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Characterization and Epidemiology of Soybean Vein Necrosis Associated Virus

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CHARACTERIZATION AND EPIDEMIOLOGY OF SOYBEAN VEIN NECROSIS ASSOCIATED VIRUS

CHARACTERIZATION AND EPIDEMIOLOGY OF SOYBEAN VEIN NECROSIS ASSOCIATED VIRUS

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ABSTRACT

Soybean vein necrosis disease (SVND) is widespread in major soybean-producing areas in the U.S. The typical disease symptoms exhibit as vein clearing along the main vein, which turn into chlorosis or necrosis as season progresses. Double-stranded RNA isolation and shot gun cloning of symptomatic tissues revealed the presence of a new tospovirus, provisionally named as Soybean vein necrosis associated virus (SVNaV). The presence of the virus has been confirmed in 12 states: Arkansas, Illinois, Missouri, Kansas, Tennessee, Kentucky, Mississippi, Maryland, Delaware, Virginia and New York. Symptomatic samples collected from eight states (AR, IL, MO, MS, KS, TN, MD and DE), were used to study the population structure of the virus. The study revealed a perfect correlation between SVND and the presence of SVNaV and a relatively homogeneous virus population indicating that the diverse symptoms observed in the field is probably caused by the different host genotypes rather than distinct virus strains. Virus epidemiology is critical for virus control and disease management. In this study, in total of 24 plant species belonging to ten families were tested by mechanical inoculation as potential alternative hosts of SVNaV and seven species belonging to five families can sustain virus replication. In order to efficiently detect SVNaV, detection protocols based on reverse transcription – PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) was developed and their sensitivity was compared with immunological test that is currently available.

This thesis is approved recommendation the Graduate Council

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Characterization and epidemiology of Soybean vein necrosis associated virus

1.1 Abstract

Soybean yield has increased steadily in recent years because of improved management and development of high yielding cultivars (Ash, 2012). Notwithstanding economic losses caused by virus diseases remain major concerns for growers given that there is no effective treatment available once plants are infected. A new virus-like disease was first observed in Arkansas and Tennessee in 2008 and was subsequently recorded in multiple states (Tzanetakis et al., 2009; Zhou et al., 2011; Mulrooney, 2011; Bergstrom, 2011). Further studies revealed that a new virus, provisionally named Soybean vein necrosis associated virus (SVNaV), was closely associated with the novel disease (Zhou et al, 2011). The broad distribution of SVNaV highlights the significance of studying its biological properties and understanding virus epidemiology and population structure. The complete genome of SVNaV was obtained and the virus was classified in the genus *Tospovirus*. Thirty seven virus isolates were collected from eight states and the nucleoprotein gene was studied to determine virus population structure. Conventional and real-time reverse transcription (RT)-PCR protocols were developed and used to determine the presence of the virus in soybean and other hosts. In total, 24 plant species were tested as putative hosts of SVNaV and eight were found to be hosts of the virus, including a widespread weed, frequently found in soybean fields.

1.2 Introduction

Soybean (*Glycine max,* L.) a member of the family *Fabaceae*, has its origins in China and is a widely-grown around the world. It was first domesticated in the 11th century (Hymowitz and Shurtleff, 2005). In North America, soybean was first introduced in Georgia in 1765 by British colonists (Hymowitz, 1970; Hymowitz and Shurtleff, 2005). Large-scale farming in the United States did not initiate until the early

1920s when it was realized that soybean was not only a good source of oil and protein, but can also improve soil quality. The warm and humid climate in the middle part of the U.S. favors soybean farming, facilitating its adaptation to the new land. Nowadays, soybean is being planted in 31 states, with Iowa, Illinois and Minnesota being the top three producers (Sinclair et al., 1999). The annual production in the U.S. is over 90 million metric tons, accounting for more than one third total yield of the world, followed by Brazil, Argentina, China, and India (Ash, 2012). The application of soybean has been extended to animal feeding and industrial use following the massive planting in different parts of the world.

Soybean is a bushy, leafy annual with height ranging from 20 cm to 2 meters. The plant has an erect stem with sparse or dense branches, which varies depending on cultivar and growing conditions. Foliage has pinnate venation with predominantly ovate shape, supported by petioles attached to the stem. In most cultivars, the whole plant, except root and flower, is covered by tan-colored pubescence, though glabrous cultivars do exist. Followers emerge in auxiliary or terminal racemes blooming from the base of the main stem towards the tip of the plant. The average number of flowers produced in a raceme varies from 2 up to 35, depending on the cultivar. There are two types of stem growth habit in terms of floral initiation, designated as indeterminate and determinate stems. In indeterminate cultivars, vegetative activity of the terminal bud is retained during most of the growing season; this activity is abolished in determinate cultivars as blooming occurs. As maturity approaches, cotyledon and leaves in the lower level turn yellow and gradually fall off. Following blossom, pods bearing one to five seeds are formed on the tip of racemes. Mature seeds are projected from straight or slightly curved pods (Carlson, 1973).

Soybean is referred to as “the magic bean” as it has an exceedingly diverse use including human consumption, livestock feeding, and industrial applications. Food products derived from soybean, such as tofu, soy sauce, and soymilk are important components of the diet in many Asian countries and have become popular in the West due to their well-balanced nutrients. Soybean oil serves as a major

vegetative oil source in daily cooking and in the food processing industry, as a component of edible oils, margarine, mayonnaise and shortening (Sinclair et al., 1999). Soybean oil is considered a healthy cooking alternative given that it is rich in unsaturated fatty acids and does not contain cholesterol. Soybean is preferred not only for its low saturated fat content but also because of the high-quality protein and water-soluble fiber. Recent studies also revealed that isoflavones, phytoestrogens abundant in soybean seeds, have health benefits because they protect against cancer (Anderson et al., 1999; Su et al., 2005). There is also increasing evidence supporting the role of soybean in preventing hypertension and lowering risks of coronary heart disease (Bazzano et al., 2001; He et al., 2005; Jenkins et al., 2001 and Zhang et al., 2005). Soybean is also a high quality ingredient in industrial lubricants, solvents, cleaners and paints, since these products are more environment-friendly compared to the traditional petroleum counterparts (Sinclair et al., 1999).

1. 3 Soybean vein necrosis disease: an emerging problem in the United States

A new virus-like symptom was first observed in Arkansas and Tennessee in 2008 and was found widely spread in other states including Illinois, Kansas, Kentucky, Missouri, and Mississippi (Tzanetakis et al., 2009; Zhou et al., 2011). First symptoms observed early June in Arkansas and Illinois were exhibited as vein clearing along the main veins, which became chlorotic and necrotic in more extended areas in the following growth stages (Fig.1.1). In order to identify virus or viruses possibly associated with this disease, symptomatic plants were tested with 16 soybean viruses or virus groups using immunology (Zhou et al., 2011). However, none of the tested viruses was associated with the symptoms leading to the assumption that another agent is the causal agent of the disease. Double-stranded RNA was extracted from symptomatic soybean leaves and a new virus, provisionally named as Soybean vein necrosis associated virus (SVNaV), was identified. Soybean vein necrosis disease (SVND) has been

observed in several other states since it was first reported and the virus is now confirmed in at least 12 states.

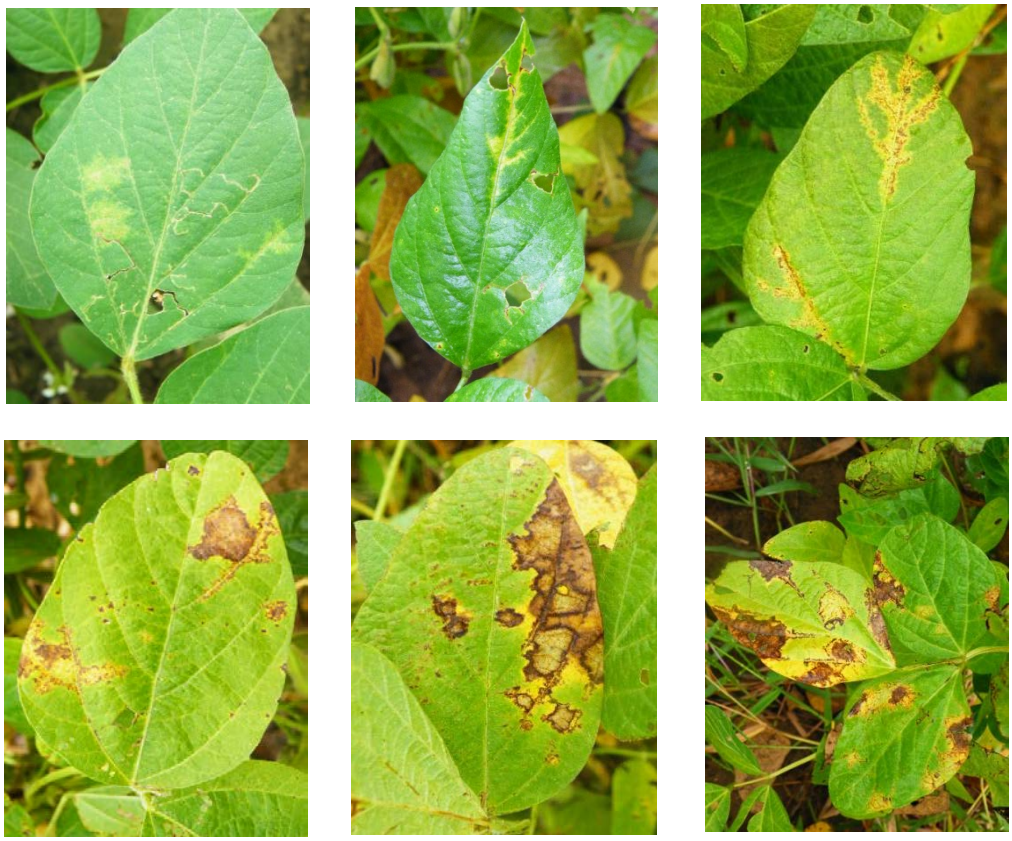


Fig. 1.1 Symptoms of SVNaV-infected soybean leaves. A-B: chlorotic lesions, C-D: necrotic lesions, E-F: expanded necrotic lesions.

1.4 Soybean vein necrosis associated virus and the genus *Tospovirus*

Phylogenetic analysis revealed that SVNaV is a new member of the genus *Tospovirus* (Zhou et al., 2011), the only plant-infecting genus in the family *Bunyaviridae*. Tospoviruses have enveloped particles with a diameter of 80-120 nm. Members in the genus share similar genomic composition, with a segmented, single-stranded RNA genome consisting of three RNAs: large (L), medium (M) and small (S) RNAs (Tsompana and Moyer, 2008). The L RNA has a single open reading frame (ORF) coding for RNA-

dependent RNA polymerase (RdRp) in the negative orientation (de Haan et al., 1991; Adkins et al, 1995; van Knipperberg et al., 2002). Both M and S fragments use the ambisense strategy for expression of the encoded proteins (de Hann et al., 1990; Kormelink et al., 1992). The M RNA encodes for non-structural protein (NSm) in the positive orientation and the precursor of two glycoproteins (Gn and Gc) in the negative polarity. NSm functions as the movement protein and affects disease development (Lewandowski and Adkins, 2005; Li et al., 2009); whereas Gn and Gc have an integrated part in particle morphogenesis and transmission (Whitfield et al., 2005; Kikkert et al., 2001; Ribeiro et al., 2008). The two ORFs of the S RNA are translated into a non-structural protein (NSs) in the positive sense that functions as a suppressor of RNA silencing (Takeda et al., 2002; Bucher et al., 2003), and nucleoprotein (NP) in the negative sense direction, involved in the encapsidation of the genomic RNA and possibly long-distance movement (Ribeiro et al., 2009).

