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Editorial

Host Range Determination and Relative Quantification of Susceptibility to Strawberry Vein Banding Virus Infection using Agroinoculation and PCR

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Abstract

Due to lack of success in mechanical inoculation, and the low efficiency of particle bombardment inoculation, *Agrobacterium tumefaciens* cells carrying a partial tandem repeat construct of strawberry vein banding virus (SVBV) were used to inoculate plants to assess the host range of SVBV. An infection rate of 75 - 100% was achieved with agroinoculation of 23 different indicators and widely grown cultivars of strawberry (*Fragarria spp*.). Infected cultivars differed in symptom severity and had variable virus titers as relatively were quantified by end-point PCR analyses. Agroinoculation of SVBV to several common herbaceous indicator plants, close relatives of strawberry in the *Rosaceae*, and to plants infected by other caulimoviruses revealed that only burnet (*Poterium sanguisorba*) was a systemic host of SVBV. SVBV-infected burnet plants were symptomless, but were positive for virus infection by PCR. An ornamental strawberry (*F. x ananassa* cv. Temptation) was the least susceptible to SVBV infection. Several cultivars (e.g., the newly released Carlsbad) were found to be more susceptible.

Keywords: *Inoculation; Detection; Screening new strawberry cultivars for susceptibility to SVBV infection*

Abbreviations: SVBV: Strawberry vein banding virus; PCR: Polymerase Chain Reactions; F: Fragaria; pSVBV-E3: Name of the SVBV DNA clone inserted in pUC8 plasmid; FPS: Foundation Plant Service

Introduction

Strawberry vein banding virus (SVBV) was first described by Frazier (1955) [1] after differential aphid transmission to susceptible wild strawberries. He described disease symptomatology, identified wild strawberry plants as suitable virus indicators, and demonstrated virus transmission by various aphids, dodder (*Cuscuta subinclusa*), and grafting. Virus-vector interactions (i.e., specificity of aphid species, acquisition access and retention times, semi-persistent manner of transmission, and transmission efficiency), and the inability to transmit the virus via sap were established by Frazier (1955) [1]. Similar studies focusing on aphid vectors of SVBV and symptomatology were used as the basis for naming the virus [2-7]. (Prentice, 1952; Schöniger, 1958; Frazier and Posnette, 1958; Frazier, 1960; Mellor and Forbes, 1960; Miller and Frazier, 1970; Frazier and Converse, 1980).

Stenger., *et al*. (1988) [8] purified and cloned the SVBV genome (pSVBV-E3). By the techniques available at that time, Stenger and coworkers were unable to demonstrate the infectivity of the clone. Rub-inoculation of the excised SVBV DNA, as a linear monomer or self-ligated circular genome, failed to result in infection. In contrast, parallel control experiments demonstrated that a clone of CaMV was infectious to turnip after mechanical inoculation [8,9].

The infectivity of the cloned SVBV genome in pSVBV-E3 and completion of Koch's postulates for SVBV was accomplished by particle-gun bombardment of UC-5 strawberry plants with gold particles coated with the viral DNA [10,11]. However, this method of inoculation was inefficient (15 - 20% infection). Agroinoculation proved to be a far more efficient inoculation procedure (100% infection) as demonstrated by Mahmoudpour [10,11].

The primary objective of this study was to utilize agroinoculation to determine the host range of cloned SVBV DNA. Multiple PCR analyses were used to screen inoculated plants for systemic SVBV infection. Inoculated plants were grouped as non-hosts or hosts for SVBV. The host plants (mostly strawberry cultivars) were further grouped according to symptom expression. Another objective of this study was to determine relative virus titer in infected strawberry plants by PCR dilution end-point analysis. The infected strawberry cultivars and indicators were ranked on the basis of relative virus titer and susceptibility to SVBV infection.

Materials and Methods

SVBV isolate and the SVBV DNA clone

The genomic clone of SVBV (pSVBV-E3, American Type Culture Collection No. 45058) in pUC8 was obtained by Stenger., *et al*. [8] using a California SVBV isolate. The SVBV isolate was maintained in *Fragaria vesca* L. var. UC-5 strawberry. Virus-infected plants were propagated by rooting runners. The cloned SVBV DNA and the virus isolate were kindly provided by T. J. Morris.

