoculum containing 10 µg of plasmid in 0·1 M phosphate buffer pH 7·0. The full-length cDNA clone was used for infection either undigested or *Nhe*I digested (170 bp upstream of the 35S promoter and 23 bp downstream of the terminator) (Fig. 1*c*). Negative control plants were mock-inoculated with buffer. After inoculation, leaves were rinsed with water and the plants maintained in a greenhouse. *Chenopodium* plants were analysed 7–30 days post-inoculation (p.i.) and *F. vesca* and *R. rosifolius* plants 1–2 months p.i. for the presence of viral antigen by TAS-ELISA and ISEM as described previously (Kaden-Kreuziger *et al*., 1995).

Particle bombardment was performed with the Biolistic PDS-1000 Particle Delivery System (Bio-Rad). 0·7 µg of pG35S-SMYEaV bound to tungsten particles was used at pressures of 450, 900 and 1350 p.s.i. (ca. 3·1, 4·1 and 9·3 MPa) for inoculation of 16-, 23-, 30- or 58-day-old *F. vesca* 'Alpine' seedlings as described by Sanford *et al*. (1991). Briefly, 7 µg plasmid DNA, sufficient for 10 deliveries, was mixed with 90 µl of M20 tungsten microcarrier particle suspension (60 µg/µl in 50% glycerol). The mixture was brought to a final volume of 400 µl containing 560 mM $CaCl_2$, 9 mM spermidine and 45% ethanol. The particles were pelleted and resuspended in 65 µl 95% ethanol. After sonication, 5 µl was pipetted immediately onto a macrocarrier, allowed to dry down and placed in the bombardment chamber of the apparatus. The seedlings were laid side by side on 0·5% water agar plates or left in soil at a target distance of 5–7 cm. Plants were then grown under greenhouse conditions.

A. tumefaciens strain LBA4404, grown in tryptic soy broth (TSB) media (Sigma) with streptomycin (50 mg/ml), was made competent as described by Nagel *et al*. (1990). Transformations using pBIN-SMYEaV were done by electroporation at standard conditions for *E. coli* as described by the manufacturer (Gene pulser: Bio-Rad). For agroinfection, bacterial cells were grown in 2·5 ml TSB with streptomycin (50 mg/ml) and kanamycin (25 mg/ml) at 28 °C overnight, collected by centrifugation (3 min, 10000 r.p.m.) and resuspended in 100 µl 10 mM $MgSO_4$ pH 6·0, either with or without 10 µM acetosyringone. 30 µl of this solution was mechanically inoculated on carborundum-dusted young leaves of 56-day-old *F. vesca* 'Alpine' seedlings. Plants inoculated by particle bombardment or agroinoculation were assayed by TAS-ELISA starting 14 days after inoculation. ELISA positive plants were also tested by ISEM (Jelkmann *et al*., 1990) using the crude antiserum no. 648 (Kaden-Kreuziger *et al*., 1995) at a dilution of 1:1000 for particle trapping and at a dilution of 1:50 for decoration. Inoculated strawberry seedlings were also observed at regular intervals for symptom expression and were compared with control plants.

■ **Aphid transmission.** Strawberry aphids were given an acquisition access period of 3–10 days on strawberries inoculated with the full-length clone by agroinfection or particle bombardment that tested positive for SMYEaV by ELISA. The inoculation access feeding time on healthy *F.*