There are eight assigned tospoviruses along with another 16 tentative species in the genus *Tospovirus* (King et al., 2011), including some of the most devastating viruses in agriculture such as *Tomato spotted wilt virus* (TSWV; Parrella et al., 2003; Pappu et al., 2009) and *Impatiens necrotic spot virus* (INSV), a major problem for the ornamental industry (Daughtrey et al., 1997). Another tospovirus that has emerged in recent years is Iris yellow spot virus (IYSV; Gent et al., 2006). IYSV has been recognized as an emerging threat to *Allium* production in several producing regions since its discovery in the 1980s; resulting in yield losses of up to 100% (Pozzer et al., 1999; Toit et al., 2004; Gent et al., 2006; Ravi et al., 2006).

Thrips are the only recognized vectors for tospoviruses in nature. More than ten species belonging to the genera *Frankliniella*, *Scirtothrips* and *Thrips* have been identified as efficient vectors of tospoviruses (Tsompana and Moyer, 2008). Transmission occurs in a persistent propagative manner but not transoverially (Riley et al., 2011). Virus acquisition is a life stage-dependent process since virus can

only be acquired by thrips in their larvae stages, especially in the first larvae. This enables thrips to transmit tospoviruses in the following life stages (Whitfield et al., 2005). Virions are acquired by probing on infected leaf epidermis and subsequently travel though foregut into the midgut, where the replication of virus occurs and infection initiates. Virus particles consequently infect muscle cells surrounding the midgut, disseminate to primary salivary glands and migrate to salivary-food canal, the avenue that particles are transmitted back to plants (Whitfield et al., 2005). Vector control has been proven difficult due to the high proliferation rate of thrips and their ability to develop resistance to insecticides. Integration of resistance genes into crops has also proven ineffective as resistance has been overcome in many crops (Tsompana and Moyer, 2008).

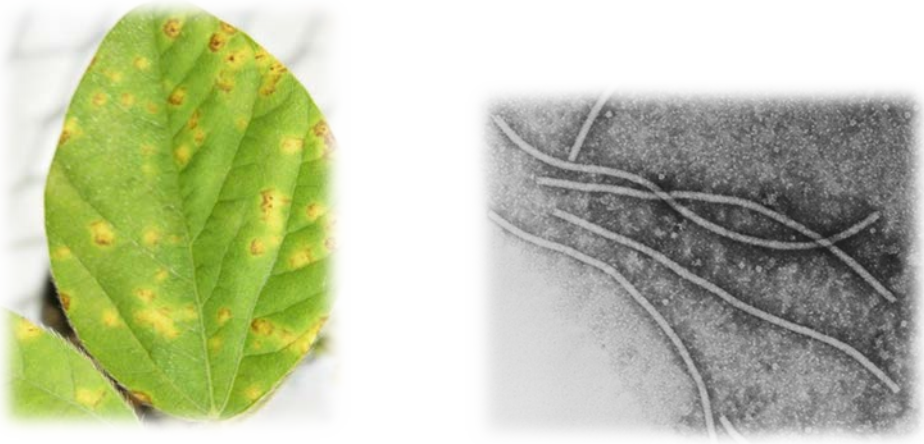
1.5 Other major viral diseases on soybean

Virus diseases pose a great threat to the soybean industry, accounting for about 10% of the annual yield reduction caused by plant diseases for the past 15 years (Wrather, 2010). Soybean has been reported to be the natural host for more than 50 viruses, among which two: *Soybean mosaic virus* (SMV) and *Bean pod mottle virus* (BPMV) are considered major problems in the field (Sinclair et al., 1999).

SMV is one of the most prevalent viruses infecting soybean around the world. The most distinct symptoms are manifested as mosaic patterns on the leaves (Fig. 1.2.A). Severe infections could lead to flower abortion, reduction in pod set, loss of seed number and weight leading to yield reduction of up to 94% (Lu et al., 2010). SMV belongs to family *Potyviridae*, genus *Potyvirus.* Members in this genus are non-enveloped, flexuous, filamentous particle of 680-900 nm long and 11-15 nm wide encapsidating the linear, positive strand RNA genome (Fig. 1.2.B). The genome size is about 10 kb, with a genome-linked viral protein (VPg) covalently linked to the 5’ and a poly (A) tail at the 3’ termini respectively (Seo et al., 2009). A single polyprotein (350 kDa) is initially translated from the genomic RNA which is subsequently

processed by multiple proteinases coded by the virus to individual, functional proteins (Vance et al., 1984). At least ten mature proteins including P1, helper component-proteinase (HC-Pro), P3, 6K1, cylindrical inclusion (CI), 6K2, VPg, NIa (nuclear inclusion a), NIb, and coat protein (CP) are translated from the genome (Urcuqui-Inchima et al., 2001; Seo et al., 2009). An additional ORF, designated as PIPO (Pretty Interesting *Potyviridae* ORF), is embedded within the P3 cistron but translated in the +2 frame (Chung et al., 2008; Fig.1.2. C). Many of the SMV proteins are multi-functional. P1 may be involved in defense mechanism of host plants (Urcuqui-Inchima et al., 2001) but its precise function still needs to be determined. HC-Pro is involved in aphid transmission, systemic movement, symptom development and RNA silencing (Urcuqui-Inchima et al., 2001; Seo et al., 2009). The short peptide 6K1 usually binds to P3, an understudied protein,that is probably involved in replication and movement of the virus. Recent study revealed that the P3N-PIPO protein is a virus movement protein coordinated with other proteins such as CI (Wen et al., 2010; Wei et al., 2011). CI is related to RNA replication and cell-to-cell movement of the virus whereas another short peptide 6K2 is associated with the targeting of pre-mature virion onto the endoplasmic reticulum (ER). NIa is the major proteinase encoded in the potyvirus genome and is involved in polyprotein maturation and RNA replication; it also interacts with NIb, the RNA-dependent RNA polymerase of the virus, and VPg. The CP is the most abundant protein within infected plant cells and is involved in RNA encapsidation, aphid transmission and virus movement (both cell-to-cell and long distance) (Urcuqui-Inchima et al., 2001).

Aphids are efficient vectors of SMV. They can acquire the virus in seconds and transmit it in a non-persistent manner (Clark et al., 2002). Over 30 aphid species have been reported to vector SMV although the vector transmission is considered as secondary virus inoculum compared to seed, considered the critical pathway for virus dissemination (Steinlage et al., 2001; Lu et al., 2010).



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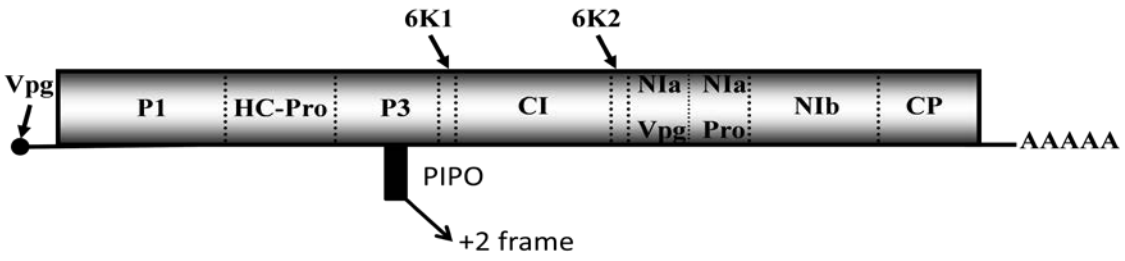
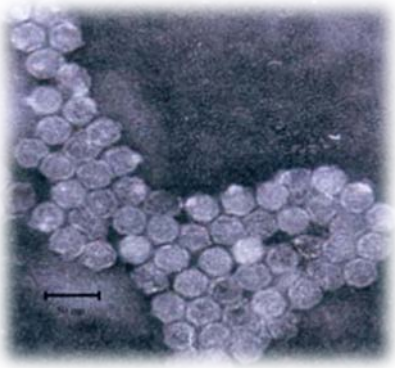


Fig. 1.2 A. SMV infected soybean. B. Potyvirus particle under electron microscopy (Brunt et al., 2006; Dallwitz, 1980; Dalwitz et al., 1993). C. Genome structure of SMV.

BPMV is the causal agent of another important disease of soybean in the U.S. that is approaching epidemic levels. BPMV belongs to the genus *Comovirus,* family *Secoviridae* and has isometric particles of 28 nm in diameter (Fig.1.3.B). The bipartite genome consists of two positive single-stranded RNA segments which are designated as RNA1 and RNA2. Both RNA segments employ a polyprotein expression strategy and have a VPg attached to the 5' and a poly A tail at the 3' termini of the genomic molecules, respectively. The 6.0 kb RNA1 encodes five proteins including a 32 KDa proteinase, a common 'replication block' consisted of type III helicase, 3C-like cysteine proteinase and type I polymerase, and a VPg found between the helicase and protease motifs. RNA2 is 3.6 kb and encodes a movement protein and two coat proteins (Giesler et al., 2002; Sanfaçon et al., 2009) (Fig.1.3.C). BPMV was first reported in Arkansas in 1951 and had been disseminated from the southern U.S. (Arkansas, the

Carolinas, Kentucky and Mississippi) to the north central region (Iowa, Illinois, Indiana, Kansas, Ohio, Nebraska; Giesler et al., 2002; Ziems et al., 2007). The prominent symptom of BPMV infection is seed coat mottling appearing as “bleeding hilum”. As in SMV infection, discolored seed is evaluated as lower grade and affects consumer acceptance (Mabry et al., 2003; Redinbaugh et al., 2010). In the field, infected soybean plants exhibit different symptoms (Fig.1.3.A; Fig1.4), including mild chlorotic mottling in upper leaves, puckering or severe mosaic foliage; more severe symptoms such as terminal necrosis and death of leaves have been reported in some cultivars (Sinclair et al., 1999; Giesler et al., 2002 and Ziems et al., 2007). Acute symptoms develop on young leaves whereas severity decreases over time. Reduced pod formation, seed size, weight and number can lead to up to 60% yield reduction (Byamukama et al., 2011 and Redinbaugh et al., 2010) and is therefore a primary concern for growers. Synergistic effects occur between BPMV and SMV and usually cause excessive reductions in yield and seed quality (Giesler et al., 2002; Pedersen, 2007). Several species of beetles feeding on leaves as well as the western corn rootworm (*Diabrotica virgifera virgifera*) and soybean leaf miner (*Odontota horni*) are reported to transmit BPMV between plants, with the bean leaf beetle (BLB; *Cerotoma trifurcata*) being the most prevalent vector. BLB transmits BPMV in a non-circulative manner and survives winters in the field (Giesler et al., 2002; Byamukama et al., 2011).



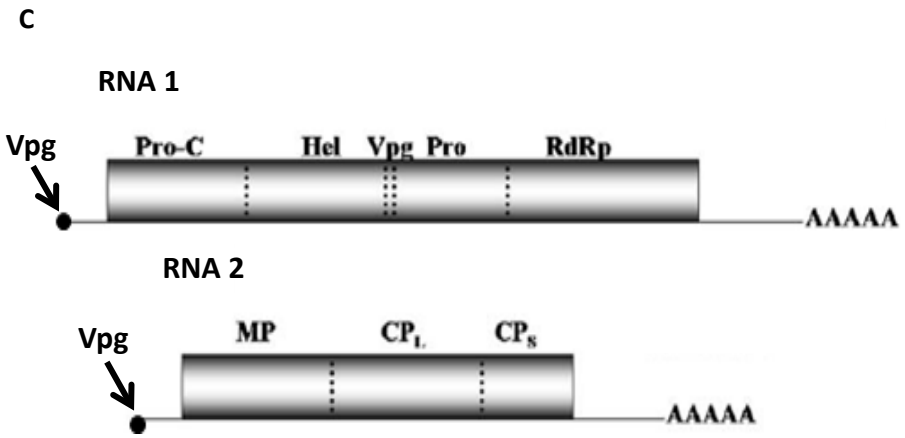


Fig. 1.3 A. BPMV infected soybean; B. Negatively stained BPMV virion (Giesler et al., 2002); C. Genome structure of BPMV

Fig.1.4 BPMV symptoms on soybean cultivars

1.6 Research goals

The goal of this thesis was to understand the basic biological features of SVNaV. This was accomplished by characterizing the virus genome, developing detection methods, analyzing population structure and distribution, and determining the alternative host range. Knowledge obtained in this study is critical for elucidating the epidemiology of the virus. For virus characterization, viral nucleic acid was isolated from symptomatic soybeans, sequenced and analyzed by comparing the SVNaV genome with those of known tospoviruses. The nucleoprotein gene of 37 isolates collected from eight states were

amplified and sequenced for diversity analysis to gain a better understanding of virus population structure. This knowledge was used to develop conventional and qPCR primers; primers that could detect all studied isolates. To determine the potential host range of SVNaV, multiple plants species mainly from dicotyledons, including major crops, vegetables and ornamental species were selected and tested by mechanical inoculation.