Preparation of inoculum

Constructing the partial tandem repeat of SVBV DNA in a binary vector (pCGN1547, McBride and Summerfelt, 1990) [12], and transforming *Agrobacterium tumefaciens* LBA4404 cells (Invitrogen®) to prepare inoculum have been described [10,11].

Agroinoculation

Agroinoculation to generate SVBV-infected strawberry plants was conducted according to Mahmoudpour [11]. Transformed LBA4404 cells were grown in 2xYT medium supplemented with 100 µg/ml of streptomycin and 10 µg/ml of gentamycin. The cultures were incubated at 28°C in a shaking incubator. Non-transformed LBA4404 cells (negative control) were grown under the same conditions, except for the absence of gentamycin. Cells were collected by centrifugation (5,000g) and suspended in 0.1 volumes of 100 mM phosphate buffer, pH 6.5-7.0. Bacterial suspensions were infiltrated into intercellular spaces of 4-weeks-old strawberry leaves using a 5.0 ml syringe with no needle. Three sites were inoculated per leaf, and 3 to 5 leaves were inoculated on each plant. Inoculated plants were maintained in a glasshouse. Four to 30 plants were inoculated per species or cultivars. Inoculation of burnet plants was repeated three times with 16 plants inoculated per experiment, and infected plants (as determined by PCR) were maintained for 6 months.

Plants used for agroinoculation

Forty-five different plants representing 23 species, 15 genera, and 7 families were used in host range studies of SVBV as listed below. The plants marked with * are primary hosts for other members of *Caulimoviridae*.

- Family *Rosaceae*
- *Fragaria spp*.

o Strawberry virus indicators

1. *F. vesca* var. UC-4 and UC-5
2. *F. virginiana* var. UC-10 and UC-11

o Cultivated strawberries, *F. x ananassa* cv.:

3-18. Aromas, Camarosa, Capitola, Carlsbad, Chandler, Cuesta, Diamante, Gaviota, Fern, Irvine, Osogrande, Pacifica, Pajaro, Parker, Seascape, and Selva.

o Ornamental strawberries

19. *F. x ananassa* cv. Temptation (Patio strawberry)
20. *F. chiloensis* (Ground cover)

- 21. *Poterium sanguisorba* (Burnet)
- 22. *Rosa sp*. (Wild rose)
- 23. *Potentilla verna* (Cinquifoyle)
- *Rubus spp.*

24. *R. argutus* var. Marion (Marion blackberry)
25. *R. argutus* var. Ollalie (Ollalie blackberry)
26. *R. idaeus* cv. Brandywine raspberry
27. *R. idaeus* cv. Fall gold raspberry
28. *R. idaeus* var. Strigosus (known as Indian summer ever-bearing red raspberry, Heritage red raspberry, and New Burch red raspberry)
29. *R. laciniatus* var. Black stain (Black stain thornless blackberry)
30. *R. subgenus* var. Eubatus (Cheyenne blackberry)
31. *R. ursinus* (known as Nectarberry blackberry, Thornless boysenberry, and Thornless loganberry)
32. *Rubus sp*. (Tayberry: raspberry x blackberry)

Family Solanaceae

- *Nicotiana spp.*

33. *N. tabacum* cv. Xanthi
34. *N. occidentalis*
35. *N. clevelandii*
36. *N. benthamiana*

- *Solanum sp. (Garden Huckleberry)*

37. *Physalis edulis* (Gooseberry)
38. *Petunia hybrida* (Petunia)*

Family Cucurbitaceae

39. *Cucumis sativus* (Cucumber)

Family *Chenopodiaceae*

40. *Chenopodium quinoa*
41. *C. amaranticolor*

Family *Composite*

42. Dahlia pinnata (Dahlia)*

Family *Cruciferae*

43. Brassica campestris cv. Just Right (Turnip)*
44. Armoracia rusticana (Horse radish)*

Family *Nyctaginaceae*

45. *Mirabilis jalapa* (Four o'clock)*

The indicator plants used in these experiments had no obvious disease symptoms prior to inoculation, and therefore, they were considered as free from infection with the known strawberry viruses. They were propagated in a glasshouse and tested negative for SVBV infection by PCR as described by Mahmoudpour [11]. Strawberry cultivars propagated from heat-treated mother plants were used as source of healthy plants for the host range studies. These plants tested negative for SVBV infection by PCR before being used. All mother plants also were tested on UC-4, UC-5, UC-10, and UC-11 indicators by graft inoculation to ensure they were free from known strawberry viruses.

