Eur J Plant Pathol (2012) 133:783–790
DOI 10.1007/s10658-012-9969-5

Generation of polyclonal antibodies and serological analyses of nucleocapsid protein of *Soybean vein necrosis-associated virus*: A distinct soybean infecting tospovirus serotype

B. Khatabi · R.-H. Wen · D. E. Hershman · B. S. Kennedy · M. A. Newman · M. R. Hajimorad

Accepted: 16 February 2012 / Published online: 26 February 2012

Abstract *Soybean vein necrosis-associated virus* (SVNaV) is a newly isolated tospovirus from field-grown soybeans in the United States. Polyclonal antibodies generated against the recombinant *Escherichia coli*-expressed nucleocapsid protein (NP) of the virus, reacted specifically with SVNaV and exhibited low, if any, cross-reactivity with other species within the genus *Tospovirus*. The serological results are in agreement with low sequence homology of the SVNaV-NP gene when compared with those of other tospovirus species, some of which are capable of infecting soybean naturally. Phylogenetic analysis utilizing NP sequences from several SVNaV isolates collected from different geographical regions and various soybean genotypes over 4 years showed close relationships, but distinct from the representatives of all the established serogroups or distinct serotypes within the genus of *Tospovirus*. All SVNaV isolates examined reacted strongly with the generated polyclonal antibodies. Collectively, our serological analyses suggest that SVNaV represents a new and distinct soybean-infecting tospovirus serotype. Furthermore, our data demonstrate that antiserum against NP has the potential to serve as a reliable probe for SVNaV detection in field-grown soybeans.

Keywords *Tospovirus* · Immunoassay · ELISA · Diagnosis · *Glycine max*

The nucleotide sequence data reported in this article have been deposited in the GenBank under accession numbers JF808207-JF808215 and JQ277450-JQ277452.

B. Khatabi · R.-H. Wen · M. A. Newman · M. R. Hajimorad (✉)
Department of Entomology and Plant Pathology,
The University of Tennessee,
Knoxville, TN 37996, USA
e-mail: mrh@utk.edu

D. E. Hershman · B. S. Kennedy
Department of Plant Pathology, University of Kentucky
Research and Educational Center (UKREC),
Princeton, KY 42445, USA

R.-H. Wen
Guangxi Key Laboratory of Subtropical Bioresource
Conservation and Utilization, College of Life Sciences
and Technology, Guangxi University,
Nanning, Guangxi 530004, China

Tospoviruses are responsible for considerable losses in a large number of agriculturally important crops worldwide (Pappu et al. 2009; Plyusnin et al. 2011). Several tospovirus species have been reported from field-grown soybeans in different parts of the world. These include *Tomato spotted wilt virus* (TSWV) (Golnaraghi et al. 2004; Nischwitz et al. 2006), *Tomato yellow ring virus* (TYRV) (Hassani-Mehraban et al. 2007), *Groundnut ringspot virus* (GRSV) (Pietersen and Morris 2002), and *Groundnut bud necrosis virus* (GBNV) (Bhat et al. 2002).

Soybean vein necrosis-associated virus (SVNaV) is a newly identified tospovirus originally isolated from field-grown soybeans in Tennessee (TN), USA, in 2008 (Tzanetakis et al. 2009). Soybean is a major

legume crop in the United States with approximately 78.1 million acres under plantation in 2010. Symptomatic soybeans exhibited vein clearing and chlorosis of leaf tissues adjacent to veins (Fig. 1a), which eventually progressed to necrosis (Fig. 1b and c). Since observation of these symptoms in TN, the presence of similar symptoms in soybean fields in other soybean growing areas of the United States, including Kentucky (KY), and isolation of the virus have been reported (Zhou et al. 2011). In KY where the seasonal timing of appearance of symptoms was followed for 3 years, time of appearance of the symptoms varied from late June to early August of each year and symptomatic plants remained sporadic within soybean fields. Zhou et al. (2011) have noted that first symptoms appeared in Arkansas and Illinois in early June.