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Chapter II

Molecular characterization of a new tospovirus infecting soybean

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2.1 Abstract

A new, widespread, disease was recently observed in soybean in the United States. The disease, named Soybean vein necrosis, is manifested by intraveinal chlorosis and necrosis and was found in almost all of the 50 fields visited over a period of three years in the Midwest and Midsouth part of the United States. A virus was isolated from symptomatic material and detection protocols were developed. More than 150 symptomatic specimens collected from seven U.S. States were tested, and all were found positive for the virus unlike 75 asymptomatic samples. Protein pairwise comparisons coupled with phylogenetic analyses indicate that the virus is a new member of the genus *Tospovirus.*

2.2 Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important crops in world agriculture. There are several viruses reported in the crop (Hartman et al., 1999), many of which cause significant losses worldwide, estimated to be well over two million metric tons annually (Wrather et al., 2010) and new virus diseases are being identified continually (Kuroda et al., 2010; Lamprecht et al., 2010; Nam et al., 2009). The United States is the leading producer of soybeans accounting for about one third of the world production with more that 30 million hectares planted and about 90 million ton harvested in 2009 (Anonymous, 2011). Many viruses, including Alfalfa mosaic, Soybean mosaic and Bean pod mottle are listed as pests of great importance for the U.S. soybean industry (Anonymous, 2011).

A disease with virus-like symptoms was observed in soybean fields in Arkansas, Kansas, Missouri, Illinois, Mississippi, Tennessee and Kentucky. First symptoms were observed in early June in Arkansas and Illinois and manifested as vein clearing. As the season progressed the affected areas became chlorotic or necrotic and in severe cases leaves died off (Fig. 2.1).

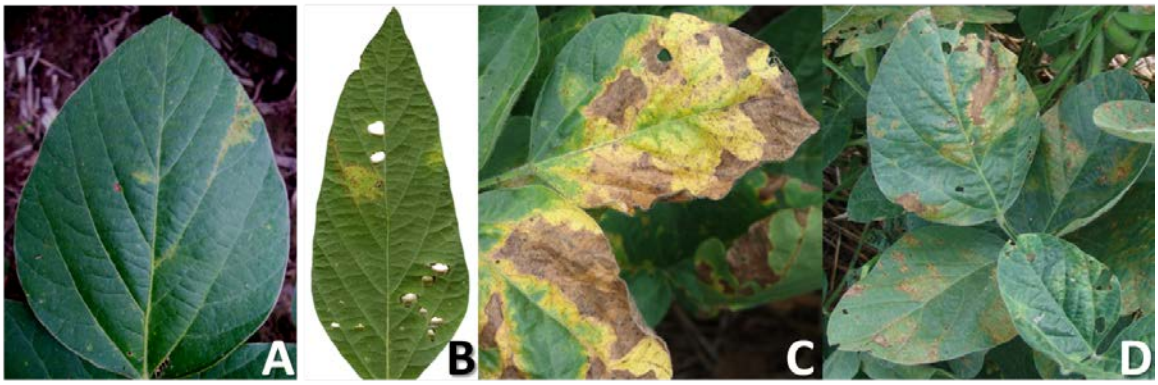


Fig. 2.1 Symptoms associated with Soybean vein necrosis associated virus infection in early (A), mid (B) and late (C) season. Panel D shows a severely affected plant in late season.

Immunological tests failed to identify an agent associated with the disease and the possibility of a new virus infecting soybean was investigated. Indeed, a new virus was found in symptomatic material and detection protocols were developed. Surveys in several states showed that the virus was present at high incidence and was 100% associated with typical disease symptoms as all of the 150 symptomatic samples tested were positive for the virus. The importance of soybean in world agriculture in association with the high prevalence of this virus in major soybean production areas in the United States made its characterization essential.

2.3 Materials and methods

2.3.1 Soybean virus testing

Samples showing soybean vein necrosis disease symptoms were tested by ELISA for 16 viruses or virus groups at Agdia, Inc. (Elkhart, IN). The viruses assayed were: *Alfalfa mosaic virus*, *Bean pod mottle virus*, *Cowpea mosaic virus*, *Cucumber mosaic virus*, *Impatiens necrotic spot virus* (INSV), *Peanut stunt virus*, *Southern bean mosaic virus*, *Soybean dwarf virus*, *Soybean mosaic virus*, *Tobacco mosaic virus*, *Tobacco ringspot virus*, *Tobacco streak virus*, *Tomato ringspot virus*, *Tomato spotted wilt virus* (TSWV), *Watermelon mosaic virus-2* and Potyvirus group.

2.3.2 Nucleic acid purification

Double-stranded RNA (dsRNA) isolation from symptomatic soybean leaves was performed using a lithium salt extraction protocol as described by Tzanetakis and Martin (Tzanetakis et al., 2008) with the following modification: the digested nucleic acids were bound to 20µl glass milk (Sigma, MO) in the presence of ethanol (instead of CF-11 cellulose) and were then eluted in 100µl TE instead of being subjected to ethanol precipitation. For detection purposes, the total nucleic acid extraction protocol of Tzanetakis et al. (2007) was used.

2.3.3 Detection and geographic distribution

Purified nucleic acids were subjected to reverse transcription using Maxima ® reverse transcriptase (Fermentas, MD) according to manufacturer's instructions using virus specific primers Ldet R and Sdet R (100nM final concentration) (Table 2.1). The purified nucleic acids made up 10% of the total reaction volume. For PCR two oligonucleotide primer sets (Table 2. 1) that amplify regions of the L (Ldet F/Ldet R) or the S (Sdet F/Sdet R) RNAs (297 and 861 nucleotides respectively) were optimized. The final concentration of the virus primers were 400nM. The PCR was performed in a Mastercycler® (Eppendorf) using Taq polymerase (Genscript, NJ) at a concentration of 1u/25µl reaction consisted of 2 min incubation at 94C followed by 30 cycles of 15 sec denaturation at 94C, 15 sec extension at 55C and 1 min sec extension at 72C. The program terminated with 10 min incubation at 72C. The validity of the tests was confirmed after sequencing at least 30 amplicons for each primer set at the University of Arkansas Sequencing Center. The geographic distribution of the virus was studied by applying virus-specific RT-PCR tests on 150 symptomatic and 75 asymptomatic samples collected from 50 production fields and variety trials from Arkansas, Tennessee, Illinois, Missouri, Kansas, Kentucky and Mississippi.

Table 2.1 List of primers used to detect, study the diversity and obtain the genome of Soybean vein necrosis associated virus. F- forward primer (sense), R- reverse primer (antisense)

Oligo Name	Sequence 5' to 3'
Detection	
LdetF	GAGCCCATAAACCTGTCTGC
LdetR	TGCCATGATGTGCTCAGATT
SdetF	CTATCTATAACCAACAGAATCAAG
SdetR	GATTAAACAGAAAACTCCTTTG
Sequencing	
LRCext	GCCTCGGGAAATTTAAGGGAGC
LRCint	GCCCTCAGGTCATGGAATGAACG
LaF	TTGTGTGTCAACATTTTCATTACT
LaR	TCTCTTTGAAACTGTGACTGCC
LbF	TAGTCTTCATGCTCATCAGATAA
LbR	TAGCGGGTATATTTACATAATGAAAC
LcF	GATCGGCACTTTGCTTGGAGA
LcR	CAGGAATCTCTGATGACATCTCTA
LendF	CCCTTCTGTTTCTTCGCAAATC
MRCext	GAGACTCAGTGATAGGATTCTGG
MRCint	CTCTCCATTGCTTTTCGGTATCA
MF	TTCTTGTACTGCTCCTGGCTGT
MR	TTGTGTAAGTTATTGTTCAGCA
MendF	TAATAGAGGCATTGATAAATATACT
SRCext	CAAATCTGCTGGAATCAATAAAT
SRCint	TGTATCTATGACACATTTCTGGT
SF	CAGGAAATGTTACTAGCATACAC
SR	GCTAAAGTTGCATCTGTGCTG
SendF	TTTCTCAAATTCTATGTCTTCTTCA

2.3.4 Genome characterization and phylogenetic analyses

DsRNA was used as template for shotgun cloning as previously described (Tzanetakis et al., 2005a). Sequences were compared with those found in Genbank using BLAST (Altschul et al.,1997) and those of tospovirus origin were used to design oligonucleotide primers to acquire the complete genome of the virus as previously described (Tzanetakis et al., 2005b; Table 2.1). The end of the virus segments were obtained after two tailing reactions as previously described (Tzanetakiset et al., 2004). Genome assembly was performed using CAP3 (Huang et al., 1999). The complete virus sequences have been deposited in Genbank under accession Nos HQ728385-7.

Sequence comparisons between the new virus proteins and their orthologs were performed using MatGAT (Campanella et al., 2003; Table 2.2). Additional *in-silico* protein analysis included identification of RNA binding domains using BindN tool at 90% specificity (Wang et al., 2006). The putative signal peptides, transmembrane domains and glycosylation sites were predicted using the SignalP 3.0 (Bendtsen et al., 2004), TMHMM 2.0 (Krogh et al., 2001), NetNGlyc 1.0 and NetOGlyc 3.1 (Julenius et al., 2005), respectively. For phylogenetic analysis the L RNA polyprotein and nucleoprotein amino acid sequences were used. Analyses were performed with ClustalW employing a neighbor-joining algorithm with Kimura's correction and bootstrap analysis consisting of 1000 pseudoreplicates (Thompson et al., 1994). Phylograms were visualized on TreeView (Page et al., 1996).

	WSMoV	CaCV	GBNV	TZSV	CCSV	MYSV	IYSV	TSWV	INSV
L RNA									
Polyprotein	28.2(44.9)	28.9(45.1)	33.9(53.3)	27.5(44.0)	30.1(47.5)	28.6(45.4)	27.6(43.2)	33.9(53.3)	37.5(57.4)
M RNA									
NSm	21.2(42.4)	24.6(43.9)	24.9(44.5)	13.4(30.8)	13.7(32.1)	23.6(42.1)	24.2(42.4)	33.6(36.7)	35.5(50.2)
Gn/Gc	22.4(39.7)	21.5(39.0)	22.3(39.9)	21.4(38.6)	21.7(39.8)	21.0(39.9)	21.5(39.4)	21.5(42.4)	24.9(44.2)
S RNA									
NSs	11.8(25.5)	11.1(26.6)	10.5(25.9)	8.3(21.4)	7.8(20.7)	9.2(19.2)	10.8(27.3)	8.0(18.5)	7.6(23.4)
NP	30.5(51.3)	30.2(52.7)	32.2(52.2)	32.1(52.7)	32.5(60.0)	33.6(52.3)	33.7(55.7)	34.1(58.9)	33.6(59.5)

Table. 2.2 Amino acid identities and similarities between Soybean vein necrosis associated virus (SVNaV) and tospovirus orthologous proteins. Polyprotein (Polyp), non-structural protein (NSm), glycoproteins (Gc/Gn), non-structural protein (NSs), and nucleoprotein (N). Identities are listed with similarities given in parentheses. Virus species and Genbank accession numbers used in comparison are as following. *Tomato spotted wilt virus* (TSWV; NC_002050, NC_002051, NC_002052); *Impatiens necrotic spot virus* (INSV; NC_003625, NC_003624, NC_003616), *Groundnut bud necrosis virus* (GBNV; NC_003614, NC_003620, NC_003619), *Watermelon silver mottle virus* (WSMoV; NC_003843, NC_003841, NC_003832), Capsicum chlorosis virus (CaCV; NC_008302, NC_008303, NC_008301), Melon yellow spot virus (MYSV NC_008307, NC_008306, NC_008300), Tomato zonate spot virus (TZSV; NC_010491, NC_010489, NC_010490), Calla lily chlorotic spot virus (CCSV; FJ822962, FJ822961, AY867502), *Iris yellow spot virus* (IYSV; FJ623474, FJ361359, FJ713700, AF001387).

