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Agroinoculation of Strawberry Vein Banding Caulimovirus: Molecular Mechanism

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Abstract

The focus of this review article is to touch up on the history and biology of Caulimoviruses and provide the necessity and molecular mechanisms involved in Agroinoculation of SVBV. Unlike other members of Caulimoviridae which all have narrow host range, strawberry the only host of SVBV presents a challenging mechanism against mechanical virus inoculation through cell death and browning reaction leading to prevention of virus entry. Naturally, the strawberry plants are infected by several aphid species and spread readily through vegetative propagation. While vein banding symptoms on wild strawberries were defined as early as near a century ago, the Koch's postulate was not feasible until late 1990's. Infectivity of the deduced SVBV sequence was confirmed by the Agroinoculation procedure and subsequently, Koch's Postulates was satisfied using the infectious SVBV clone. Unlike SVBV, in other members of Caulimoviridae, manual inoculation of purified virions or even naked genome using an abrasive agent was enough to initiate the infection and demonstrating the infectivity of the cloned DNA as well as the Koch's postulate. After decades of delay, a genetic construct carrying a linear 1.25mer genome of SVBV in a binary vector was transformed into *Agrobacterium tumefaciens* with a disarmed Ti-plasmid. The inoculation of these bacterial cells was led to incorporation of excised T-DNA including the SVBV genome into host chromosomes. The expression of transformed cells generated circular genomic dsDNA which yielded systemic infection and developing corresponding symptoms defined a decade earlier. This mechanism is now available to screen the new cultivars for virus susceptibility in strawberry as well as extending to woody plants hosting other viruses. The corresponding data demonstrated by the author will be presented in this reviewed.

Keywords: *Caulimoviridae; Caulimoviruses; Strawberry Vein Banding Virus; Virus Infection; Replication and Expression; Molecular Mechanisms of Agroinoculation*

Abbreviations

SVBV: Strawberry vein banding virus; CaMV: Cauliflower Mosaic Virus; ORF: Open Reading Frame; ATF: Aphid Transmission Factor; TAP: Transactivator Protein; pSVBV-E3: Name of the SVBV DNA clone inserted in pUC8 plasmid

Background Review

Caulimoviridae and Caulimoviruses

Caulimoviridae is the small family of plant viruses consisting of a single genus of Caulimovirus and 8 members with semi-similar characteristics and differing in their host range, transmission and distinct properties. Even though the members of this genus or family were all identified sometimes during the last century, they were grouped together after numerous contributions made in studying and revealing the molecular characteristics of these viruses. The extensive studies were devoted for Cauliflower Mosaic Virus (CaMV) with

its significant contribution in plant biology and breeding transgenic crops through using its strong and well-known 35S promotor which appeared in 1000's of publications. The initial symptoms were first defined in cauliflower hence the name of virus family and genus were derived from. However, the main host which included most of the studies turned out to be turnip and most studies were carried in cultivar known as "Just Right". Unique characteristics of this virus which are distanced significantly from other common plant viruses will be discussed below. Other members were grouped in this family and genus after revealing their molecular identities. Further studies gradually have distanced these members from each other.

Viral Expression and Infection among Caulimoviruses

Naturally, the virion particles of SVBV and aphid-transmissible Caulimoviruses are transmitted into the host cells by aphids. Experimentally, the virus particles may enter the cells after passing the cell wall through mechanical means of inoculations. Once in cytoplasm, genomic DNA carrying the single stranded nicks enters passively into the nucleus due to the affinity of the DNA into nucleus after decoating procedure. Ligation activity which is found only inside the nucleus repairs the nicks and generates intact double stranded circular genome. The resulted plasmid-like genome supercoils and interacts with histone proteins to form a mini-chromosome harboring 42 ± 1 nucleosomes [1]. Interaction of host RNA polymerase II with 19S and 35S promotor sites leads into synthesis of two RNA molecules. The synthesis of 19S mRNA starts at upstream of start codon of the ORF VI and terminates beyond the stop codon corresponding with the 19S promotor regulation and downstream signals for termination of RNA synthesis.

These data have not been delineated precisely in SVBV and not clearly described in CaMV. To transcribe 35S RNA, RNA polymerase II spans the 35S promotor region and starts the synthesis prior to reaching at multiple short ORFs and ORFVII. These ORFs are also overlapped in 19S RNA and do not lead to any detectable protein synthesis. Apparently, their role is to interact and influence ribosome which is defined as ribosomal shunt. After completing the first round of transcription and generating the full-length RNA, transcription continues beyond the starting point for ~180 nucleotides hence generating terminal redundancy and leading to a single-stranded overhang at the alpha nick during synthesis of genomic DNA (see below). Like any other mRNA, both RNA products are transported into the cytoplasm after 5' capping and 3' polyadenylation [1-3]. There is no report or evidence for splicing the 35S RNA or generating shorter mRNA molecules before exporting from the nucleus.