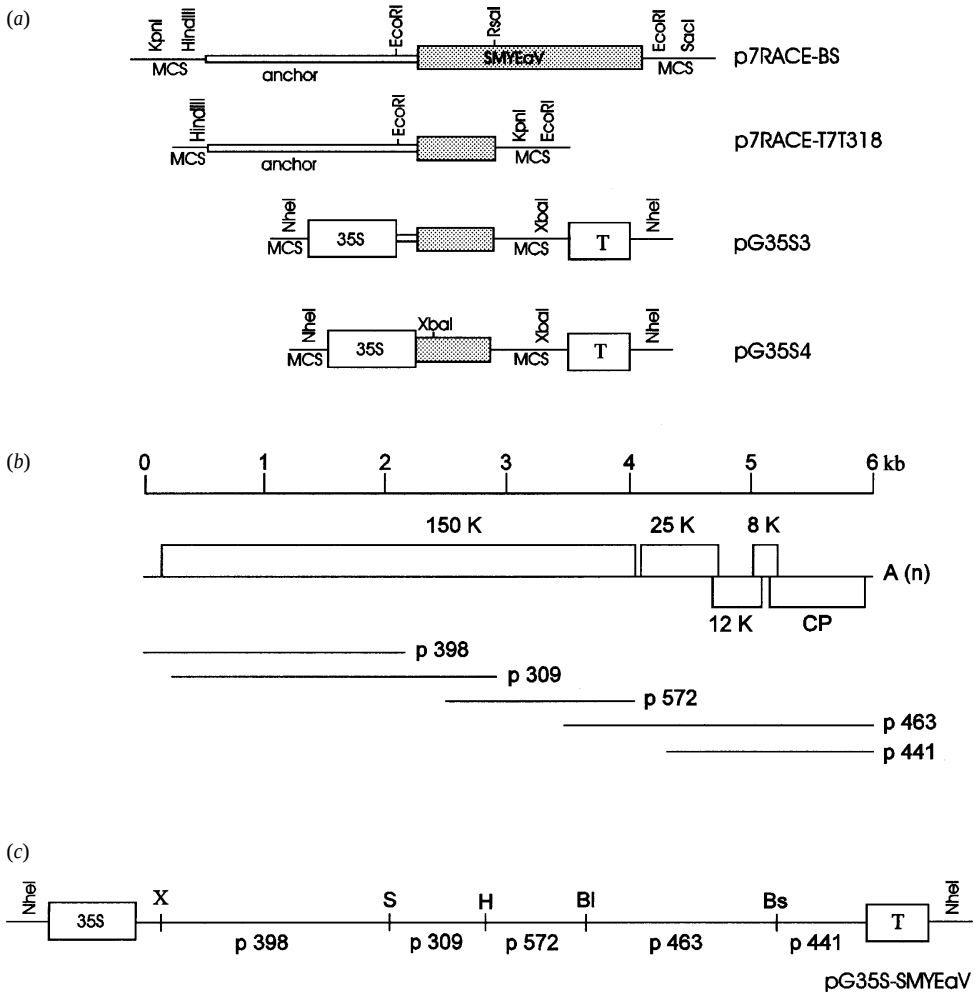

Fig. 1. Schematic representation of the SMYEaV full-length cloning strategy. (*a*) 5′ end subcloning and mutagenesis: a PCR product consisting of the RACE kit anchor and the 5′ end of SMYEaV was cloned into the MCS (*Eco*RV) of pBluescript M13+ vector; subsequent cloning with *Hin*dIII and *Rsa*I into pT_7T_318 yields p7RACE pT_7T_318; this construct was *Eco*RI digested, filled in with Klenow polymerase, *Kpn*I digested and ligated to pG35S1 to yield pG35S3; mutagenesis was performed to remove non-viral nucleotides between the promoter and the virus sequence. 35S, CaMV 35S promoter; T, CaMV terminator; MCS, multiple cloning site; thin lines represent parts of the vector sequence. (*b*) Structure of the SMYEaV genome and cDNA fragments involved in the construction of the full-length clone (pG35S-SMYEaV). (*c*) Schematic map of the full-length clone; restriction sites used in the construct were *Xba*I (X), *Sac*II (S), *Hpa*I (H), *Bln*I (Bl) and *Bst*EII (Bs).

vesca Alpine′ seedlings was 3–5 days. For the transmission tests, 5–10 aphids were used per plant (Krczal, 1980). After the inoculation access period the plants were sprayed with an insecticide. Virus detection by TAS-ELISA was performed 12, 29 and 60 days after the inoculation access period.