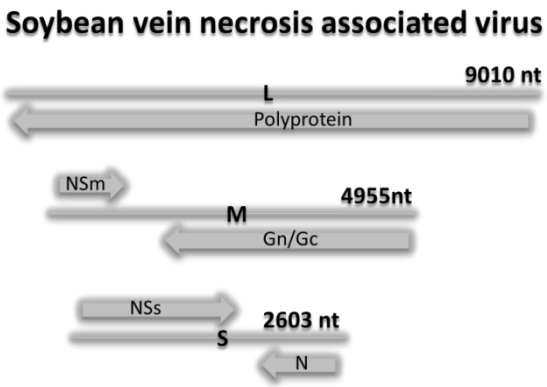
2.4 Results

Soybean vein necrosis symptoms were observed in all but one of the 50 fields surveyed signifying the importance of the disease and the need to study it. Although disease symptoms have not been associated with any previously described soybean viruses, testing for 16 viruses and virus groups minimized the possibility of mixed infections with synergistic effects resulting in leaf necrosis. No known virus was found consistently associated with vein necrosis symptoms in these tests.

Shotgun cloning revealed the presence of a tospovirus, SVNaV, and a new endornavirus, endogenous dsRNA molecules found in several plants but never associated with disease symptoms (Valverde et al., 2007). Notwithstanding, testing for the endornavirus did not reveal any association between the dsRNA molecule and disease (Tzanetakis, unpublished). Detection protocols were developed and used to assess the presence of the virus is a wide geographic area including major soybean producing areas in the United States. All 150 samples with typical soybean vein necrosis symptoms tested positive with two detection primer sets targeting two virus segments. Seventy-five asymptomatic plants from fields with the virus did not yield any amplicons. This perfect association of virus and symptoms indicates that the virus is likely the cause of the disease and thus the name Soybean vein necrosis associated virus (SVNaV; Fig. 2.2A) is proposed.

SVNaV was fully sequenced and revealed several typical and atypical characteristics for members of the genus *Tospovirus*. All SVNaV segments have the highly conserved *Tospovirus* 5' terminal sequence ($AGAGCA_{1\text{-}6}$) predicted to be crucial as replication and transcription signals (Sherwood et al., 2000). SVNaV L RNA is 9010 nucleotides (nt), the longest among sequenced tospoviruses. Similar to other tospoviruses, the first and last 19 nts are complementary leading to the circularization of the molecule by the formation of a panhandle structure (De Haan et al., 1991; Fig. 2.2B). SVNaV L RNA has a single open reading frame (ORF) in the negative orientation starting at nt 8980 and terminating at nt 185 encoding for a putative polyprotein of 336 KDa. Typical RNA-dependent RNA polymerase (RdRp) domains (fingers, palm and thumb) forming an open "U-shape" crevice and conserved sequences reported in the family *Bunyaviridae* and other segmented negative-strand viruses were identified in the protein (Roberts et al., 1995). Motif A $(DXXKWS)_{539\text{-}544}$, and motif C $(SDD)_{665\text{-}667}$, are present in the palm domain, involved in divalent metal cation binding (Lukashevich et al., 1997). Mutation of the Asp in the two motifs (motif A: D_{539}, motif C: D_{667}) abolished polymerase activity in *Bunyamwera virus* (Jin et al., 1993). The Gly_{528}, conserved in almost all viral RdRp, is present in motif B $(QGXXXXXSS)_{527\text{-}535}$ (Kolakofsky et al., 1991) and may, because of its mobility, play a crucial role in RNA binding. Lys_{712} (motif D) is presumably involved in catalytic activity of the enzyme because of its proximity in the tertiary structure of the protein to motif A Asp_{539} as determined for *Lassa fever virus* (Lukashevich et al., 1996). Motif E $(EFXSE)_{721\text{-}725}$, is involved in cap scavenging and has endonuclease activity (Kolakofsky et al, 1991). Region F, the NTP-binding site, is subdivided into three domains; F1: $(KX)_{451\text{-}452}$, F2: $(KXQR)_{459\text{-}462}$ and F3:$(TXXDRXIY)_{463\text{-}470}$ (Bruenn et al., 2003). Several RNA binding domains participating in the formation of the RNA replication complex and maintaining nucleotides in specific positions were identified, including $TSSSGSK_{2900\text{-}2906}$ and $KWSKPKKKKKPKAKPKKSKKKHNK_{2908\text{-}2931}$, at the C-terminus of the protein (Kukkonen et al., 2005).

A



B

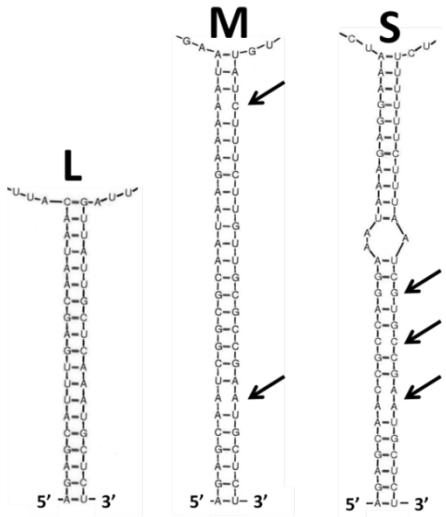


Fig. 2.2 A. Genome organization of Soybean vein necrosis associated virus. Abbreviations: Non-structural protein M RNA (NSm), glycoproteins (Gc/Gn), non-structural protein S RNA (NSs), and nucleoprotein (N); **B.** Hybridization of the untranslated region termini of the genomic RNAs (L, M, S) of Soybean vein necrosis associated virus using mfold (Zuker, 2003). Arrows point to mismatches found in the stems at the termini of the molecules.

The M RNA is 4955 nt and similar to the L segment, the end sequences are complementary; but instead of the 19 nt in the L RNA, the first and last 27 nt form the panhandle structure, with two mismatches (Zuker 2003; Fig. 2.2B). The segment codes an ORF in the positive and one in the negative

orientation. ORF1 initiates at nt 58 and terminates at nt 1008 coding for a non-structural protein (NSm) of 36 kDa. The protein is probably involved in cell-to-cell movement as the highly conserved $LXDX_{40}G$ motif of the '30K movement protein superfamily' was identified in several orthologs (Silva et al., 2001; Melcher et al., 2000; Mushegian et al., 1993), but SVNaV has an Ile instead of Leu at the beginning of the motif ($IXDX_{40}G_{165\text{-}208}$). The 'P/D-L-X motif' and phospholipase A2 catalytic site, present in some tospovirus NSm including TSWV, *Groundnut bud necrosis virus* (GBNV), Chrysanthemum stem necrosis virus (CSNV) and *Tomato chlorotic spot virus* (TCSV), are absent from the SVNaV ortholog. A glycosylation site was predicted at N_{195}. ORF2 starts at nt 4863 extending to nt 1276 and codes for the precursor of the virus glycoproteins. As secretory proteins, the transportation of the mature glycoproteins from the cytosol to the endoplasmic reticulum may be directed by a signal peptide such as the one predicted to be cleaved from the N-terminus of the protein. The 26 amino acid (aa) peptide is primarily composed of basic and hydrophobic amino acids. The putative signal cleavage site for the mature proteins (Gn/Gc), predicted after alignment of tospovirus orthologs, is found between Cys_{378} and Ser_{379} yielding the 43 kDa Gn and 91 kDa Gc (Kormelink et al., 1992). N-Glycosylation sites of Gn were predicted at N_{25}, N_{229} and N_{343}, respectively; whereas three transmembrane domains are located between aa 6-28, 317-339 and 349-371. The RGD Gn domain, predicted to play a critical role in virion-cell attachment was found at position 29-31 (Kormelink et al., 1992; Cortez et al., 2002). A series of conversed domains present in *Tospovirus* Gcs are also resent in the SVNaV ortholog, including a highly conserved Lys_{702}, a motif consisted of T-X-$T_{714\text{-}716}$, followed by $CTGXC_{730\text{-}734}$ and $TSXWGCEEXXCXAXXXGXXXGXC_{754\text{-}776}$ (Cortez et al., 2002). A transmembrane domain is found between Gc aa 77-99, leaving a long amino acid tail inside the virus particle. N-glycosylation modifications are predicted at three positions: N_5, N_{20} and N_{171} whereas no O-glycosylation site was predicted in either of the glycoproteins.

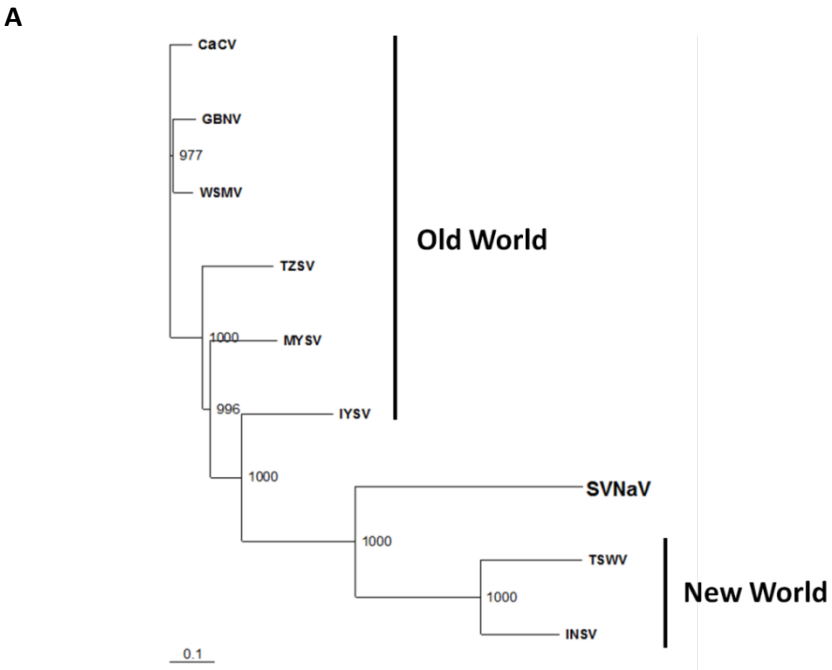
The S RNA is 2603 nt, has the first and the last six nucleotides complementary and alike the other two RNAs its untranslated region is highly structured (Fig. 2.2B). Similar to the M RNA, the S RNA encodes an ORF in the positive and one in the negative orientation. ORF1 initiates at nt 59 and terminates at nt 1381 encoding a 50 kDa non-structural protein (NSs), a putative silencing suppressor (Takeda et al., 2002). The conserved $GK_{178-179}$ motif is present in the Walker motif A where K_{179} is believed to interact with the ATP/ADP phosphate (Caruthers et al., 2002). The SVNaV NSs Walker motif B ($DEXX_{148-151}$) is located upstream motif A, similar to *Watermelon silver mottle virus* (WSMoV), GBNV and Capsicum chlorosis virus. The conserved $DE_{148-149}$ is presumably involved in Mg^{2+} binding and ATP hydrolysis. Lokesh et al. (2010) recently reported NSs to be a biofunctional enzyme: its NTPase activity hydrolyses ATP required by Dicer to process and unwind siRNA. It also functions as phosphatase removing the phosphoryl group from the 5' end of dsRNA making it unidentifiable by Dicer. Whether the SVNaV NSs employs the same strategy and functions as an RNA silencing suppressor remains to be studied. ORF2 spans from nt 2533 to nt 1700, encoding for a putative nucleoprotein (N) of 31 kDa. N may be essential for RNA synthesis together with the L RNA polyprotein (Kukkonen et al., 2005). Similar to the TSWV ortholog, the C terminal motif $KKDGKGKKSK_{264-273}$ was predicted to bind RNA (Kainz et al., 2004) whereas other discrete amino acids, including PSN_{7-9}, RK_{51-52}, RY_{54-55} and KK_{73-74} may interact with the virus RNAs to prevent premature termination caused by base pairing of the newly synthesized RNA strands and to protect it from degradation (Kukkonen et al., 2005).