The 19S mRNA, which is transcribed at lower efficiency compared to 35S mRNA, is translated through cellular machinery to produce transactivator protein (TAP). This protein products form aggregates of viral inclusion bodies or so called viroplasms which are sites of virus replication, assembly and initial infection. TAP is synthesized through both 19S RNA and 35S RNA. The 35S RNA is similar to polycistronic mRNA carried in bacterial cells and many types of RNA viruses. It is used as template for both protein synthesis and virus replication. To synthesize each copy of dsDNA genome, a full copy of 35S RNA has to be degraded. Translation procedure and synthesis of viral proteins are mediated through interaction of TAP. This protein is also essential for viral infection and forming viroplasms for viral replication [4].

The detailed mechanisms involved in protein synthesis from 35S RNA including alternate splicing, ribosomal shunt, termination/re-initiation and proteinase events have not been clearly described yet in intensely studied CaMV. Massive aggregation of ORF VI product (TAP) forming viroplasms and incorporation of 420 subunits of coat protein (ORF IV products) to form a single virion demonstrate that the regulatory effects on expression of viral genome through two 19S and 35S RNA molecules are biased. The movement proteins and insect transmitting factors (ORF I, II, III) are not needed in abundance for viral infection. This implies that 35S RNA is not translated as polyprotein undergoing cleavage by proteinase activity. The third abundant protein is expressed through ORF V which is a functional protein with several motifs and activities similar to replicase enzyme seen in retroviruses. Due to complicated replication of double-stranded genomic DNA, presumably several units of replicase acts simultaneously. Multiple priming events in absence of repair mechanism in the cytoplasm leave variable numbers of single stranded nicks behind. There is no evidence of sequence specificity in determining priming sites during synthesis of -/+ DNA strands.

Genomic Replication of Caulimoviruses and Infection Cycle

Once the multi-function virus replicase is synthesized, it takes multiple roles in synthesis and replication of virus genome. Due to activity of this enzyme and replication mode of Caulimoviruses, these viruses are also regarded as Para-retroviruses with similarities to HIV representing retroviruses. Unlike retroviruses, the members of para-retroviruses do not integrate into the host genome. According to later studies this classic distinction or universality among Caulimoviruses is under question (see below).

First of all, the 35S RNA is used as template for synthesis of first negative DNA strand of virus (cDNA) by reverse transcriptase or RNA-dependent DNA-polymerase activity of the viral replicase. To initiate this polymerase activity, due to base complementarity, cellular formyl methionine tRNA binds to its complementary sequence near 3' end of 35S RNA and acts as primer for synthesis of cDNA. Once the DNA synthesis initiated, the tRNA is removed and the new DNA : RNA hybrid is recognized by RNAase H activity of the replicase which starts degrading the RNA template at its 3' end following polymerase activity of leading replicase [1].

Due to terminal redundancy of the 35S RNA and complementarity of cDNA to other end of the RNA, these two ends anneal and form a circular molecule. A short stretch of cDNA which is synthesized from the extra portion of 35S RNA (excess to the full-length genome) remains as ssDNA overhang. The gap between initial primer (fmet-tRNA) and cDNA known as alpha nick remains to be fixed in the nucleus in absence of ligation activity in cytoplasm. Completion of cDNA synthesis prepares it to serve as template for synthesis of second strand of DNA through DNA-dependent DNA-polymerase activity. These enzymatic activities of replicase protein are conducted simultaneously by multiple units of enzyme hence creating 2 - 3 unfilled nicks due to priming and initiating the synthesis at several locations. Therefore, internal priming and leaving a nick behind does not have to wait for complete synthesis of negative strand. The newly synthesized genome is encapsidated with 420 subunits of coat protein forming 45 - 52 nm icosahedral particles. Variable sizes of virions have been reported among the members with triangular number of T = 7 inside the viroplasm. The new virus particles are ready for cell to cell and long distance movement for systemic infection of the host plant [5]. Some particles are acquired by transmitting aphids to spread the infection for other plants. In early infection of virus, alternatively, the newly synthesized genomes are imported into nucleus to be repaired and used as template for further RNA production. Infected nucleus would carry up to 1000 copies of supercoiled genome [1].

A Brief History in Biological Studies of SVBV

SVBV genome consists of a double-stranded (ds) DNA genome [6,7] of 7,876 base pairs encapsidated in icosahedral particles of approximately 45-nm diameter [8,9]. It is transmitted in a semi-persistent manner by several aphid species and has a narrow host range that includes only strawberry [10]. SVBV was classified as a member of *Caulimoviridae* [7-9]. 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 was not unequivocally demonstrated.

Study on strawberry virus and virus-like diseases in California was initiated as early as 1922 [11]. Indexing methodology for virus detection which is still in use was established [12,13]. Initial research on strawberry viruses (including SVBV), their symptomatology, and their transmission by aphids also was conducted in Great Britain [14-18].