Symptomatology and virus detection

Inoculated plants were maintained in a glasshouse for at least 3 months. To enhance symptom expression, most leaves (including inoculated ones) and branches were removed from plants four weeks' post-inoculation. All plants were tested for SVBV infection by PCR according to "Method I" 3-to-5 times post-inoculation as described by Mahmoudpour [11]. Infected plants were grouped as symptomatic and asymptomatic, and ranked on the basis of relative virus titer as quantified by PCR. For PCR analysis, DNA was extracted from all the *Rosaceae* plants to reduce the effect of inhibitors. Plant homogenates from non-Rosaceous plants were either directly used in PCR or subjected to nucleic acids isolation before PCR.

Analysis of virus titer in strawberry cultivars

PCR analyses were used to study the relationship between symptom expression and the relative virus titer in different strawberry cultivars according to Mahmoudpour [11]. Serially diluted DNA extracts were prepared to determine the dilution end-point for the PCR. To determine the virus titers, composite samples representative of entire plants was prepared from older, middle-aged, and young leaves of the infected plants. For each leaf type, 3 - 4 leaf disks/leaf, 3 leaves per plant and 3 plants per host were pooled in 250 mg samples. To identify dilution end-point for each pooled sample, PCR was conducted with 6 template concentrations. The entire PCR product (20 ul) was examined by agarose gel electrophoresis. PCR-amplified SVBV DNA fragments were detected as EtBr-stained bands in 1% agarose gels and photographed by a digital camera with an exposure time of 2 sec. The brightness of each band was rated visually as described by Mahmoudpour [11]. This indirect virus-titer quantification method was used to group the cultivars in order of their relative susceptibility to SVBV infection. Duncan's New Multiple Range Test (P = 0.05) was used to rank susceptible cultivars.

Results and Discussion

Bacterial infiltration of host plants with LBA4404 cells carrying a partial tandem repeat construct of SVBV provided 75 to 100% infection rate as revealed 4 weeks' post-inoculation. Control plants inoculated with non-transformed cells remained symptomless and were negative for SVBV infection as tested by PCR. All the host and non-host plants inoculated with SVBV in this study are shown in (Tables 1,2).

SVBV Host Plants	
Rosaceae	
Non-Strawberry Host	Cultivated Strawberries
Poetrium sanguisorba	*Fragaria* x *ananassa* cvs.
Strawberry virus indicators	Aromas, Camarosa, Capitola, Carlsbad, Chandler, Cuesta, Diamante, Gaviota, Fern, Irvine, Osogrande, Pacifica, Pajaro, Parker, Seascape, and Selva
Fragaria vesca vars. UC-4 & UC-5	
F. virginiana vars.UC-10 & UC-11	
Ornamental Strawberries	
F. x *ananassa* cv. Temptation	
F. chiloensis	

Table 1: *List of plants determined to be hosts of SVBV. Plants were inoculated by infiltration of Agrobacterium tumefaciens LBA4404 cells transformed with pCGN1547SVBV1.25mer. All plants were tested positively for SVBV infection through end-point PCR analyses. Control plants were inoculated with non-transformed cells. These plants remained symptomless and tested negative by PCR.*

SVBV Symptomatic Hosts	
Virus-Indicator Strawberries	Cultivated Strawberries
Fragaria vesca var. UC-4 & UC-5	*Fragaria* x *ananassa* cvs. Gaviota, Carlsbad, Cuesta, Pacifica, & Selva

Table 2: *List of susceptible strawberries infected by SVBV, which express vein banding symptoms. No symptom or virus-infection was detected in non-infected control plants.*

Particle gun inoculation of cloned DNA on UC-5 strawberries established biological activity of the cloned DNA in pSVBV-E3 thereby completing Koch's Postulates [10,11]. Engineering the construct pCGN1547SVBV1.25mer to locally transform plant cells with SVBV genome by *Agrobacterium*-mediated gene transformation system provided a highly efficient inoculation procedure (i.e., 100% efficiency). This procedure was then used to determine the SVBV host range in several plant species. SVBV hosts were shown to be limited to members of the genus *Fragaria*. No other host plant was found outside this genus, except burnet (*Poterium sanguisorba*), which became systemically infected by the virus, but remained asymptomatic.