2.5 Discussion

SVNaV is a distinct member of the genus *Tospovirus* as it shares minimal similarity with any of the established species in the genus - all SVNaV proteins exhibit less than 40% amino acid identity to their orthologs. Many of the characteristic tospovirus protein motifs were identified in the SVNaV orthologs

whereas certain unique features like the very large polyprotein and the inverted Walker motif in NSs add to the complexity of the genus.

When SVNaV is compared to other tospoviruses using the L RNA polyprotein-RdRp, the hallmark gene for any virus evolution study and a excellent predictor of tospovirus phylogeny (Kainz et al. 2004), it becomes evident that the virus occupies the phylogenetic space between the New and Old World tospoviruses, forming a new clade in the genus (Fig. 2.3). The nucleoprotein was also used to assess the phylogenetic placement of SVNaV and its position in *Tospovirus* evolution stands but more clades appear with the most pronounced being that of Peanut chlorotic fan-spot/Peanut yellow spot viruses. These two viruses are not fully characterized and thus their phylogenetic placement remains uncertain.



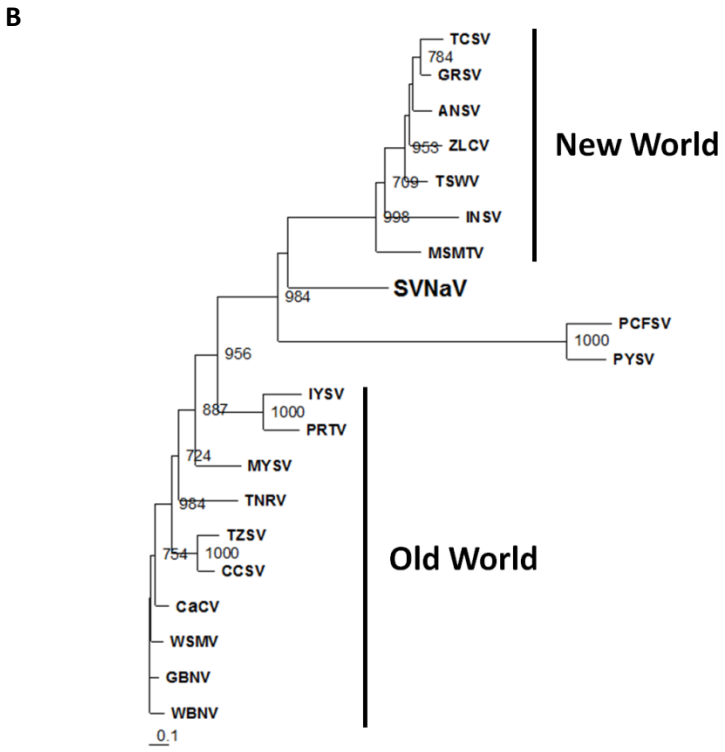


Fig. 2.3 Unrooted phylograms using the amino acid sequences of **A.** the L RNA polyprotein and **B.** the nucleoprotein of Soybean necrosis associated virus (SVNaV) and other members of the genus *Tospovirus*. Abbreviations and Genbank accession numbers (Polyprotein in plain text, nucleoprotein in italics): TSWV, *Tomato spotted wilt virus*, D10066, *NP_049361*; INSV, *Impatiens necrotic spot virus*, X93218, *NP_619709*; GBNV, *Groundnut bud necrosis virus*, AF025583, *NP_619701*; WSMoV, *Watermelon silver mottle virus*, AF133128, *NP_620771;* CaCV, Capsicum chlorosis virus, DQ256124, *YP_717923;* MYSV *Melon yellow spot virus*, AB061774, *YP_717921;* TZSV, *Tomato zonate spot virus*, EF552435, *YP_001740044;* CCSV, Calla lily chlorotic spot virus, FJ822962, *AAW58115;* IYSV, Iris yellow spot virus, FJ623474, *ACN62253;* PCFSV, Peanut chlorotic fan-spot virus, *AAC99405*; WBNV, Watermelon bud necrosis virus, *ADD83166*; ZLCV, *Zucchini lethal chlorosis virus*, *AAF04198*; PYSV, *Peanut yellow spot virus*, *AAB94022*; TCSV, *Tomato chlorotic spot virus*, *AAL07433*; GRSV, *Groundnut ringspot virus*, *AAM47011*; ANSV, Alstroemeria necrotic streak virus, *ACZ18222*; MSMTV, Melon severe mosaic tospovirus, *ABX72231*; PRTV, Polygonum ringspot tospovirus, *ABO31117*; TNRV, Tomato necrotic ringspot virus, *ACK99533*. The bootstrap values are based on 1000 pseudoreplicates and support of under 70% is not shown as considered unreliable. Bars represent 0.1 amino acid changes per site.

Tospoviruses cause significant losses and inferior quality of produce in vegetables, legumes and ornamentals among other crops around the world. Some members of the family (i.e. TSWV and INSV) infect both dicots and monocots with host ranges that exceed several hundred species causing losses in the billion U.S. dollar range (Daughtrey et al., 1997; Pappu et al., 2009). The prevalence of disease and virus in almost all fields visited makes it a potential threat to the soybean industry.

Whereas this communication presents the molecular characterization of SVNaV, a virus found closely associated to Soybean vein necrosis, fulfillment of Koch postulates are needed before proving that it is the causal agent of the disease. We are also working on the biological characterization and epidemiology including potential vectors, alternative hosts and genetic diversity, aspect of the virus biology that will help better understand the disease and assist in the implementation of disease control strategies.

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Chapter III

Epidemiology of Soybean vein necrosis associated virus

3.1 Abstract

Soybean vein necrosis associated virus (SVNaV) is widespread in major soybean-producing areas in the U.S. The presence of the virus has been confirmed in 12 states: Arkansas, Illinois, Missouri, Kansas, Tennessee, Kentucky, Mississippi, Maryland, Delaware, Pennsylvania, Virginia and New York. Symptomatic samples, collected from eight states (AR, IL, MO, MS, KS, TN, MD and DE), were used to study the population structure of the virus. The nucleoprotein gene was analyzed given that the gene represents the most diverse region of the tospovirus genome. The study revealed a relatively homogeneous virus population indicating that the diverse symptoms observed in the field is probably caused by the different host genotypes rather than distinct virus strains. In order to efficiently detect SVNaV, detection protocols based on reverse transcription – PCR (RT-PCR) and quantitative reverse transcription – PCR (qRT-PCR) were developed and their sensitivities were compared with immunological test. In total, 24 plant species belonging to ten families were tested by mechanical inoculation as potential alternative hosts of SVNaV. The alternative host range and vector identification validated the conclusion that SVNaV is a distinct tospovirus given that it replicates in some but not all common indicator plants used for tospoviruses diagnosis.

3.2 Introduction

Tospoviruses are some of the most devastating plant viruses affecting global agriculture. *Tomato spotted wilt virus* (TSWV), the type member of the genus, has a host range exceeding 1000 species in

more than 100 plant families, causing losses measured in the billions of dollars. Other members in the genus such as *Impatient necrotic spot virus* (INSV) and Iris yellow spot virus (IYSV) also infect a large number of economically important species and are becoming major threats for global agriculture. Previous studies showed that several tospoviruses including TSWV, *Groundnut bud necrotic virus* (GBNV), *Groundnut ringspot virus* (GRSV) and Tomato yellow fruit ring virus (TYFRV) and now Soybean vein necrosis associated virus (SVNaV) (Reddy et al., 1983; Golnaraghi et al., 2001; Pietersen et al., 2002; Golnaraghi et al., 2004; Golnaraghi et al., 2007; Zhou et al., 2011) infect soybean naturally or experimentally. The wide distribution of SVNaV in the U.S. highlights the importance of studying its epidemiology including population structure, natural vector(s) and alternative host range, with the goal of better understand the virus and the disease and develop appropriate measures as to minimize the losses caused by the virus.

Thrips are the only known vectors for tospoviruses. More than ten species in the genera *Frankliniella*, *Scirtothrips* and *Thrips* can transmit over 15 virus species (Riley et al., 2011). Virions are acquired by thrips in their first and second larvae stages and propagate in a persistent-circulative manner. Glycoproteins on the surface of the viral particle interact with receptors of the vector's midgut membrane, initiating entry of the particle in the insect cells. New virus progenies are assembled within the vector and transmitted to plants through infected saliva. *Frankliniella occidentalis* (western flower thrips) is one of the most efficient tospovirus vectors transmitting TSWV, GRSV, INSV, *Tomato chlorotic spot virus* (TCSV) and Chrysanthemum stem necrosis virus (CSNV) (Riley et al., 2011). However, the observation that *Sericothrips variabilis* (soybean thrips), *Frankliniella fusca* (tobacco thrips) and *Frankliniella tritici* (eastern flower thrips) are the most abundant species in soybean fields in the mid-southern of the U.S. (Freeman et al., 2002) indicates that these species may play important roles in SVNaV transmission.

As a perennial crop, soybean is planted in Arkansas after the middle of April and harvested in middle to late fall. The wide spread and continuous presence of SVNaV in soybean fields from 2008 to 2012 suggests that other alternative host(s) may exist, hosts on which the virus can survive and overwinter in the absence of soybean. Therefore, several weeds species collected from soybean fields were tested as part of this study; in addition, other species including major crops, grown close to soybean fields or are planted before or after the soybean harvest season, vegetables, and ornamental plants were tested for their susceptibility to SVNaV.

The objectives of this study were to: (1) analyze the population structure of SVNaV; (2) determine the alternative host range of SVNaV and (3) develop rapid and sensitive detection methods for SVNaV.

3.3 Materials and methods

3.3.1 Sample collection

Soybean leaves with typical SVNaV symptoms (Fig. 1.1) were collected from multiple fields in AR, IL, MO, KS, TN, MS, MD and DE between 2008 and 2012. Samples were sealed in air tight bags and kept on ice until processed for detection.

3.3.2 Total nucleic acids isolation

Total nucleic acids were isolated as described previously (Tzanetakis et al., 2007). Briefly, 50 mg of symptomatic leaf tissue was ground in 1 ml RNA extraction buffer (200 nm Tris-HCl, pH8.5, 300 mM lithium chloride, 1.5% lithium dodecylsulphate, 10 nM ethylenediaminetetraacetic acid (EDTA), 1% sodium deoxycholate, 1% NP-40) and 1% (v/v) 2-mercaptoethanol (98%, 14 M) was added just before use. Potassium acetate (1 ml; 5.8M: 3.8 M potassium, 5.8 M acetate) was added and then 600 ml

mixture was transferred to a new 1.5 ml tube and spun down at 13000 g for 10 min. Supernatant (700 µl) was then transferred to another 1.5 ml tube, mixed with an equal amount of 100% isopropanol and kept at -20°C for 30 min. Nucleic acids were precipitated by centrifugation at 13000 g for 20 min and bound to 20 µl silica milk in the presence of 500 µl RNA wash buffer (10 µM Tris-HCl, pH7.5, 0.5 mM EDTA, 50 mM NaCl, 50% ethanol). The bound nucleic acids was washed twice with wash buffer to eliminate inhibitors for the downstream reactions, dried with a speedvac (Thermo Fisher, PA, U.S.) and subsequently eluted in 150 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After centrifuging at 13000 g for 2 min, 100 µl supernatant was collected and kept at -80°C before use.