Norman Frazier, a distinguished entomologist at the UC Berkeley and a pioneer researcher of SVBV was successfully able to isolate strawberry viruses by using multiple and differentiated species of aphids and maintain them in separate wild species of strawberries as early as 1950's and this work continued into the late 1980's [10,19-21]. He described virus disease symptomatology, identified wild strawberry plants as suitable virus indicators, and established that the causal agent could be transmitted by various aphids, dodder (*Cuscuta subinclusa*), and grafting. Virus-vector interactions (i.e., specificity of aphid species as vectors, acquisition access and retention times, semi-persistent mode of transmission, and transmission efficiency) and the inability to transmit the virus via sap were also demonstrated [10]. However, there was no means available at the time to prove that disease symptoms in isolated viruses maintained in wild strawberries were not due to mixed infection.

In other early studies on aphid transmission and symptomatology of vein banding disease, the disease and the virus were described under different names and descriptions [19,20,22-25]. Differential isolation of SVBV strains and other strawberry viruses by aphid transmission experiments was studied in more detail by Frazier [19], where he reported four strains of SVBV. Twelve different species of aphids were shown to vector SVBV under natural and experimental conditions [26,27].

Kitajima and coworkers [8] reported strawberry vein banding disease in Brazil and studied the morphology of SVBV particles by electron microscopy. Based on the characteristics of the virus inclusion bodies and the size and isometric morphology of the virus particles, it was concluded that SVBV belonged to virus family of *Caulimoviridae*. Kitajima and coworkers further noted the presence of cytoplasmic inclusion bodies in leaf mesoderm and parenchyma cells surrounding the vascular system. However, inclusion bodies were absent from the phloem, xylem, and epidermal cells. They also failed to detect the virus by EM in leaf-dip preparations or by light microscopic analysis of stained epidermal strips. Because of the restricted host range of SVBV (observed only in wild and commercial strawberries), Kitajima and coworkers suggested that the virus should be considered distinct from CaMV. Morris and coworkers reached the same conclusion based on partial purification of the virus and serological studies through using CaMV antiserum [9]. SVBV was subsequently confirmed to be a serologically distinct member of the *Caulimovirus* group [9,21]. This conclusion implies a distinct gene sequence encoding capsid protein in CaMV and SVBV.

Stenger and coworkers [6] purified and cloned an approximately 8.0-kb ds-DNA genome of the virus into a pUC8 cloning vector to generate pSVBV-E3. The complexity of the isolation protocol used to recover the viral DNA reflected the difficulties of isolating nucleic acids from strawberry tissue. Using this protocol less than 1 µg of viral DNA was recovered per kg of infected tissue. Infectivity of the cloned SVBV DNA was not established due to failure of inoculation. Rub-inoculation of the cloned, excised and self-ligated SVBV DNA on to strawberry leaves did not result in development of vein banding symptoms. In parallel experiments, an infectious clone of CaMV was readily transmissible to turnip via mechanical inoculation [6,28]. Cloned SVBV DNA labeled with ^{32}P was used as DNA probe for detection of the virus in dot blot hybridization and to construct restriction and physical maps.

SVBV was detected in the Czech Republic [29] and the complete nucleotide sequence of the pSVBV-E3 was determined [30]. Unlike CaMV genome which was shown to consist of 8031 bp [31] the SVBV sequence was consisted of 7,876 bp derived from sequencing SVBV-E3 clone [30]. The sequence data was confirmed by this author while demonstrating the infectivity of the Stenger's pSVBV-E3 clone [32]. Comparison of SVBV sequence with that of CaMV [33] provides that the SVBV genome is 155 bp shorter. Unlike CaMV, where an extra ORF of VII with an undetectable protein has been introduced, such unknown ORF has not been seen or reported in SVBV sequence (Figure 1). The recombinant plasmid pSVBV-E3 was used as a probe for non-radioactive dot blot hybridization and the sequence was used to design primers for PCR detection of SVBV [34,35]. Using the sequence of the clone and previous data the genomic organization was delineated and the assignment of SVBV as a distinct *Caulimovirus* species substantiated [7]. Comparison of a 431-bp PCR-amplified DNA fragment corresponding with coat protein from six SVBV isolates collected in the Czech Republic and the United States did not yield significant sequence differences. So, it was concluded that they were all isolates of the same virus. It was speculated that since the virus was first described in California and Oregon, it must have been introduced into Europe via importation of infected plant materials [36].

An important step in biological characterization of any pathogenic agent in plant or animal systems is the demonstration of Koch's postulates established in 1876. This serves to prove that a given agent, associated with a disease, can induce the symptoms of the disease. According to the conventional rules, the pathogenic agent causing the disease should: 1) be associated with the disease; 2) be isolated from diseased tissue and grown in pure culture; 3) and when inoculated to healthy hosts should produce the same symptoms or disease; and 4) be re-isolated from that newly diseased host. With the failure in demonstration of Koch's postulates, an organism cannot unequivocally be proven to be the causal agent of a disease.

Over time, Koch's postulates have been redefined, but the basic concept remains. Prior to the discovery of viruses as pathogenic agents, most pathogens (e.g., bacteria and fungi) were isolated and cultured in artificial media before they were used to inoculate healthy hosts.