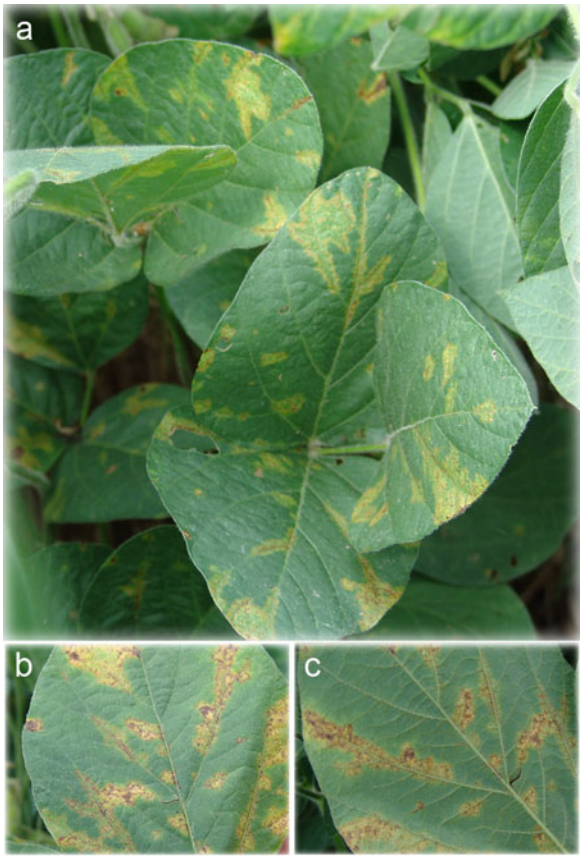

Fig. 1 **a** Field-grown soybean cultivar Asgrow 5304 showing typical symptoms associated with *Soybean vein necrosis-associated virus* disease in Tennessee, USA. Note the irregular chlorotic lesions surrounded by necrotic halo zones around the leaf veins. **b** and **c** Vein necrosis on upper and lower surfaces of an infected soybean leaf, respectively

Organization of SVNaV genome is similar to other members of the genus *Tospovirus* within the family *Bunyaviridae* (Zhou et al. 2011). *Tospovirus* is the only genus in the family *Bunyaviridae* for which its members are capable of infecting plants. Member species are defined based on vector specificity, host range, serological properties and nucleoprotein (NP) gene characteristics (Chen et al. 2010; Plyusnin et al. 2011). According to the 9th report of the International Committee on Taxonomy of Viruses, the genus *Tospovirus* contains a total of eight definitive species with an additional 15 tentative species (Plyusnin et al. 2011).

Tospoviral virions, in general, consist of a core nucleocapsid protein (NP) encapsulating viral genome surrounded by a host-derived lipid membrane with two virally encoded glycoproteins (Plyusnin et al. 2011). NP is the most abundant structural protein of tospoviruses, and as such it has served well as a candidate for generation of antibodies. Indeed the majority of tospoviral monoclonal and polyclonal antibodies are directed against this structural protein of the virus (Chen et al. 2005; Jain et al. 2005; Wu et al. 2009). Current classification of tospoviruses based on serological relationship of NPs, has divided them into three related serogroups including *Iris yellow spot virus* (IYSV), *Tomato spotted wilt virus* (TSWV) and *Watermelon silver mottle virus* (WSMoV) (Chen et al. 2010). In addition, there are three distinct serotypes with no clear serological relationship with the established serogroups. These serotypes include *Impatiens necrotic spot virus* (INSV), *Peanut yellow spot virus* (PYCS), and *Peanut chlorotic fan-spot virus* (PCFV) (Chen et al. 2010; Chu et al. 2001; de Avila et al. 1993; Jain et al. 2005; Pappu et al. 2009). Another tospovirus species, *Melon yellow spot virus* (MYSV), was originally identified as a distinct serotype (Cortez et al. 2001; Kato et al. 2000); however, in a subsequent study by Chen et al. (2010), serological relationships based on NP between MYSV and WSMoV were demonstrated. More recently, a few additional serologically-distinct tospovirus species have also been identified (Bag et al. 2010; Dong et al. 2008; Hassani-Mehraban et al. 2005, 2010; Seepiban et al. 2011).

Comparative serological analyses of the NP of SVNaV with other tospovirus species, especially those infecting soybean, were the aim of this study. Due to the instability of virions of tospoviruses (Best 1968), the recombinant *Escherichia coli*-expressed NP of SVNaV was utilized as an immunogen to produce polyclonal antibodies. Subsequently, the generated

antibodies were utilized for comparative serological analysis and development of an immunoassay for detection of SVNaV in soybean.