3.3.3 cDNA synthesis and virus detection

RNase-free DNaseI was used to degrade DNA in purified total nucleic acids according the manufacturer's instruction (Fermentas, MD, U.S.). Briefly, 5 U (1 U/µl) of DNaseI was added to 50 µl nucleic acids along with 10 µl 10×buffer, 20 U RNase inhibitor (40 U/µl, RiboLock, Fermentas) and nuclease-free water to a final volume of 100 µl. After incubating at 37°C for 1 h, the enzyme was deactivated by adding 10 µl 0.5 M EDTA. RNA was then collected by 20 µl glass milk in the presence of 1.5 ml RNA wash buffer. The amount and quality of RNA were evaluated with a spectrophotometer (Nanodrop, Thermo Fisher Scientific, DE, U.S.). Samples with good quality (260/280 > 2.0) were used for downstream reactions. Reverse transcription was performed in a 25 µl reaction consisting of template RNA, 1 µl dNTPs (10 mM), 0.5 µl Ldet F and Sdet R (20 µM for each, Table 2.1), 5 µl 5×reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 50 U reverse transcriptase (200 U/µl, Maxima, Fermentas) and 6 U RNase inhibitor (40 U/µl, RiboLock, Fermentas). The reaction was carried out in a thermocycler (Thermo C-1000, Thermo Fisher Scientific) with incubation at 50°C for 60 min followed by enzyme deactivation at 80°C for 5 min. The synthesized cDNA was used as template for detection PCR. Detection primers Ldet F and Ldet R designed based on the

RdRp gene was used in a 25 µl PCR consisting of 0.5 µl dNTPs (10 mM), 0.5 ul reverse and forward primers (20 uM for each), 0.2 U *Taq* polymerase (5 U/ul, Genscript) and 2.5 ul cDNA template. The reaction was initiated by denaturation at 94°C for 2min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 10 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. Amplification products were visualized in 2% agrose gel in 0.5×TBE (40 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH7.2) post-stained with GelRed®(Biotium, CA, U.S.).

3.3.4 Virus diversity analysis

All samples with typical vein necrosis symptoms were tested positive for SVNaV. Isolates collected from eight states (AR, IL, MO, MS, KS, TN, MD and DE) were used for the population structure analysis. Primers (Sdet F/Sdet R) (Table. 2.1) were employed to amplify the complete N protein gene. The PCR program included initial denaturation at 94°C for 2min, followed by 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 50°C for 10 s, extension at 72°C for 40 s, followed by a final extension at 72°C for 10min. Amplification products were visualized in a 1% agarose gel in 0.5×TBE post-stained with GelRed®(Biotium).

Amplicons were purified using the Quick Clean®5M PCR purification kit (Genscript, NJ, U.S.) according to manufacturer's insrtructions. The amount and quality of the PCR products was determined using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, DE, U.S)and ligated into pCR®2.1 vector (Original TA cloning kit, Invitrogen, NY, U.S.) following manufacturer's instructions. Briefly, a 5 µl ligation reaction consisted of DNA (volumes used vary among samples), 0.5 µl vector, 0.5 µl 10×ligation buffer, 0.5 µl T4 DNA ligase and water was performed at 14°C overnight. The ligation product (2 µl) was used to transform 25 µl α-select *E. coli* competent cells (Gold Efficiency, Bioline, MA, U.S.). The transformation reaction was kept on ice for 15 min before heat shock (42 °C for 45 s). The mixture was immediately chilled on ice for 2 min and 300 µl SOC medium (2% tryptone, 0.5% yeast extract, 10 mM

NaCl, 3.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added. Cells was shaken at 37 °C for 1 h and then plated onto LB agar plates (1% tryptone, 0.5% yeast extract, 10 mM NaCl and 1.5% Agar) containing 40 µM X-gal (bromo-chloro-indolylgalactopyranoside), and kanamycin (50 µg/µl). Plates were incubated at 37 °C overnight. White colonies were selected and grown overnight in 2 ml LB broth (1% tryptone, 0.5% yeast extract, 10 mM NaCl) containing 50 µg/µl ampicillin. Colony PCR (25 µl reaction consisted of 2.5 µl 10×buffer, 0.5 µl dNTP (10 mM), 0.5 µl M13 forward and reverse primers (20 µM for each), 1.25 U *Taq* polymerase (5 U/µl, Genscript) was used to select colonies with the insert of interest. The reaction was performed at 94 °C for 5 min as initial denaturation followed by 40 cycles of 94 °C for 30 s, annealing at 50 °C for 10 s, 72 °C for 40 s and final extension at 72 °C for 10 min. Plasmids were extracted from colonies with the inserts of the expected size and were sequenced using M13 forward and reverse primers at the DNA Resource Center of the University of Arkansas.

Sequences were visualized using Sequence Scanner Software v1.0 (Applied Biosystems, CA, U.S.) and ambiguous nucleotides were corrected manually. The contigs of the complete N protein gene of 37 isolates (with a minimum 3x nucleotide coverage) were obtained by using CAP3 sequence assembly program (Huang and Madan, 1999). Corrected sequences were aligned with ClustalW 2 (Larkin et al., 2007) and analyzed at both the nucleic acid and amino acid levels.

3.3.5 Alternative host range

Twenty four plants species belonging to ten families were evaluated as alternative hosts of SVNaV (Table. 3.2). Those species include major crops, indigenous weeds, vegetables and ornamental plants, previously reported as common hosts for tospoviruses (Cho et al., 1987; Stobbs et al., 1992). Lesions tested positive for SVNaV were collected from soybeans at the R3 to R6 stages and were ground in a pre-cooled mortar with chilled, fresh phosphate buffer (0.1 M, pH7.2) containing 0.1% (v/v) 2-mercaptoethanol at 1/10 (w/v) ratio. The paste was rub-inoculated on leaves dusted with carborundum

(600 meshes) to assist virus delivery. Inoculated plants were kept in the greenhouse for symptom development. Inoculated plants were tested for virus infection when the virus-like symptom first appeared, whereas for species that did not show any symptoms, PCR testing was performed at least 4 weeks post-inoculation.

3.3.6 Comparison of detection sensitivity

The alignment of 37 virus isolates revealed the most conserved region in the N protein gene which was subsequently used to design conventional and real-time PCR primers. Two different regions of the gene (region1: RNA 3 nt 1778-2126) and (region 2: RNA 3 nt 2318-2412) were selected for PCR and qPCR primer design, respectively (Table. 3.1). Wobble bases were used in the Taqman® qPCR probe and primers given that there was no region with 100% identity between all isolates.

To compare the sensitivity of PCR and qPCR detection protocols, DNaseI-treated RNA with different amount (1 ng, 10 ng and 100 ng) was used as template in reverse transcription as described above. The PCR was carried out in 25 µl consisting of 0.5 µl dNTPs (10 mM), 0.5 µl SVNV-NP F/SVNV-NP R (20 µM for each), 0.2 U *Taq* polymerase (5 U/µl, Genscript) and 2.5 µl cDNA template. In order to determine the detection limit, the reaction was performed at different number of cycles. The program started with pre-denaturation at 94 °C for 2 min, followed by 20 or 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 10 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. Using the same cDNA as template, a 20 µl qPCR mix including 10 µl TaqMan universal PCR Master Mix (Invitrogen), 2 µl primer/ Taqman® probe mix (5 µM/2.5 µM), 2 µl cDNA and 6 µl water was performed in a BioRad CFX96 Real-Time PCR detection system with the activation of AmpliTaq Gold at 95 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 15 s and annealing/extension at 55 °C for 60 s.

Table. 3.1 List of oligonucleotide primers and probe used in the population structure study and the sensitivity comparison between PCR and qPCR of Soybean vein necrosis associated virus. F – forward primer, R-reverse primer.

Primer/Probe name	Nucleotide sequence (5'-3')
SVNV0624 F	NCAGCGAGTTCTACAANACCC
SVNV0624 R	CACATGTTGACNATAGCNTTTCTG
SVNV0624 probe	TGGGTCGCAAATTCAGGTANGCATCT
SVNV-NP F	ACTTGTGCAAGCTTATGGT
SVNV-NP R	GAAATGATTCCAATCTGTTC

Double antibody sandwich ELISA (DAS-ELISA) was performed as described by Clark et al. (1977). Briefly, SVNaV antibody stock (AC diagnostics, AR, U.S.) was diluted 1:200 with coating buffer according to manufacturer's instruction. One hundred microliter of diluted antibody was added to each well of polystyrene plate and incubated at room temperature for 3 – 5 h. The plate was then emptied and tapped dry before adding 200 µl blocking buffer, which was then incubated at room temperature for 1 h. During the incubation, 100 mg SVNaV infected leaf tissue was ground thoroughly in 2 ml sample buffer and diluted into 1:50, 1:100 and 1:200 for further use. In each dilution, three replicates were set up for all independent samples. After tapping dry the plate, 100 µl plant sap was added in each well and left overnight at 4 °C. The plate was sealed within air-tight bag to prevent the evaporation. Plate was then emptied and washed three times with wash buffer. After final wash, diluted coating conjugate was added to each well and incubated at room temperature for at least 4 h. The plate was then washed thoroughly as above to ensure that all the unbound conjugate was removed from the plate to minimize background reactions. One tablet of p-nitrophenyl phosphate (5 mg, Sigma, U.S.) was completely dissolved in 10 ml substrate buffer and 100 µl were added to each well. Incubation was done at room temperature until color develops.

3.4 Results

3.4.1 Geographical distribution of SVNaV

Screening of surveyed samples by molecular detection methods revealed the presence of SVNaV in 12 states (AR, IL, MO, KS, TN, KY, MS, MD, DL, PA, VA and NY). Over 400 samples were tested during 2008-2012, with all of the more than 300 samples with vein necrosis disease symptoms testing positive for the virus. All asymptomatic leaves were SVNaV-free.

3.4.2 Virus population structure

Comparison of the complete N protein gene of 37 isolates collected from eight states did not reveal significant diversity within or among states. Sequence alignment using ClustalW revealed high similarity ranging from 98 to 100% at both the nucleic acids and amino acids levels, indicating that SVNaV has a homogeneous population structure within the geographic area studied. Phylogenetic analysis did not reveal a distinct geographical pattern in isolate clustering (Fig. 3.1). All N protein gene sequences generated during this work were deposited in Genbank through accession numbers HQ728355-84, HQ728386 and JQ946869-946874.