This practice is not applicable for obligate pathogens such as viruses. The chemical purification of virus particles and, later, the cloning of viral genomes were substituted for growing the pathogen in culture. Failure to obtain suitable quantities of infectious particles and/or the failure of the virus to multiply to high levels was overcome by cloning the full-length viral genome [37]. Demonstrating the infectivity of the cloned viral nucleic acid in the appropriate host plant is the final step necessary to complete Koch's postulates for viruses. The purified particles of SVBV were never shown to be infectious. The infectivity of the genomic clone (in pSVBV-E3) could not be demonstrated [6]. Therefore, Koch's postulates for SVBV remained to be demonstrated by this author; see below.

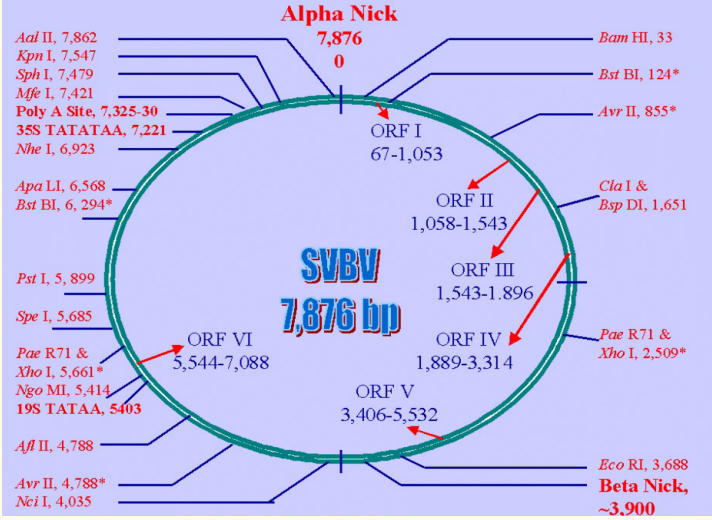

Figure 1: *Physical map of the strawberry vein banding virus genome, showing locations of restriction enzymes that cleaves once or twice (as marked with *). Putative promoter sequences of gene VI (19 S RNA) and the full-length genomic (35 S RNA), transcriptional termination site (Poly A), and single-stranded nicks in the genome are shown in bold.*

Biological and molecular studies of SVBV and other strawberry-infecting viruses were hindered by the properties of the host plant. Strawberry is rich in viscous substances (polymeric carbohydrates) and phenolic compounds which oxidize and turn brown (as known as browning reaction) in the presence of oxygen [38]. Furthermore, no suitable SVBV alternate host was identified to facilitate its biological studies. Thus, procedures for obtaining purified virus or cytoplasmic proteins belonging to the virus in quantities needed for molecular characterization of the virus genome and virion proteins, and for producing specific antibodies was obscured by the difficulties associated with chemical composition of strawberry tissues. The infectivity of the pSVBV-E3 clone was established by this author, thereby fulfilling Koch's postulates for the virus [32,39,40]. The procedures that allowed for these results were efficient inoculation methods in strawberry including DNA delivery by particle gun bombardment and Agroinoculation. Improved protocols for partial recovery of virions and a viral DNA paved the road for efficient diagnosis via PCR [32,40,41].

SVBV purification from strawberry tissues is limited by relatively low initial titer [40], and by significant reduction in virus recovery due to co-purification of host-derived contaminants. Partially purified virion preparations were used to isolate viral DNA and analyze the coat proteins [40]. Urea and Triton-X-100 were used in grinding buffers to dissolve the cell membranes and disrupt the virus inclusion bodies through extended periods of stirring in purification of SVBV and other Caulimoviruses [6,9,42]. However, this method was not suc-

cessful for isolation of SVBV from strawberry [6,9]. This was probably due to browning reactions that occurred during the long stirring periods and also due to the sedimentation of other host-derived contaminants. On the other hand, a commonly used concentration step in virus purification is polyethylene glycol (PEG) precipitation of plant viruses from the clarified extract [37]. This method was also tried for SVBV isolation from the strawberry extract, and it was unsuccessful. The products obtained by PEG precipitation contained strawberry contaminants that did not dissolve in the buffer. The DNA recovered from PEG suspension produced a smear in agarose gel analysis, and no SVBV DNA was detected by Southern hybridization.

Viral Genome and 35S Promotor

Genetic structure of SVBV is diagrammatically depicted in Figure 1. The double-stranded circular-DNA virus consists of 7,876 bp and six open reading frames (ORF). According to close similarity to genome of CaMV, ORF I to ORF VI encodes movement protein (MP), aphid transmission factor (ATF), non-sequence specific DNA-binding protein (also defined as second ATF), capsid protein (CP), multi-functional enzymatic protein acting as RNA/DNA dependent DNA polymerase (replicase)/RNAase H/proteinase [1,3,43] and lastly transactivator protein (TAP) through ORF VI. The 19S promotor or TATAA box at position 5,403 is responsible for synthesis of 19S mRNA which includes only ORF VI, apparently for over-expression of TAP and initiation of viral infection, which is the site of virus replication and encapsidation in the cytoplasm of infected cells. The abbreviation "S" represents sedimentation coefficient deduced from high-speed centrifugation under density gradient separation of macromolecules based on their molecular mass. The well-known 35S promotor starts with the TATATAA box positioned at 7,221-7 of the genome along with a stretch of poly A at 7,325-30. The promotor has been shown to overlap with ORF VII in CaMV as well as including several short open reading frames, apparently associated with gene expression in terms of ribosomal shunt, but no protein or activity has been documented so far for these open reading frames [44].