PCR analyses did not reveal any signals from non-inoculated leaf samples collected from other plants, indicating that these were not hosts. Samples taken from inoculated leaves at or around inoculation sites of non-host plants produced strong PCR signals. These signals were presumed to be originated from the bacterial infections, resulting in the limited transfer of SVBV into wounded cells. Symptoms on inoculated leaves observed in non-host plants were not related to virus infection, as similar symptoms were also produced on the plants inoculated with non-transformed LBA4404 cells.

Unlike strawberry, which had high infection rate, agroinoculation of burnet (*Poterium sanguisorba*) resulted in 25% infectivity. PCR-amplified SVBV DNA fragments from infected burnet plants were consistently lower in intensity than those from infected strawberries. No symptoms were observed in the infected burnet plants. Initial systemic infection (as detected by PCR) or symptom development was not enhanced by trimming old leaves as observed in strawberry. Only weak PCR signals were observed three months' post-inoculation in terminal leaves, with relatively stronger signals detected in the older leaves. The virus could still be detected in these plants 5-6 months' post-inoculation.

Vein banding symptom expression was used in these studies to compare different host plants. It was found that the severity of symptom expression was directly correlated with host susceptibility. Mild to moderate vein banding symptoms were detected only in susceptible indicators and strawberry cultivars (Figure1). Symptoms usually were not persistent, but would reappear in young leaves emerging after trimming of the old basal leaves. Most strawberry cultivars did not express any vein banding symptoms. Table 3 lists only the symptomatic hosts. The other infected cultivars and indicators, including UC-10 and UC-11 remained symptomless throughout the study. UC-5, UC-4, and Carlsbad showed stunted growth and non-expanded leaves after prolonged infection with SVBV.

All infected strawberry cultivars and indicators were assayed by PCR to examine the relative virus titer. According to the collected data in this study, (i) variable virus titers were found in different strawberry cultivars; (ii) virus titers in older symptomatic leaves were the highest; (iii) virus titers in terminal leaves were higher than those in asymptomatic old leaves. The summary of PCR analysis of virus titer in 18 different strawberry cultivars and the UC-5 indicator is described in (Table 4). In this analysis, only young terminal leaves from three plants per cultivar were collected and assayed. A detailed study of virus titer in different types of tissues in selected strawberry cultivars has been reported [11]. Using these data (Table 4), the cultivars were categorized based on their susceptibility to SVBV infection.

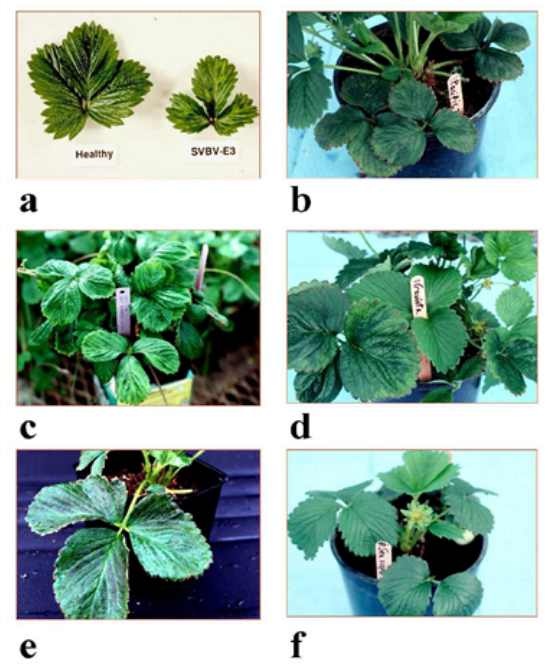

Figure1: *Illustration of selected strawberry cultivars and indicators infected with SVBV. a) UC-5 infected and healthy leaves, b) Pacifica, c) UC-4, d) Gaviota, e) Carlsbad, and f) Seascape.*