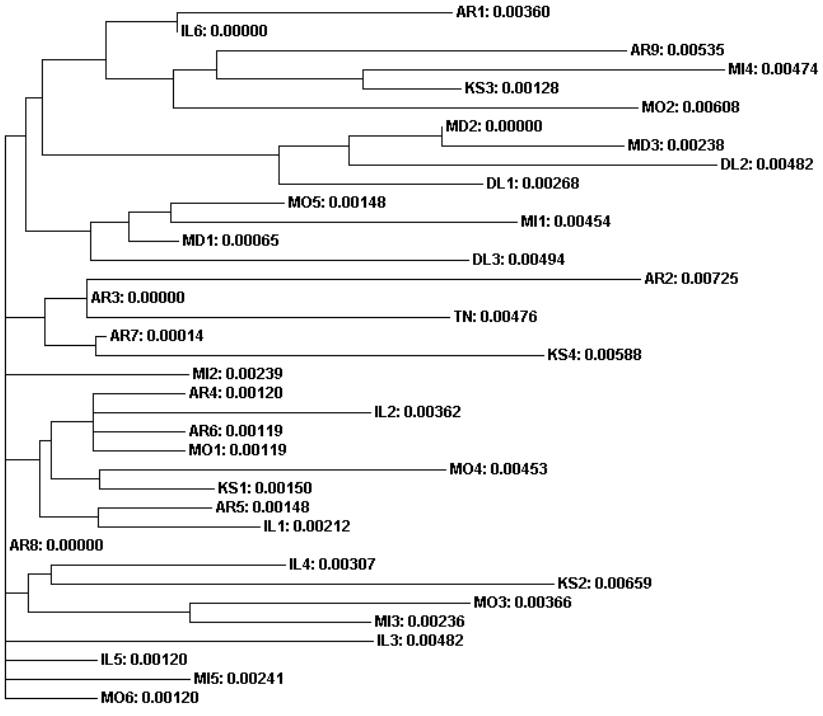

Fig. 3.1 Phylogram of the N protein gene of 37 Soybean vein necrosis associated virus (SVNaV) isolates. Numbers represent genetic distances of individual isolates. AR1, 728355; AR2, 728356; AR3, 728357; AR4, 728358; AR5, 728359; AR6, 728360; AR7, 728361; AR8, 728362; AR9, 728363; IL1, 728364; IL2, 728365; IL3, 728366; IL4, 728367; IL5, 728368; IL6, 728369; MO1, 728370; MO2, 728371; MO3, 728372; MO4, 728373; MO5, 728374; MO6, 728375; MS1, 728376; MS2, 728377; MS3, 728378; MS4, 728379; MS5, 728380; KS1, 728381; KS2, 728382; KS3, 728383; KS4, 728384; TN, 728386; MD1, JQ946869; MD2, JQ946870; MD3, 946871; DL1, 946872; DL2, 946873; DL3, 946874.

3.4.3 Alternative host range

Mechanical transmission revealed susceptibility of ten species to SVNaV (Table. 3.2). Commonly used tospovirus indicator plants such as *Nicotiana benthamiana*, *N. tabacum*, *N. glutinosa*, *Gomphrena globosa*, *Chenopodium quinoa* and *Dahlia variabilis* were included in the study. However, only three *Nicotiana* spp. sustained virus replication. Among all tested species, local lesions were first observed on *N. benthamiana* (Fig. 3.2 A), *N. tabacum*, *N. glutinosa*, cowpea and mug bean 5-7 days post-inoculation and the presence of the virus was detected at the same time. The virus moved systemically on the *Nicotiana* spp. and the systemic symptoms manifested 2-3 weeks post-inoculation (Fig. 3.2 B).The presence of the virus was also detected in symptomless chrysanthemum, pumpkin and morning glory. All plant species used in the test were grown from seeds in the greenhouse.

Table.3.2 Plant species used in alternative host test of Soybean vein necrosis associated virus and test results.

Family	Species	Plants tested	SVNaV positive plants
Amaranthaceae	Gomphrena	10	0
	Palmer amaranth	10	0
	Spinach	11	0
Asteraceae	Chrysanthemum	11	2
	Dahlia	10	0
Brassicaceae	Cabbage	11	0
Chenopodiaceae	Quinoa	10	0
	Album	10	0
Convolvulaceae	Morning glory	10	1
Cucurbitaceae	Cucumber	10	0
	Pumpkin	11	3
Leguminosae	Peanut	12	0
	Cowpea	80	60
	Mug bean	10	4
	Green bean	11	0
Malvaceae	Cotton	10	0
Poaceae	Rice	10	0
	Wheat	10	0
	Corn	10	0
Solanaceae	*N. benthamiana*	80	65
	N. tabacum	10	8
	N. glutinosa	10	7
	Tomato	11	0
	Pepper	10	0

Fig. 3.2 Soybean vein necrosis associated virus symptoms on *Nicotiana benthamiana*. A. Local lesion (indicated by black arrow). B. Systemic infection.

3.4.4 Analytical sensitivity of RT - PCR, qRT - PCR assays and ELISA

Detection sensitivity using PCR primer set SVNV-NP F/R and qPCR primer set SVNV0624 F/R (Table. 3.1) was compared using three biological replicates; each with 10-fold RNA dilution series. Primer sets were designed from the N gene given the extensive information on the sequence variability of the region. RNA used in reverse transcription was standardized as 100 ng, 10 ng and 1 ng for each sample. Equal amount of cDNA was then used as templates in both PCR and qPCR. The analytical sensitivity for conventional PCR is about 4 pg after 30 PCR cycles whereas the virus was detectable when using 400 pg of total RNA in 20 PCR cycles (Fig. 3.3). Quantitative PCR (standard curve equation: y=-3.376x +28.822) with reaction efficiency E=97.8% and R^2 =0.991 (Fig. 3.4) was less sensitive than conventional PCR given that the virus was detectable in 24-25 cycles in the 400 pg samples (Fig. 3.4), however, the virus was detectable in 28 to 30 cycles in the 4 pg samples, similar to conventional PCR. DAS-ELISA test using polyclonal antibody against N protein (Khatabi et al., 2012) showed that the virus was only detectable using undiluted plant tissue following the standard protocol (Fig. 3.5).

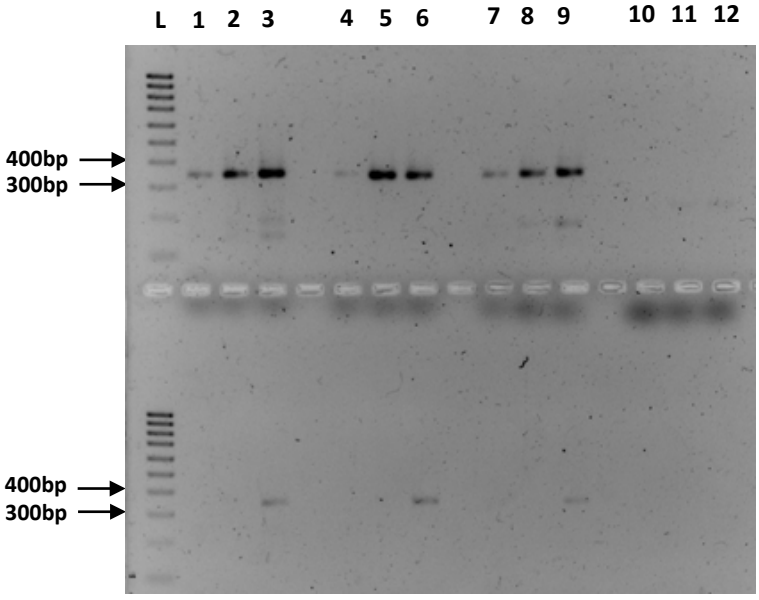

Fig. 3.3 RT-PCR detection of Soybean vein necrosis associated virus (SVNaV). Upper lane, detection after 30 PCR cycles; lower lane, detection after 20 PCR cycles. L, DNA ladder; lane 1-9, SVNaV positive

samples; lane 10-12, negative controls. Lane 1, 4, 7 and 10: 4 pg of total RNA; lane 2, 5, 8 and 11 are samples: 40 pg total RNA; 3, 6, 9 and 12: 400 pg total RNA.

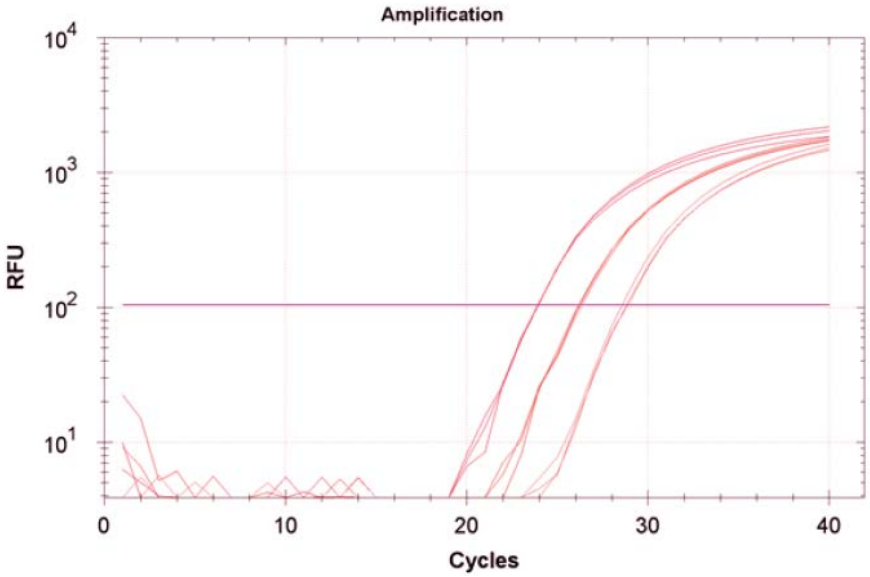

Fig. 3.4 Quantitative reverse transcription-PCR detection of Soybean vein necrosis associated virus Curve. 1: 400 pg total RNA; curve 2: 40 pg total RNA; curve 3: 4 pg total RNA. Each concentration contains three replicates.

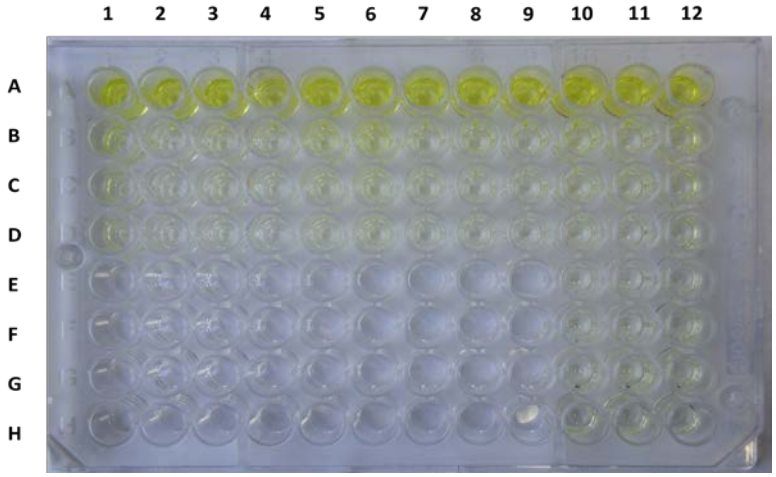

Fig. 3.5 Double antibody sandwich ELISA (DAS-ELISA) test of Soybean vein necrosis associated virus. Line A to D represents different plant sap concentrations: A: undiluted; B: 1: 50; C: 1: 100; D: 1: 200. Line E to H: negative control with the same concentrations as positive samples. Row 1 to 12 represents four positive samples, each sample contain three replicates.

3.5 Discussion

SVNaV is a newly-identified, widespread tospovirus infecting soybean in the major producing areas of the U.S. No previous information is available on the biological properties of the virus, impeding further exploration concerning its transmission and effects in yield. This study aims to elucidate important aspects of the virus epidemiology including the virus population structure and alternative hosts.

Among tospoviruses that have been identified in recent years, SVNaV is of special interest given its wide geographic distribution in the U.S. To date, the presence of the virus has been confirmed in at least 12 states (Tzanetakis et al., 2009; Bergstrom, 2011; Mulrooney, 2011; Zhou et al., 2011). In addition, the typical SVNaV symptoms have been recently observed in soybean plants in Oklahoma (personal communication, Damicone). The high incidence of SVNaV is rare among soybean viruses. Only few viruses such as *Soybean mosaic virus*, *Bean pod mottle virus* and *Alfalfa mosaic virus* have been reported to cause damages in large scale (Sinclair et al., 1999; Wrather, 2010). SVNaV is likely to be another virus that leads to considerable losses to the soybean industry, a compelling reason for studying the virus epidemiology.