Soybean plants with typical SVNaV symptoms were collected from fields in TN and KY, USA, during growing seasons 2008 to 2011. Isolates TN1 and TN2 were collected in 2008 from TN. Isolates KY1 to KY3 were collected in 2009, KY4 to KY7 in 2010, and KY8 to KY10 in 2011. Soybean cultivars from which these isolates were collected included Asgrow 4630, Asgrow 4703, Asgrow 4707, Asgrow 5304, Southern States RT4996N, Southern States LL511N, Pioneer 94B73, Pioneer 94Y30, and Steyer 4430. The infected leaf tissues were stored at −80°C.

Inoculum for sap-inoculation was prepared by grinding the symptomatic leaf tissues in the presence of ice-cold 0.1 M phosphate buffer, pH 7.0 containing 0.2% sodium sulphite (w/v) and 1% (v/v) 2-mercaptoethanol (Mandal et al. 2008) and rub-inoculated onto carborundum-dusted (600 mesh) leaves of *Nicotiana glutinosa* L., *N. benthamiana* as well as various soybean genotypes including Williams, Lee68, and Asgrow 5304. The inoculated plants were kept in a growth chamber operating at 22°C with a photoperiod of 16 h and monitored for the appearance of symptoms up to 4 weeks postinoculation and screened for the presence of SVNaV by either RT-PCR or ELISA.

For cloning of the NP gene, total RNA from naturally infected soybean leaf tissues was isolated using the QIAGEN RNeasy Mini Kit (QIAGEN, Santa Clara, CA, USA). The first-strand cDNA was generated using 10-mer random primers in the presence of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Specific PCR primers utilized to detect SVNaV in the suspected leaf tissues were SVNaV-f1 (5′-AGATATAAAGTTGAGACACTATC-3′) and SVNaV-r1 (5′-TGCAACACATCCGGAACTCTG-3′) corresponding to nucleotide sequences 2553–2575 and 1636–1657 of small RNA from TN isolate, respectively (Zhou et al. 2011; GenBank accession no. HQ728387). The PCR program consisted of a cycle of 2 min at 95°C; 30 s at 94°C, 30 s at 56°C, 1 min at 72°C, 32 cycles; followed by final extension for 10 min at 72°C. In order to express NP, while cDNA generated with random primers served as a template, the entire NP gene of KY1 isolate was amplified in the presence of primers SVNaV-f2 (5′-**CCT**GAATTCATGCCACAAACAGCAGG-3′) and SVNaV-r2 (5′-**TTA**GCGGCCGCTAAACAGAAAACTCC-3′) (*Eco*RI and *Not*I sites are underlined whereas non-SVNaV sequences are bold-faced) corresponding to nucleotide sequences 2516–2533 and 1688–1703 of small RNA from TN isolate, respectively (Zhou et al. 2011; GenBank accession no. HQ728387). Subsequently, the amplicons were digested with *Eco*RI and *Not*I restriction enzymes (New England BioLabs, Beverly, MA, USA), and ligated in-frame with 6 × His-tag in the bacterial expression vector pET-28a (+) (Novagen, Madison, WI, USA). Following verification of the sequences by sequencing, the construct was transferred into *E. coli* strain BL_{21} (DE_3) (Stratagene, La Jolla, CA, USA). Expression of the recombinant protein was induced in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega, Madison, WI, USA). The recombinant poly-histidine tagged expressed protein was purified using Ni-NTA affinity column chromatography under native condition according to the QIAexpress protocol (Qiagen). Subsequently, the purified protein was subjected to SDS-PAGE analysis followed by amino acid sequencing using MALDI-TOF-MS/MS at the Iowa State University-Protein Facility (Ames, IA, USA).

Polyclonal antibodies against the recombinant SVNaV-NP were raised in a rabbit through a contract with Covance Inc. (Denver, PA, USA). Rabbit received a total of 1.75 mg of purified SVNaV-NP via 10 injections over a period of 6 months. Initially, 250 μg of the purified recombinant protein emulsified with Freund's complete adjuvant (1:1 v/v) was administered. Subsequent injections (seven times with 125 μg protein per injection) were administered at 21-day intervals in the presence of incomplete Freund's adjuvant. Finally, two booster injections with a total of 625 μg of SVNaV-NP were given each with a rest period of 1 month in between. Blood samples were taken at various intervals and the antisera were evaluated by antigen coated indirect-ELISA (AC-IELISA) (Malapi-Nelson et al. 2009) using bacterially expressed purified SVNaV-NP (0.1 μg/well) or sap from naturally infected soybean leaf tissues (1:10 w/v in carbonate buffer, pH 9.6) as antigens. For antiserum titration, serial dilutions were prepared in phosphate buffer saline (PBS; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat dry milk. To evaluate sensitivity of anti-SVNaV serum in detecting the virus in infected soybean tissues, serial dilutions of sap extracts in carbonate buffer, pH 9.6 was probed with a constant dilution of the antiserum (1:2000). Absorbance values exceeding mean