These detail studies which have not been fully delineated yet have not been described in respect with SVBV genome and its replication and expression. Therefore, the promotor region of 35S in SVBV (Figure 1) has been considered to hypothetically extend for maximum length of 656 bp before reaching at alpha nick. This promotor is responsible for synthesis of more than full-length pre-genomic and polycistronic 35 mRNA which spans entire length of the genomic DNA by starting at some where before the alpha nick position and terminating after a short stretch (180 nucleotides in CaMV) beyond the starting position which leads to a terminal redundancy [1,4]. According to reports made on CaMV, the 35S RNA includes a 5' cap and continues with transcript of ORF VII before reaching to position of alpha nick and starting point of first open reading frame. Both RNA are transcribed by host RNA polymerase II under the control of corresponding promotors with specific sequences recognized by the enzyme. Both RNA are poly-adenylated at their 3' sites [1]. Strong activity of 35S promotor region has made it to be used in numerous applications and reports in plant biology and gene transformation to dicotyledonous plants by providing significant level of gene expression. There are two single stranded nicks in the genome of SVBV at positions 0 and ~3900 known as alpha and beta nicks. The position of these single-stranded nicks have been shown to be at different positions in isolates of well-studied CaMV and may be created at random positions based on priming effect of simultaneous activities of multiple viral replicase.

Sequence analysis of SVBV also revealed transcriptional elements known to be present in other Caulimoviruses (Figure 1). A 35S promoter sequence (TATATAA) was identified at position 7,221, and transcriptional termination sequences or poly A signals (AATAAA) were located at positions 7,325 and 7,590. In contrast, a single such sequence is known for CaMV. The termination sequence is presumed (see below) to be same for both the 19S and 35S RNAs. If this is the case, the messenger RNA (mRNA) for gene VI would have a 3' non-coding sequence of 337 nucleotides, and the genomic RNA (35S RNA) would have 115 nt at 3'-terminal redundancy. If the second termination sequence is active, then these 3' noncoding sequences would be greater (502 and 370 nt, respectively). In CaMV, the 3' non-coding sequences are 268 and 207 nt, respectively. In FMV, these sequences are 162 and 174 nt, respectively [45]. Promoter sequences for the SVBV 19S RNA (TATAA) were located at positions 5,335 and 5,404. The second site is the more likely promoter in SVBV, because the coding sequence of gene VI starts at position 5,544 and this would result in a small (141 nt) 5' non-coding sequence. In CaMV, the 19S RNA leader sequence is 45 nt, whereas that of FMV is 77 nt. The restriction map was constructed before engineering the pCGN1547-SVBV1.25mer construct.

This map allowed for the efficient generation of the multimeric construct, which was required for generation of the SVBV Agroinoculation system. This infectious construct and information from this physical map will allow for further molecular genetic studies of SVBV.

Agarose gel and Southern blot hybridization analyses 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. DNA recovered from virus particles previously treated with DNase I provides evidence that the isolated DNA recovered by this procedure was likely derived from virions as opposed to being free-viral or host DNA purified in this procedure. Enzymatic digestion of SVBV DNA (along with pSVBV-E3 as control) with two restriction enzymes at unique sites using *Bam* HI and *Eco* RI, and hybridizing these bands with pSVBV-E3-derived DNA probe provided further evidence for identity of the viral DNA. These DNA fragments co-migrated in the gel, and similar restriction sites were present in both DNAs [32,39,40].

However, considering the current sequence information, the single alpha nick in negative strand at position zero is adjacent to *Bam* HI site (position 33) not to *Eco* RI site (position 3,688) as reported before. Only a single nick could be detected in the beta (+) strand roughly at 3,900-position. In this analysis, Bam HI digestion (33 bases from the alpha nick) produced a pair of bands migrating between 3.6 and 4.2-kb. This could happen only on linear genomes, physically or chemically broken at beta nicks. In absence of breakage, the virus genome is seen as a linear 7.9-kb band when digested with Bam HI. No other smaller band was found to indicate additional nicks in beta strand [32,39,40].