The diversity analysis of a particular virus species reflects the overall population structure of the virus, providing evidence on how the virus reacts to different selection pressures and how it evolves from other related viruses. The homogeneous population structure of SVNaV revealed in this study suggests that the intensity of symptoms observed within and among states is probably due to the soybean genotypes. A less likely hypothesis is that SVNaV has evolved relatively recently and there have not been sufficient time to accumulate mutations. The compatibility of SVNaV with commonly used

soybean cultivars in multiple states demonstrates that the virus is quite adaptive to different genotypes. If the selection pressure from host and environment remains the same, it is highly possible that SVNaV can be disseminated to larger areas under conducive conditions.

The alternative host study revealed several species that are susceptible to SVNaV. The high percentage of plants susceptible to the virus is consistent with previous studies pointing to the wide host range of tospoviruses (Cho et al., 1987; Stobbs et al., 1992). Other than soybean, two other legume species (cowpea and mug bean) are susceptible to the virus. Given that thrips are the only natural vector of tospovirus and the composition of thrips species in fields of different legume species in the midsouth are almost identical (McLeod, 2008), it is highly possible that SVNaV will be a new threat not only to the soybean industry but also to the production of other legume species.

Morning glory, an indigenous weed species in soybean fields can sustain SVNaV replication being asymptomatic. Morning glory is a perennial weed species commonly found in temperate regions and may function as the reservoir for SVNaV in the field. However, more weed species growing close to soybean fields need to be collected and evaluated as potential SVNaV hosts. Knowledge of the alternative hosts is very important for the development of a successful management scheme so as to reduce the occurrence of SVNaV.

The detection sensitivity comparison between conventional RT-PCR and qRT-PCR revealed that conventional RT-PCR has a higher sensitivity. To avoid the effect due to different degrees of reiteration of target genes, N gene was used in primer/probe design for both techniques. The conclusion we obtained is consistent with previous studies in which qPCR had higher sensitivity than PCR under some circumstances (Hafez et al, 2005; Hierl et al., 2004; Scotter et al., 2005), including viruses detection (Li et al., 2003). Several reasons may account for this result: First, the nature of the target gene and the selected PCR primers and probe play important roles in determining PCR sensitivity (Bastien et al.,

2008). It is highly possible that the genomic region we used in designing PCR primers and qPCR primers/probe have variable affinity in annealing to the template cDNA even in the same target gene. This is supported by previous studies showing that reaction sensitivity differentiated among the variety of primers designed for the same target up to 100-fold (Lachaud et al., 2002). Genetic variability mentioned by Bastien and co-works (2008) would be another important element in explaining lower sensitivity of qPCR in our study. Polymorphic bases were used in the Taqman® qPCR probe and primers. The sequence mismatches may have led to suboptimal annealing between primers/probe and the target, which may, in turn, have contributed to the reduction of the sensitivity of the technique (Bastien et al., 2008). Given that the goal of our study is to detect all available isolates in an efficient manner, the universality of the detection is more important than the detection limit given that the virus could easily be detected after 30 PCR cycles when the norm for most plant viruses is 40 PCR cycles. Under this circumstance, the qPCR primer/probe we employed is the most appropriate combination although other more sensitive options may exist but cannot be applied to all virus isolates. In addition, the master mix used in the qPCR also open fewer chances for optimizing the reaction, in which the concentration of Mg^{2+}, *Taq* polymerase, primer/probe and cDNA template are critical for better detection sensitivity (Bastien et al., 2008).

DAS-ELISA test using polyclonal antibody against SVNaV N protein produced by Khatabi and co-workers (2012) was able to detect the virus only when the plant sap are not diluted (1/20 w/v), but not in any dilution suggesting the currently available antibodies do not provide the most sensitive tool for detection.

Identification of vector is an important aspect in virus epidemiology. We are still under the way to elucidate the natural vector of SVNaV. Another question worth exploring in the future is how minor differences (less than 2%) among different virus isolates could affect virus pathogenesis. A recent study

showed that other than encapsidation, a well-known function of N protein, the protein is also involved in long-distance movement of the viron and possibly foliar symptoms (Zhang et al., 2012). It is still unknown whether point mutations occurred in particular positions of the N protein or any other areas of the genome, could contribute to differential disease symptoms, as the cases revealed in some potyviruses, where mutations in the coat protein can affect interactions between virion and aphids, whereas mutations in the HC-Pro can suppress viral particle assembly (Blanc et al., 1998; Varrelmann and Maiss, 2000).

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Chapter IV

Conclusions

4.1 Abstract

Soybean vein necrosis disease (SVND) is a new disease prevalent in major soybean producing areas in the U.S. No information was available on the basic biological characteristics of the causal agent of the disease, limiting the understanding of the disease epidemiology and the effects on soybean yield. This study revealed that a new virus provisionally named Soybean vein necrosis associated virus (SVNaV), belonging to the genus *Tospovirus*, family *Bunyaviridae,* was closely associated with SVND. Given that soybean is a crop of major economic importance and the high incidence of SVND, having been confirmed in at least 12 states across the nation, it was urgent to understand the epidemiology of SVNaV. This study was focused on the characterization of SVNaV and important aspects of the virus epidemiology, including population diversity, alternative hosts as well as the development of rapid and sensitive detection protocols. The diversity analysis revealed a relatively homogeneous virus population, indicating that the diverse disease symptoms observed in the field are probably due to the different soybean genotypes. Eight plant species belonging to five families are able to sustain virus replication, including indigenous weed species in soybean fields, species that may serve as virus reservoirs. Detection protocols with high sensitivity was developed; providing an efficient tool to monitor the occurrence of SVND in the field and contributing to the study of this new prevalent disease.

4.2 Soybean vein necrosis associated virus – a distinct tospovirus infecting soybean

The study of a new disease, SVND, led to the discovery of a new virus infecting soybeans in major soybean producing areas in the U.S. The presence of the virus, SVNaV, is closely associated with vein clearing and necrotic symptoms. SVNaV has a typical tospovirus genomic structure, which consists of three segmented single-stranded RNAs, using negative and ambisense translation strategies,

respectively. Sequence comparison of putative proteins coded by the virus and *in-silico* analysis classified SVNaV as a new *Tospovirus*, however, SVNaV has several features that distinguish it from other members of the genus.

The comparison between all SVNaV proteins (RdRp, NSm, Gn/Gc, NSs and NP) with orthologs of nine accepted tospovirus species showed minimal similarity between species ranging from 8% -37%, with the N protein sharing 52% similarity to Bean necrotic mosaic virus (BeNMV), the most closely related tospovirus sequenced to date (de Oliveira et al., 2012). Therefore, according to the International Committee for Virus Taxonomy, SVNaV is a new tospovirus as the N protein, a widely accepted descriptor in classification of tospovirus species, has less than 90% identity to recognized species of the genus (Goldbach and Kuo, 1996; Chu et al., 2001; King et al., 2011).

In-silico analysis identified characteristic functional domains or motifs of the family *Bunyaviridae* in the SVNaV proteins. Three major domains (fingers, palm and thumb domains) that are typical in polymerases are present in putative L RNA RNA-dependent RNA polymerase. Conserved motif of the "30 K movement protein superfamily" was identified in the NSm providing evidence that the protein is involved in virus movement; whereas putative signal cleavage sites and transmembrane domains were found in the glycoprotein precursors; common features in tospoviruses. However, further analysis indicated that the putative proteins coded by SVNaV have multiple unique characters compared with other members in the genus. SVNaV has a large L RNA consisted of 9010 nt and codes for a 336 Kda polyprotein. In the NSm, instead of Leu, an Ile initiates the highly conserved $LXDX_{40}G$ motif of the "30K movement protein superfamily"; the 'P/D-L-X motif' and phospholipase A2 catalytic site identified in several tospoviruses does not exist in SVNaV. The NSs protein Walker motifs A and B, critical in the NTPase activity are present in SVNaV, however, unlike other viruses, motif B precedes motif A.

The nucleoprotein is the most abundant tospovirus protein (Chu et al., 2001) and has multiple critical functions in the virus lifecycle (Whitfield et al., 2005; Ribeiro et al., 2009). As the most diverse region of tospovirus genome, N protein gene is used in analyzing phylogenetic relationship of the genus. RdRp gene was also employed in our study due do its functional significance for the virus. The analysis based on RdRp using all genes available in Genbank revealed that SVNaV stands in a distinct branch sharing identical genomic distances between American and Eurasian tospovirus clades. However, the phylogenetic tree extracted from all available N protein sequences including *Peanut yellow spot virus* (PYSV) and Peanut chlorotic fan spot virus (PCFSV) showed that the unique position of SVNaV was compromised because other than SVNaV, the phylogram also reflected the distinctiveness of PYSV and PCFSV, concurring with previous studies (Pappu et al., 2000; Chu et al., 2001). The different results obtained from RdRp and NP were mainly caused by different completeness of tospoviruses in the Genbank, where N proteins of tospoviruses are extensively studied and therefore sequence data from more species is accessible than RdRp sequences in the database.

The low similarity of SVNaV N protein gene shared with other members in the same genus (less than 52%) indicates that instead of being introduced from other countries, this new virus is possibly inherent in the U.S. The population structure lacking in heterogeneity revealed indicated host genotype rather than virus isolate is likely accounting for different symptom intensity.

In conclusion, SVNaV is a new member of the genus *Tospovirus* with several distinct characters. Although the correlation between SVNaV and SVND needs to be attested by Koch's postulates, the uniqueness of SVNaV in evolutionary history of the genus highlights the importance of further exploration on this arena.

4.3 Significance of Soybean vein necrosis associated virus

Soybean is a natural host of SVNaV and the occurrence of SVND has been confirmed in several major production areas across the U.S. The high similarity of N protein gene among all sequenced isolates revealed a relatively homogeneous population. The prevalence of the virus in different states indicates SVNaV may be adaptive to multiple commercial cultivars grown in the U.S. However, a thorough study is needed to evaluate the yield loss caused by the new virus in the different genotypes.

The alternative host range showed that SVNaV infects some but not all commonly used tospovirus indicators, an observation that is consistent with the notion that SVNaV is a new tospovirus. Several dicotyledonous species belonging to the families *Solanaceae* and *Leguminosae* can sustain virus replication with high efficiency. Three *Nicotiana* species (*N. benthamiana*, *N. tabacum*, *N. glutinosa*) proved to be good indicators because they gave conspicuous local and systemic symptoms in a relatively short time frame. Given the fact that two other legumes (cowpea and mug bean) are alternative hosts for SVNaV, it is possible that the virus can emerge as a problem to legume production of the U.S. Another important finding in this study is that an indigenous weed species – morning glory can sustain virus replication without exhibiting obvious symptom suggesting that it may serve as asymptomatic virus reservoir in the field when soybean is absent during winter and early spring. However, the function of morning glory species in SVNaV transmission still needs to be confirmed by other aspects including (1) whether the SVNaV vector feeds on it and (2) their relative abundance in soybean fields. Other susceptible species include chrysanthemum and pumpkin indicates SVNaV may have a wide alternative host range although infections may be asymptomatic.

4.4 Future work

In the future, it would be necessary to complete important aspects of SVNaV epidemiology that have not been covered in the study. First, Koch's postulates need to be fulfilled in order to prove SVNaV is the causal agent of SVND. Second, the identification of virus vector, the determination of vector transmission efficiency and basic biological characteristics of the vector are critical for the implemantation of control measures and disease management. Third, more weed species in soybean fields should be tested for their susceptibility to SVNaV. Monocotyledons should also be included in the study given that some tospovirus species can naturally infect both dicotyledons and monocotyledons (Kritzman et al., 2000).

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