value of background (i.e. reaction with sap extract from healthy soybean) + 3X standard deviation were considered positive for ELISA. The ability of anti-SVNaV serum to detect the virus in soybean infected tissues by western-immunoblotting was also evaluated where five leaf discs (~1 cm each) were collected from the symptomatic areas of leaves of field-grown soybean plants, pulverized in the presence of liquid nitrogen and homogenized in 1 ml of extraction buffer [0.625 M Tris–HCl pH 8, 2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol] (Harlow and Lane 1988). The homogenate was briefly vortexed, boiled for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant recovered, mixed with equal volume of loading buffer [0.25 M Tris–HCl pH 6.8, 8% (w/v) SDS, 30% (v/v) Glycerol, 0.02% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol)], boiled for 10 min and 10 μl of each sample loaded per lane. Western-immunoblotting was done essentially as described by Towbin et al. (1979).

Serological relationship between SVNaV and other tospovirus species infecting soybean or representatives of distinct serotypes or serogroups were evaluated by AC-IELISA. These included GRSV, INSV, *Tomato chlorotic spot virus* (TCSV), TSWV and TYRV. Polyclonal antibodies against these tospoviruses, as well as their homologous antigens, were all supplied by AC Diagnostics (AC Diagnostics Inc., Fayetteville, AR, USA). Positive samples consisted of plant extracts from tospoviruses infected plant tissues were used as supplied.

Calculation of amino acid sequence identity was done by GeneDoc version 2.6.2002 (http://www.psc.edu/biomed/genedoc/). Multiple sequence alignment was performed using Clustal X Version 2.0 (Larkin et al. 2007). Phylogenetic tree was constructed by MEGA software version 4.0 based on the neighbour joining (NJ) method and the interior reliability of each branch by the bootstrap value with 1,000 replicates (Tamura et al. 2007).

The PCR amplicons harbouring the entire NP gene from SVNaV with an expected size of 834 nucleotides encoding 277 amino acids was obtained from the field-grown symptomatic soybean leaf tissues (data not shown). The recombinant *E. coli*-expressed NP was obtained and purified under native conditions. Based on SDS-PAGE analysis, the purified expressed protein was a single polypeptide with a molecular mass of 33 kDa (Fig. 2). This was slightly larger than the predicted size of the NP based on the deduced amino acid sequences of the gene (30.8 kDa). This anomaly in size is likely due to fusion to His-tag and being expressed as a recombinant protein. The observation that bacterially expressed NP was recognized by monoclonal antibody against histidine-tag in western-immunoblotting (data not shown) provided supports for the recombinant nature of the purified protein. Nevertheless, amino acid sequencing using MALDI-TOF/TOF MS/MS unequivocally confirmed the identity of the purified recombinant protein as SVNaV-NP (data not shown). Therefore, it was subsequently utilized as an immunogen for generation of polyclonal antibodies.

The antiserum obtained in the last bleeding was utilized for polyclonal antibody characterization, serological analyses and immunoassay development. The dilution end point of the antiserum to detect 0.1 μg purified recombinant SVNaV-NP in AC-IELISA was 1:64,000. However, when the antiserum was titrated against sap extract derived from naturally infected soybean leaf tissues, its dilution end point was 1:8,000. The antiserum at a dilution of 1:2000 was sensitive enough to detect as low as 0.1 ng of bacterially expressed purified NP in AC-IELISA and could detect SVNaV in sap extract from naturally infected soybean leaf tissues diluted up to 1:512. The antiserum reacted weakly (O. D. 405 nm 0.21±0.07) with sap extract from healthy