Lack of a specific antibody to SVBV hindered the identification of the coat protein by Western-blot analysis. The same virion products used in DNA isolation and Southern blot hybridization analysis were used in SDS-PAGE analysis of SVBV coat protein [39,40]. Three 45 - 60 kDa-protein bands were observed in preparations obtained from the infected plants. These were presumably subunits of SVBV capsid protein produced by proteolytic cleavages of the full-length polypeptide. Multiple coat protein bands (37, 39, 44, and 57 kDa), all cross reacting with antibody raised against 37-kDa protein [46] have been reported for CaMV [47]. The predicted molecular weight of the full-length SVBV coat protein is 56.0 kDa (474 residues), compared with 56.7 kDa (489 residues) for CaMV. In addition to the presence of numerous basic amino acids in the coat protein of SVBV (55 lysine and 30 arginine residues) and CaMV (57 lysine and 22 arginine residues), it has been shown in CaMV that coat protein subunits and virion surface are heavily glycosylated [48] and phosphorylated [49]. Therefore, these subunits move slower in the gel, and estimation of exact sizes is difficult. Proteolytic activity of the replicase protein (protease domain), found also in virus shells, cleaves the native coat protein into required subunits [50] used in assembly of virions consisting of 420 subunits [5]. The exact sizes of these subunits and cleavage sites remain unclear.

Mechanical Inoculation

Mechanical or "rub" inoculation was conventionally used as default method of virus inoculation to demonstrate Koch's Postulate or generating infected plants for further studies using herbaceous hosts. Suspensions of virus particles or naked genomic DNA in a phosphate buffer are used to inoculate the young leaves of healthy (non-infected) plants using carborundum or celite as abrasive. Leaves were mildly sprinkled with an abrasive and gently rubbed by a finger carrying a drop of inoculum. The abrasive assisted virus entry into the cells by breaking the cell walls. Plants are maintained in humidified chambers or glasshouses to heal the wounds and establish virus infection. In strawberry and woody plants of *Rosacea*, the rub inoculation has been failed due to wounding response and browning reaction. In other word, necrotic tissue would not support virus replication. That is why the alternate hosts and herbaceous plants have been used in mechanical inoculation. Lacking alternate host to support infection of SVBV has been a hurdle in its biological studies.

Preparation of pCGN1547/SVBV1.25mer Construct and Agroinocula

Agroinoculation of viruses had been demonstrated previously for Caulimoviruses [51], geminiviruses [52], and potexviruses [53]. This method provided infection rate of 100% in strawberry plants with SVBV. Thus, it was established that this method could be used in efficient transmission of another virus to a host plant that is immune to manual inoculations [32,40]. To Agroinoculate strawberry

plants and investigating alternate hosts for SVBV infection [39,40,54] pCGN1547 [55], a binary vector carrying T-DNA with a limited multiple cloning site (MCS) and a gentamicin resistance gene was used to carry 1.25mer genome of SVBV. To engineer the 1.25mer construct, a 2-kb fragment of SVBV genome was excised from pSVBV-E3 (cloned in pUC 8) by digesting with *Pst* I (position 5,899) and *Bam* HI (position 33). Figure 2 illustrates the steps involved in this genetic construction [39,40]. This fragment was inserted into the multiple cloning sites (MCS) of the pCGN1547 (Figure 3) digested with *Pst* I and *Bam* HI. The ligation mix was used to transform *E. coli* DH5 alpha cells. Transformed cells were screened for inserts on nutrient broth plates supplied with l0 ug/ml of gentamicin in the presence of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) and IPTG (isopropylthio-ß-D-galactoside) according to Sambrook and coworkers [56]. Restriction sites used in these constructs are shown in Figure 1. The full-length DNA insert was released from pSVBV-E3 by Eco RI digestion, followed by re-ligation to obtain circular or linear multimeric SVBV genomes. This product was digested with *Pst* I to generate a ~7.9-kb linear genome having the promoter region in its 5' end. The mixture of ligation/restriction products (multiple forms of DNA fragments) was then combined with intermediate construct (pCGN1547/2.0-kb SVBV) digested with *Pst* I. pUC8 recombinants were eliminated by selection in presence of gentamicin.

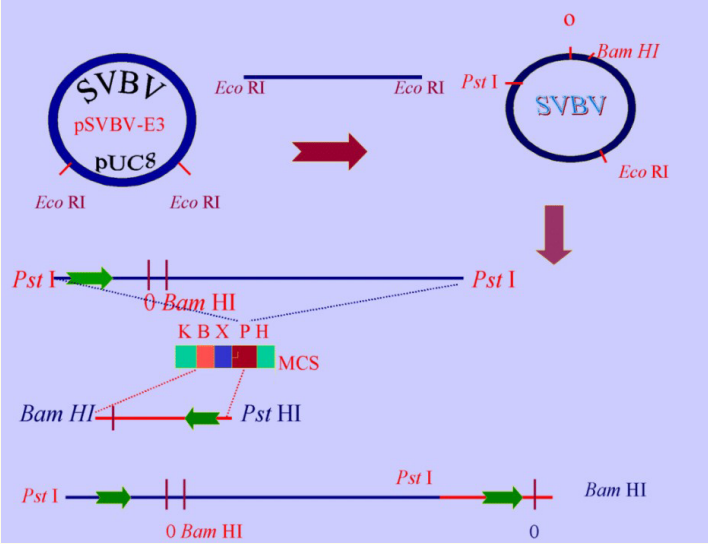

Figure 2: *Schematic representation of engineering genetic construct of SVBV1.25mer derived from pSVBV-E3 to be used in Agroinoculation.*