Results

cDNA cloning and infectivity assays

The 5′ end determination of the SMYEaV genomic RNA obtained by applying the AmpliFINDER RACE Kit (Clontech) revealed four nucleotides (nt 1–4) in addition to the sequence previously reported (Jelkmann *et al.*, 1992) (EMBL database accession number D01227). Furthermore, nucleotides 5 and 6 (previously nos 1 and 2) were found to be TC instead of AT. The total nucleotide sequence of SMYEaV consists of 5970 nucleotides. The six SMYEaV 5′ terminal nucleotides, confirmed in two independent experiments, consist of GAAATC. The full-length construct (pG35S-SMYEaV) was tested for infectivity by mechanical inoculation and particle bombardment, or by agroinoculation after insertion into pBIN19.

Local infections of mechanically inoculated *C. quinoa* and *C. foetidum* leaves were monitored by TAS-ELISA 12–20 days p.i.; A_{405} readings reached values of 0·4–1·2, whereas negative controls were 0·03. Infections were detected in 21 out of 22 *C. quinoa* and in four out of 22 *C. foetidum* plants. All plants remained symptomless and no systemic infection was recorded. Filamentous particles, identical in size and morphology to those trapped from *R. rosifolius* infected with the MY-18 isolate and from strawberry control plants infected with the D-74 isolate, were trapped in ISEM tests from plant sap of *C. quinoa*

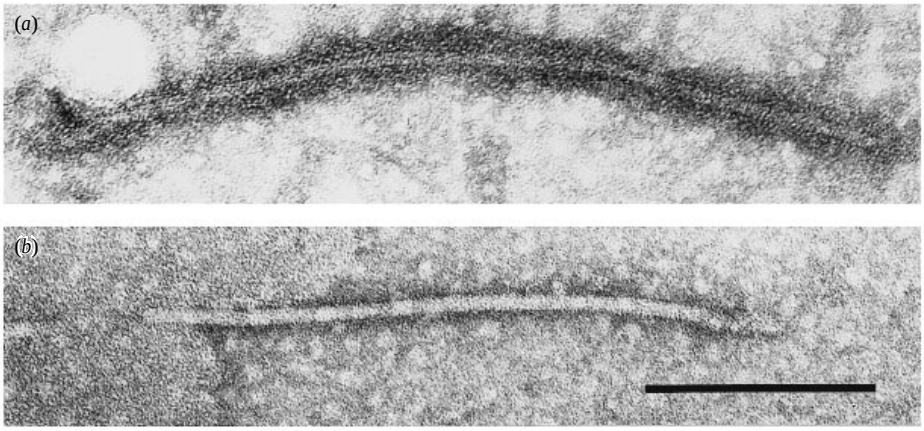

Fig. 2. Electron micrographs of virus particles from (*a*) *C. quinoa* mechanically infected with pG35S-SMYEaV, trapped and decorated with SMYEaV coat protein fusion protein-specific antiserum; (*b*) *F. vesca* 'Alpine' positive control plants infected with the D-74 isolate, trapped with SMYEaV coat protein fusion protein-specific antiserum. Bar, 200 nm.

and *C. foetidum* plants. In decoration tests the particles reacted strongly with the coat protein fusion protein-specific antiserum (Fig. 2). Similar infectivity results were obtained whether the plasmid was circular, or linearized with *Nhe*I to cut out the viral sequence flanked by the 35S promoter and the polyadenylation signal. No infections were detected after mechanical inoculation of *F. vesca* 'Alpine', *R. rosifolius*, *C. murale* or *C. foliosum* seedlings.

Of the *F. vesca* 'Alpine' plants inoculated by particle bombardment, only 55 of the 100 plants laid on water agar during inoculation survived the treatment, whereas 26 of 30 bombarded while in soil survived. Local and systemic virus infections were detected by ELISA in a total of 21 out of 81 strawberry plants at 20–100 days p.i.; A_{405} readings ranged from 0·1–1·1 for inoculated plants and averaged 0·05 for healthy controls. The ELISA results were confirmed by ISEM tests, which revealed virus particles with the same morphology as those obtained from mechanically inoculated *C. quinoa* and from aphid-infected strawberry control plants containing the D-74 isolate. Infected strawberry plants were severely stunted and declined 2–3 months after inoculation. The newly emerging leaves became successively smaller, were cupped and showed slight marginal chlorosis and subsequent leaf senescence. Leaves that were fully developed during inoculation remained unaffected. The symptoms were essentially the same as those obtained by graft inoculation of 3-month-old *F. vesca* 'Alpine' seedlings with the D-74 isolate, but were more severe. Only eight out of 55 plants that were placed on agar during bombardment were infected with SMYEaV compared with 13 out of 26 plants kept in soil during bombardment. The rate of infection was apparently independent of seedling age and delivery pressure.