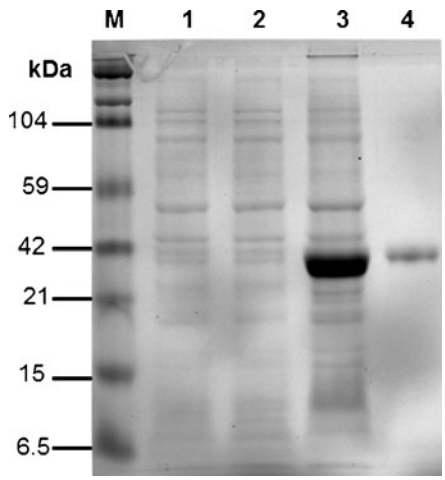

Fig. 2 SDS-PAGE analysis of total protein extracted from bacterial cells with empty pET-28a vector (lane 1), those from bacteria cell carrying pET-28a harboring *Soybean vein necrosis-associated virus* (SVNaV) nucleocapsid protein (NP) gene from KY1 isolate prior and after IPTG induction (lanes 2 and 3, respectively), and Ni-NTA affinity column purified SVNaV-NP (lane 4). Pre-stained protein markers (Bio-Rad Laboratories, Hercules, CA) were loaded in lane M. The proteins were resolved on 12% SDS-polyacrylamide gel and stained with coomassie brilliant blue. The sizes of protein standards are given at the left

soybean leaf tissues at various sap dilutions up to 1:512 tested. The antiserum showed broad specificity against SVNaV as it detected all virus isolates in AC-IELISA (data not shown) demonstrating close serological relationships among geographically distinct isolates from various soybean genotypes. Furthermore, the antiserum was efficient in detecting SVNaV in the infected leaf tissues by western-immunoblotting (Fig. 3).

The NP genes of all 12 ELISA-positive SVNaV isolates collected in this study were RT-PCR amplified and directly sequenced. Comparative analysis showed high levels of sequence identity (98–100%) among all the isolates at both nucleotide and amino acid levels (data not shown), including isolate TN that its entire genome sequence is published (Zhou et al. 2011; GenBank accession no. HQ728387). On the other hand, the NP of SVNaV isolates shared low degree of amino acid sequence identity with those of other soybean infecting tospovirus species or representative of serogroups or distinct serotypes. These included TYRV (31%); TSWV (30%); GRSV (30%); GBNV (30%); WSMoV (28–29%); INSV (28%); and PCFV (17–18%). In general, the threshold value of NP amino acid sequence identity for a distinct tospovirus is less than 90% (Plyusnin et al. 2011). Thus, any tospovirus with the NP amino acid sequence identity less than 90% with those of other known tospoviruses should be considered as a distinct species (Plyusnin et al. 2011; Jones 2005). Hence, these data confirm that SVNaV is a distinct tospovirus species (Zhou et al. 2011). A

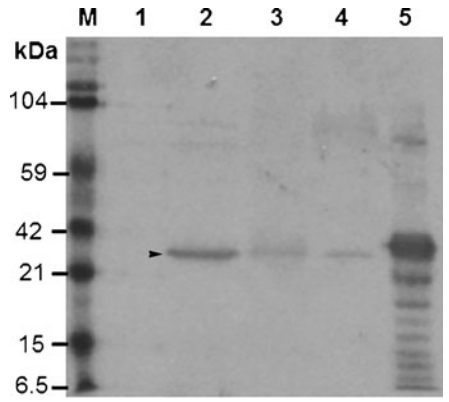

Fig. 3 Western-immunoblotting detection of three isolates of *Soybean vein necrosis-associated virus* (SVNaV). Total protein extracted from SVNaV-infected soybean leaf tissues with isolates KY1, KY4 and TN2 were loaded in lanes 2 to 4, respectively, and that of healthy soybean in lane 1. Lane 5 was loaded with 25 ng of purified bacterially expressed nucleocapsid protein (NP). Prestained markers (Bio-Rad) were loaded in lane M. The proteins were resolved on 12% SDS-polyacrylamide gel and electroblotted. A dilution of 1:4000 of the antiserum raised against bacterially expressed SVNaV-NP was used to probe the blot. The arrow head points to NP position