SVBV Non-Host Plants	
Herbaceous plants	Hosts of caulimoviruses
Chenopodiaceae	*Composite*
Chenopodium quinoa,	*Dahlia pinnata*
C. amaranticolor	(dahlia mosaic virus)
Cucurbitaceae	*Cruciferae*
Cucumis sativus	*Brassica campestris* (cauliflower mosaic virus)
Solanaceae	*Armoracia rusticana* (horseradish latent mosaic virus)
Nicotiana occidentalis	*Nyctaginaceae*
N. tabacum cv. Xanthi	*Mirabilis jalapa*
N. clevelandii	(mirabilis mosaic virus)
N. benthamiana	*Solanaceae*
	Petunia hybrida
	(petunia vein clearing virus)

Rosaceae	Other plants
Potentilla verna	*Solanaceae*
Rosa sp.	*Solanum sp.*
Rubus idaeus	*Physalis edulis*
R. laciniatus	
R. subgenus	
R. argutus	
R. ursinus	

Table 3: *List of plants determined to be non-hosts of SVBV. Plants were inoculated by infiltration of Agrobacterium tumefaciens LBA4404 cells transformed with pCGN1547SVBV1.25mer. All plants tested negatively for SVBV infection by PCR with SVBV primers.*

Relative Susceptibility to SVBV infection in Terminal Leaves of Strawberry Cultivars					
Temptation	0.7 a	Aromas	2.6 cdef	Carlsbad	3.3 efg
Irvine	1.0 ab	Ground Cover	2.7 def	Diamonte	3.4 efg
Chandler	1.3 abc	Selva	2.7 def	UC-5	3.8 fg
Parker	1.3 abcd	Capitola	2.9 efg	Osogrande	3.9 fg
Fern	1.5 abcd	Pajaro	2.9 efg	Pacifica	4.3 g
Seascape	2.2 bcde	Cuesta	3.0 efg		
Gaviota	2.2 bcde	Camarosa	3.1 efg		

Table 4: *Relative titer of SVBV among different strawberry cultivars based on PCR dilution end-point assay with DNA extracts from terminal leaves. Values were generated based on visual rating of the intensity of PCR-amplified SVBV DNA fragments in EtBr-stained agarose gels. The numbers represent the mean value of 3 replicates per treatment tested for 6 DNA concentrations. Numerical values were computed by Super ANOVA, and grouped according to Duncan's New Multiple Range Test at 5% significance level.*

Trimming the leaves from infected burnet plants did not induce the appearance of symptoms, as observed in strawberry plants. Because of the low virus titer, and difficulty in propagation, burnet is clearly not a suitable alternative host for virus purification and biological studies.

The PCR dilution end-point analysis was extended to the cultivated strawberries (*F. x ananassa*). This analysis demonstrated a variation in virus titer among these cultivars (Table 4). The pattern in terms of virus distribution in the leaves of the various cultivars was similar to those of UC-5 [11] and there was also a direct correlation between symptom expression and virus titer (data not shown). In other words, symptom expression was correlated with susceptibility to SVBV infection (i.e., cultivars with symptoms were most susceptible whereas those without symptoms were less susceptible).

Conclusion

It is important to note that after the California Strawberry Industry established virus elimination program, strawberry breeding programs have focused on improving cultivars for higher yield, marketability, and resistance to prevalent fungal pathogens. Selection for these phenotypic characteristics may have been achieved at the expense of losing genes conferring resistance to pests and pathogens. At

meantime, selection for virus resistance or identification and elimination of susceptible cultivars has not been conducted, because virus inoculation and disease evaluation in strawberry was not feasible to accomplish. Therefore, it is possible that genes for resistance to infection by strawberry viruses have been eliminated through different breeding selections. Analysis of susceptibility of strawberry cultivars lends some support to this theory. It is also interesting to note that the weakest PCR signals, detected from SVBV-infected plants, were those from the patio strawberry, selected in the Great Britain and marketed by seeds as an ornamental plant (Unwins Seeds®). There was also variability with respect to SVBV susceptibility among other strawberry cultivars originated in California.

According to this study, there was a trend of increasing susceptibility in newly released cultivars compared to the cultivars introduced in earlier years (Figure1). This report along with author's previous reports [10,11] presents methodologies for inoculation, diagnosis and evaluation of SVBV infection in strawberry. These procedures will enable strawberry researchers to conduct field experiments on etiological and epidemiological studies of SVBV, and to screen cultivars for SVBV resistance in breeding programs. Development of these procedures may also facilitate the investigation on the other strawberry viruses [12].

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Conflict of interest

I declare that there is no financial interest or any conflict of interest exists.

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