A pool of selected recombinants carrying the 10-kb insert was screened for the correct orientation (*Pst* I 0-*Pst* I 7,876-*Bam* HI 9,886) of 7.9-kb insert by restriction (Bam HI digestion) and gel analyses. Recombinants giving rise to digestion products running at the 7.9-kb position were selected at this point. The infectivity of this construct was ascertained by particle gun inoculation of 12 UC-5 plants (one replicate only) before proceeding with Agroinoculation. Electro-competent *A. tumefaciens* LBA4404 cells (Invitrogen®) were transformed with salt-free pCGN1547/SVBV1.25mer construct (cleaned with plasmid isolation kit, QIAGEN, Inc.®) by electroporation of cells at 2.0 millivolts. Cells were plated on 2xYT agar [56] supplied with 10 ug/ml of gentamicin after shaking in SOC medium (supplied by Invitrogen, Inc.®) for 4 h at 28°C. Colonies were picked after 48 h and screened for the presence of the 24-kb construct by gel electrophoresis and PCR analysis according to conventional methods used for screening recombinant *E. coli* [56].

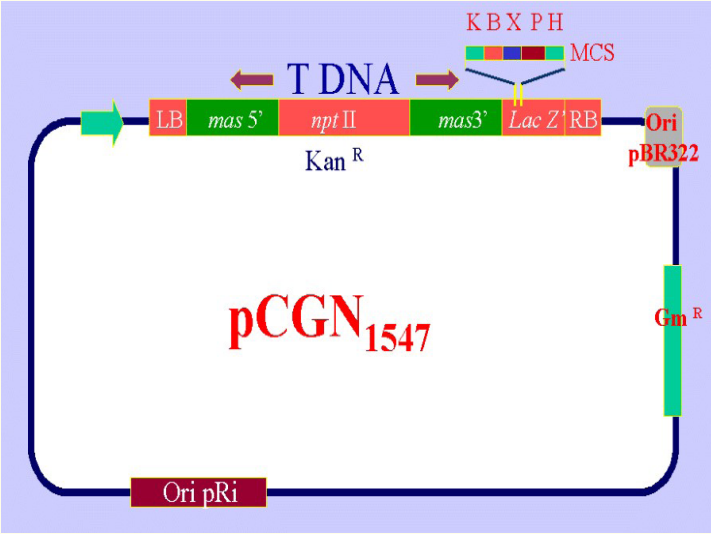

Figure 3: *Diagrammatic illustration of binary vector pCGN1547 depicting its major components which acts in presence of disarmed Ti-plasmid found in Agrobacterium tumefaciens LBA4404 (Invitrogen®).*

Agroinoculation of SVBV

Transformed LBA4404 cells were cultured 10 - 24h at 28˚C by shaking at 100 rpm in liquid 523 medium [10 g sucrose, 8 g casein (acid hydrolysate), 4 g yeast extract, 2 g K_2HPO_4, and 0.3 g of $MgSO_4.7H_2O$ (dissolved separately) per liter, pH 7]. The medium was supplied with 100 ug/ml of streptomycin and 10 µg/ml of gentamicin. The incubation was terminated when the culture reached at OD650 of 0.5. Non-transformed cells (control) were grown under the same conditions in the absence of gentamicin. Cells were pelleted by spinning at 5,000 g for l0 min and re-suspended in 0.1 x original volume of 100 mM phosphate buffer, pH 6.5-7. Ice-cold bacterial suspension was forced into intercellular spaces of young leaves through stomatal openings using a 5-ml syringe (with no needle), the outlet of which was pressed against the lower leaf surface while supporting the upper surface with a finger. Multiple sites were inoculated in each plant. Inoculated plants were maintained in a glass-house [40,57].

Mechanisms of SVBV Infection through Agroinoculation

Infiltration of *A. tumefaciens* LBA4404 suspension inserts the bacterial cells into young leaves through stomatal openings and intercellular spacing. Water soaking, 5 - 10 mm in diameter, is the immediate sign of bacterial entry. Successful infiltration leads to local infection of plant cells with *A. tumefaciens* LBA4404. These bacterial cells carry disarmed Ti-plasmid and are transformed with 1.25mer construct of linearized SVBV (Figure 2) inserted into binary vector pCGN1547. Wild type Ti-plasmid with tumor inducing T-DNA is illustrated in Figure 5 along with corresponding genes which mediate the process of tumor induction known as crown gall. Expression of genes carried in disarmed Ti-plasmid mediate excision of T-DNA or transfer DNA located between left and right borders (LB and RB) as depicted in Figure 3 (describing the molecular mechanism of these gene mediations are beyond the focus of this brief). The Linear SVBV1.25mer construct was already inserted into multiple cloning sites (MCS) located in T-DNA. The excised T-DNA exits the bacterial membranes and cell wall and enters into the cytoplasm of infected plant cells by mediation of specific proteins expressed from the same plasmids. Figure 3 also illustrates other segments of the vector. The transformed gene expression is under control of mannopine synthase (mas) promoter in both directions. Alternatively, 35S RNA promotor is used in binary vectors to support the expression of foreign genes. The SVBV construct carrying the similar 35S and 19S promotors did not need any external promotor.