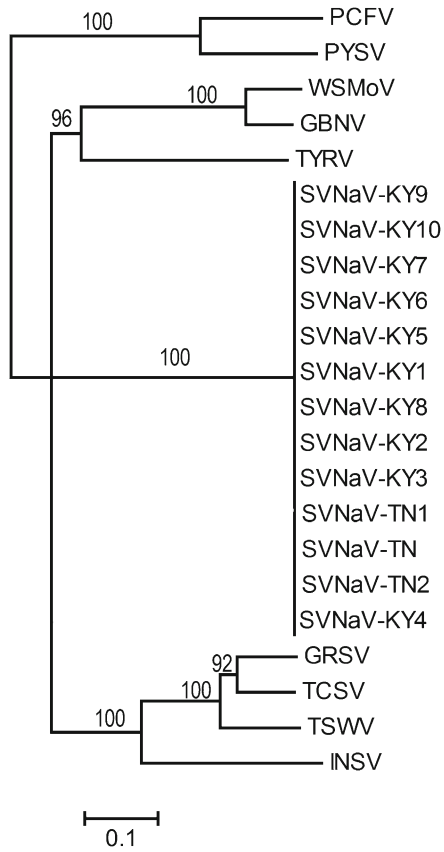

Fig. 4 Phylogenetic tree based on amino acid sequence of nucleocapsid protein (NP) of *Soybean vein necrosis-associated virus* (SVNaV) from thirteen isolates and those representing serogroups or distinct serotypes within the genus *Tospovirus* (Chu et al. 2001). These include *Groundnut ringspot virus* (GRSV; GenBank accession No. L12048), *Groundnut bud necrosis virus* (GBNV; U27809), *Impatiens necrotic spot virus* (INSV; X66972), *Peanut chlorotic fan-spot virus* (PCFV; AF080526), *Peanut yellow spot virus* (PYSV, AF013994), *Tomato chlorotic spot virus* (TCSV; S54325), *Tomato spotted wilt virus* (TSWV; D13926), *Tomato yellow ring virus* (TYRV; HQ154131) and *Watermelon silver mottles virus* (WSMoV; U78734). It should be noted that GRSV, TSWV and TYRV infect soybean naturally. Isolates KY1 to KY10 were collected in Kentucky (USA) during 2009 to 2011 while TN, TN1 and TN2 originated from Tennessee (USA) collected in 2008. Note that regardless of the origin, all SVNaV isolates grouped together with the TN isolate (GenBank accession no. HQ728387) and occupied the same distinct position compared with the other tospoviruses examined. Phylogenetic analysis was done with MEGA 4. Bootstrap values are indicated at branches, and values below 70% were collapsed

phylogenetic tree based on amino acid sequence of NP from all SVNaV isolates collected in this study as well as TN isolate (Zhou et al. 2011) revealed that all were grouped together and occupied the same position distinct from the other species examined (Fig. 4). Hence, this further confirms that SVNaV is a phylogenetically distinct species from all the other tospovirus species studied here and elsewhere (Zhou et al. 2011). Our phylogenetic data also demonstrate that all the SVNaV isolates, regardless of the geographical origin, soybean genetic background and time of collection, occupied the same position.

The serological relationships between SVNaV and GRSV, INSV, TCSV, TSWV and TYRV were examined in AC-IELISA (Fig. 5). As expected, each of the antisera exhibited the strongest reaction with its homologous antigen (Fig. 5). There was low, if any, serological cross-reactivity between SVNaV antiserum and the other tospovirus species examined. Likewise, antisera to other tospoviruses cross-reacted poorly or not at all with two SVNaV isolates tested (Fig. 5). These observations indicate that SVNaV is a distinct serotype. Furthermore, they are in complete agreement with the low amino acid sequence identity observed between SVNaV-NP and other tospoviruses (Fig. 4; Zhou et al. 2011). As expected and shown in Fig. 5, cross-reactivity was observed among GRSV, TCSV, and TSWV, which are all members of TSWV serogroup (Feldhoff et al. 1997; Jain et al. 2005; Lovato et al. 2004). INSV is a distinct serotype belonging to INSV serogroup (Chen et al. 2010). Antiserum to TYRV did not cross-react with any of the other tospoviruses tested (Fig. 5). TYRV is considered as a distinct tospovirus species as well (Hassani-Mehraban et al. 2005, 2010). The antigenic uniqueness of SVNaV