Agroinfection of 2-month-old *F. vesca* 'Alpine' seedlings resulted in symptoms 13 days p.i. on the newly emerging leaves (Fig. 3). Symptoms were identical to those observed after biolistic inoculation of strawberry plants. The inoculated leaves remained symptomless. Control infections with *A. tumefaciens* LBA4404 resulted only in healthy, growing strawberry plants. All inoculated plants survived the treatment. Virus was detected by ELISA in 41 out of 66 inoculated plants, in both locally and systemically infected leaves. The addition of acetosyringone did not influence the rate of infection. Agroinfection of *R. rosifolius* seedlings with *A. tumefaciens*: pBIN-SMYEaV resulted in systemic infection of seven out of nine plants. No symptoms were observed in these *R. rosifolius* plants or in controls carrying the MY-18 isolate following graft inoculation.

Aphid transmission

Transmission tests to *F. vesca* 'Alpine' test seedlings were made with *C. fragaefolii* to demonstrate whether SMYEaV is vector-transmissible by itself or whether a helper mechanism is necessary for transmission. Eight out of 17 control plants aphid-inoculated with isolate D-74 displayed typical symptoms 2 weeks p.i. and tested positive in ELISA. In contrast, all 89 test plants inoculated by aphids that had fed on *F. vesca* 'Alpine' plants infected with MY-18 by agroinfection or particle bombardment remained virus-free. The A_{405} readings of the control plants 1 month after transmission were 0·3–1·15, while the values for healthy *F. vesca* 'Alpine' averaged 0.05. The ELISA tests were continued until 80 days p.i.

Discussion

In this paper we describe the cloning and use of a full-length cDNA clone of SMYEaV which is infectious *in vivo* to investigate the aetiology of strawberry mild yellow edge disease, vector transmission of the virus and different inoculation techniques to infect strawberries. A cDNA library representing all but the six 5′-terminal nucleotides of SMYEaV was previously sequenced (Jelkmann *et al.*, 1992). The six 5′ end nucleotides of the genomic RNA were found to be GAAATC, thus differing at position 5 from the conserved 5′

Fig. 3. Symptoms of strawberry mild yellow edge disease in *F. vesca* 'Alpine' seedlings after infection with *A. tumefaciens* LBA 4404:pBIN-SMYEaV, (*a*) 20 and (*b*) 90 days p.i. Left to right: mock-inoculated control plant; two plants infected with cloned material.

end motif GAAAAC identified in the potexviruses plantago asiatica mosaic (PlAMV; Z21647); foxtail mosaic (FMV; M62730); narcissus mosaic (NMV; D13747); clover yellow mosaic (CYMV; D29630); white clover mosaic (WClMV; X06728); bamboo mosaic (BaMV; D26017); potato X (PVX; M72416); and potato aucuba mosaic (PAMV; S73580). Other exceptions to date are reported for papaya mosaic potexvirus (PMV; D00580) with a G in position 6 and cassava common mosaic potexvirus (CsCMV; U23414) with a C in position 1.