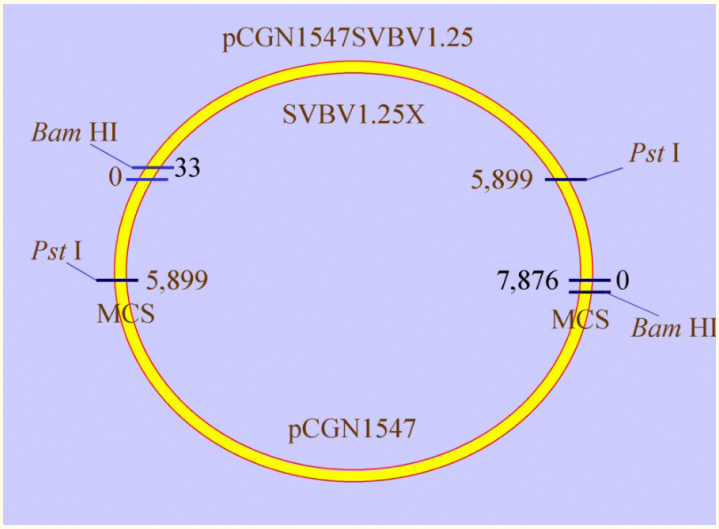

Figure 4: *Simplified presentation of finalized genetic construct to be transformed in Agrobacterium tumefaciens LBA4404 (Invitrogen®) cells for the purpose of Agroinoculation.*

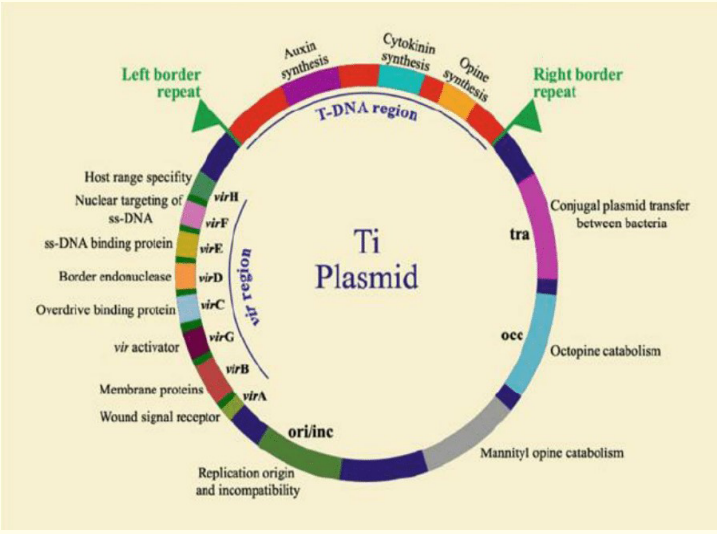

Figure 5: *Genetic map of octopine-type Ti plasmid (Modified from Ream 2002 and Özcan., et al. 2004).*

Due to affinity of naked DNA to the nucleus, the excised T-DNA passes through the nuclear pore and enters the nucleus and in a random fashion integrates into plant genome. The strong promoters at both side of the T-DNA enable gene expression through transcription by polymerase II. In absence of these promotors the SVBV construct contains its own 35S RNA promotor. Adding a 2.0 kb segment of SVBV (Figure 2) enables the polymerase to transcribe 35S RNA which is longer than the entire genome. In addition to 35S RNA, 19S RNA is transcribed as well under control of its own promotor. Both of these RNAs are ready to be exported into the cytoplasm and initiate genomic

replication, expression of viral proteins and leading to local and systemic infection. The relevant molecular mechanisms of natural virus infection were described earlier. Systemic infection in all inoculated plants was confirmed by symptomatology and PCR analysis after removing initial inoculated leaves two weeks after Agroinoculation [32,40,58-60].

Conclusion Remarks

So far, the hurdles affecting SVBV research i.e. chemical complexity of strawberry which was introduced in this review prevents precise molecular biology of SVBV. Agroinoculation was used as means of infecting strawberry plants and seeking for alternate host plants. The only herbaceous host identified by this author is burnet (*Poterium sanguisorba*) which is not suitable for biological studies due to low virus titer and its instability of systemic infection [40,54,57]. The mechanisms of natural infection of SVBV as well as through transforming by *A. tumefaciens* were described in this review. Similar constructs and means of inoculation could be extended to woody plants or members of *Rosacea* which express browning reaction and prevent virus entry. This method enables researchers to study virus biology and assess cultivars of interests in respect with virus resistance and susceptibility.

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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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