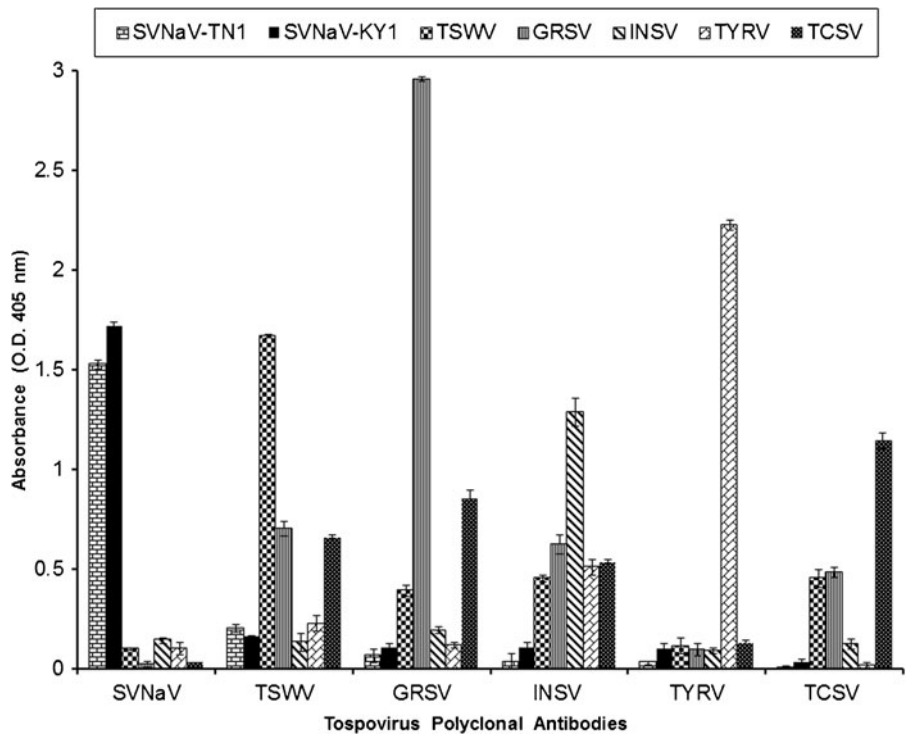

Fig. 5 Serological relationship between *Soybean vein necrosis-associated virus* (SVNaV) and other tospoviral species based on antigen coated indirect-ELISA using polyclonal antibodies as probes. Serological relationships among the nucleocapsid proteins of SVNaV isolates TN1 and KY1, *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), *Tomato yellow ring virus* (TYRV), and *Tomato chlorotic spot virus* (TCSV) are shown. It should be noted that in addition to SVNaV, TSWV, GRSV and TYRV are capable of infecting soybean naturally. The extract in carbonate buffer, pH 9.6 (1:10 w/v) from naturally infected soybean leaf tissues with TN1 or KY1 isolates of SVNaV served as positive control. Extracts from infected plant tissues with each of other tospoviruses were supplied by AC Diagnostic and used as recommended by the supplier. A dilution of 1:1000 of each of the antisera served as probes. The optical density (O. D. 405 nm) value was recorded at 60 min following substrate hydrolysis. Data shown are net absorbance values for means of duplicate ELISA wells after deducting absorbance values for healthy soybean sap extract. Bars indicate standard deviation

among tospoviruses capable of infecting soybean naturally offers an opportunity to detect the virus reliably in the infected field-grown soybeans immunologically even though the plants may be co-infected by other tospoviral species (Bhat et al. 2002; Golnaraghi et al. 2004; Hassani-Mehraban et al. 2007; Nischwitz et al. 2006; Pietersen and Morris 2002).

There are currently two different assays available for detection of SVNaV in the infected soybean tissues; RT-PCR (Zhou et al. 2011) and AC-IELISA (present study). Our AC-IELISA is capable of detecting the virus only in the symptomatic tissues and our attempts to detect the virus in non-symptomatic tissues of the same infected soybean leaves failed (data not shown). It is interesting to note that RT-PCR also amplified SVNaV sequences only from the symptomatic areas of the infected leaves (data not shown). These observations suggest that possibly SVNaV is not capable of moving efficiently in the infected soybean leaves and likely the virus is not well-adapted to soybean. Despite a number of attempts, we did not succeed in transmitting SVNaV by sap-inoculation to any of the inoculated plants including to the same soybean genotype (Asgrow 4630) from which one of the isolate was collected. Under the same inoculation conditions, an isolate of TSWV consistently infected two tobacco species inoculated (data not shown) suggesting our inoculation method was optimal for transmission of tospoviruses that are known to be mechanically transmissible (Plyusnin et al. 2011). This observation further supports the possibility that SVNaV is not a well-adapted virus to soybean and may have been introduced to soybean recently from another host. The observation that SVNaV isolates collected from different soybean genotypes and from different geographical regions over a period of 4 years have a high level of NP sequence identity also supports the likelihood that soybean is a recent host of SVNaV.