The strategy of producing an *in vivo* infectious potexvirus

cDNA driven by the CaMV 35S promoter, as reported for PVX (Baulcombe *et al.*, 1995), was chosen to circumvent disadvantages of *in vitro* transcripts such as RNA degradation and the general requirement for 5′ cap and 3′ polyadenylation (Boyer & Haenni, 1994). Most important, we anticipated difficulties in inoculating strawberry plants with this unusual aphid-transmissible potexvirus unless it was vector driven by agroinoculation. The technique has been used for beet western yellows luteovirus (BWYV), which is transmissible by aphids but not by mechanical means (Leiser *et al.*, 1992). Transmission of viruses from herbaceous host plants to strawberry is difficult and not reliable (Jha, 1961; Greber, 1979). There are rare reports of back-transmission of strawberry viruses. Jha (1961) was able to transmit only one of three arabis mosaic virus (AMV) isolates to strawberry plants, and this with some difficulty. This is in agreement with our results, showing a high rate of local infections in *C. quinoa* and *C. foetidum* plants, but failure of mechanical inoculation of strawberries and *R. rosifolius*. It is reasonable to assume that the release of phenolic compounds or change in pH during mechanical inoculation prevents infection. Both effects have been proven to have a crucial impact on back-transmission to fruit plants, resulting from phenolic compounds which oxidize to quinones when extracts of infected leaves are exposed to air or low pH (Cropley, 1964; Fulton, 1966; Vetten, 1977).

Several reports on the use of *in vitro* transcripts (Boyer & Haenni, 1994) or cloned cDNA under control of the 35S promoter (Gal-On *et al.*, 1995) report failure or great reduction of infectivity when extra 5′ nucleotides were present between the promoter and the viral sequence. Therefore, in this study non-viral nucleotides were removed by site directed mutagenesis at the 5′ end, as reported for 35S constructs of three potyviruses (Maiss *et al.*, 1992; Gal-On *et al.*, 1995; Fakhfakh *et al.*, 1996). Particle bombardment (Gal-On *et al.*, 1995) and agroinoculation (Leiser *et al.*, 1992; Prüfer *et al.*, 1995), both rarely used techniques for inoculation of infectious cloned cDNA of RNA viruses under control of the CaMV 35S promoter, were proven to be useful techniques for inoculation of strawberry seedlings. Whereas Fakhfakh *et al.* (1996) reported difficulties with small tobacco plants and used detached leaves, our results demonstrate the usefulness of small strawberry plants placed in the vacuum chamber of the particle gun apparatus. Although biolistic inoculation resulted in lower infection rates compared to agroinoculation, this technique requires no additional cloning of the construct into a binary vector and requires less stringent conditions for biological safety.

Strawberry mild yellow edge disease is defined by its symptom expression on sensitive indicator plants (Converse *et al.*, 1987). The symptoms on *F. vesca* 'Alpine' indicator plants induced by particle bombardment or by agroinoculation with the full-length infectious cDNA clone were indistinguishable from those obtained after aphid or graft transmission of the D-74 controls, and it is thus concluded that SMYEaV is the sole causal agent of strawberry mild yellow edge disease. It is therefore suggested that the virus is renamed strawberry mild yellow edge potexvirus (SMYEPV).

The experiments described in this paper allowed strawberry seedlings to be infected with SMYEPV alone, with certainty, for the first time. This virus was hitherto only transmissible by aphids or by grafting, and both techniques would also have transmitted any assistor virus which may have been present. Unless point mutations in the infectious construct were the cause of non-transmissibility, failure of aphid transmission of SMYEPV indicates that a helper mechanism is necessary for vector transmission, as previously suggested by Jelkmann *et al.* (1990). Aphid transmission of SMYEPV may be mediated by heterologous encapsidation with a luteovirus in a mixed infection. Members of the umbravirus genus are commonly encapsidated by the coat protein of a helper luteovirus – lettuce speckles mottle virus (LSMV) (Falk *et al.*, 1979); carrot mottle virus (CMoV) (Waterhouse & Murant, 1983); and groundnut rosette virus (GRV) (Hull & Adams, 1968; Rajeshwari *et al.*, 1987) – but this has not been reported for a potexvirus.

Both the inoculation techniques described for infection of strawberries will be of particular use for the study of gene functions of SMYEPV, e.g. the irregularity in initiation of translation of ORF2 (Jelkmann *et al.*, 1992) and its implication in distribution of the virus which is probably limited to phloem companion cells (Jelkmann *et al.*, 1990). The technique will also be of general use for investigating other poorly characterized strawberry viruses such as strawberry mottle (SMoV) and strawberry crinkle (SCV) (Frazier *et al.*, 1987; Mellor & Krczal, 1987).

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