Due to difficulty in sap-inoculation of SVNaV, we have not been able to reproduce the symptoms observed under the field conditions experimentally. Hence, the fulfillment of Koch's postulate that SVNaV is indeed the sole causal agent of soybean vein necrosis disease requires a breakthrough in experimental transmission of the virus. Nevertheless, we have consistently detected SVNaV in symptomatic leaf tissues from field-grown soybean exhibiting typical disease symptoms. This has been noted by Zhou et al. (2011) as well.

Our serological data presented in this paper are in complete agreement with a recent report that SVNaV was defined as a new and distinct tospovirus species based on nucleotide sequences (Zhou et al. 2011). Our phylogenetic analysis of SVNaV isolates from KY and TN demonstrate little variation among NP of these isolates regardless of the geographical origin or the genetic background of soybean cultivars and year of collection. This observation supports our conclusion that polyclonal antibodies against the NP of SVNaV coupled with AC-IELISA has the potential to serve as a quick, reliable and economical assay for large scale surveys of soybean fields for the presence of the virus, provided symptomatic tissues with typical disease symptoms are screened. This immunoassay also has the potential to play an essential role for screening of soybean germplasm for finding sources of resistance to SVNaV once an optimal method for the experimental transmission of the virus is in place.

Acknowledgements We wish to thank AC Diagnostics (AC Diagnostics Inc., Fayetteville, AR, USA) for providing antisera against tospoviruses and their homologous antigens and two anonymous reviewers for their constructive comments. This research was supported by a grant from Tennessee soybean promotion board.

References

Bag, S., Druffel, K. L., & Pappu, H. R. (2010). Structure and genome organization of the large RNA of iris yellow spot virus (genus *Tospovirus*, family *Bunyaviridae*). *Archives of Virology, 155*, 275–279.

Best, R. J. (1968). Tomato spotted wilt virus. *Advances in Virus Research, 13*, 65–146.

Bhat, A. I., Jain, R. K., Varma, A., & Lal, S. K. (2002). Nucleocapsid protein gene sequence studies suggest that soybean bud blight is caused by a strain of groundnut bud necrosis virus. *Curret Science, 82*, 1389–1392.

Chen, T.-C., Hsu, H.-T., Jain, R. K., Huang, C.-W., Lin, C.-H., Liu, F.-L., et al. (2005). Purification and serological analyses of tospoviral nucleocapsid proteins expressed by *Zucchini yellow mosaic virus* vector in squash. *Journal of Virological Methods, 129*, 113–124.

Chen, T.-C., Lu, Y.-Y., Cheng, Y.-H., Li, J.-T., Yeh, Y.-C., Kang, Y.-C., et al. (2010). Serological relationship between Melon yellow spot virus and *Watermelon silver mottle virus* and differential detection of the two viruses in cucurbits. *Archives of Virology, 155*, 1085–1095.

Chu, F.-H., Chao, C.-H., Peng, Y.-C., Lin, S.-S., Chen, C.-C., & Yeh, S.-D. (2001). Serological and molecular characterization of *Peanut chlorotic fan-spot virus*, a new species of the genus *Tospovirus*. *Phytopathology, 91*, 856–863.

Cortez, I., Saaijer, J., Wongjkaew, K. S., Pereira, A.-M., Goldbach, R., Peters, D., et al. (2001). Identification and characterization

of a novel tospovirus species using a new RT-PCR approach. *Archives of Virology, 146*, 265–278.

De Avila, A. C., de Haan, P., Kormelink, R., Resende, R. de O., Goldbach, R. W., & Peters, D. (1993). Classification of tospoviruses based on phylogeny of nucleoprotein gene-sequences. *Journal of General Virology, 74*, 153–159.

Dong, J.-H., Cheng, X.-F., Yin, Y.-Y., Fang, Q., Ding, M., Li, T.-T., et al. (2008). Characterization of tomato zonate spot virus, a new tospovirus in China. *Archives of Virology, 153*, 855–864.

Feldhoff, A., Kikkert, M., Kormelink, R., Krczal, G., Goldbach, R., & Peters, D. (1997). Serological comparison of tospoviruses with polyclonal antibodies produced against the main structural proteins of tomato spotted wilt virus. *Archives of Virology, 142*, 781–793.

Golnaraghi, A. R., Shahraeen, N., Pourrahim, R., Farzadfar, S., & Ghasemi, A. (2004). Occurrence and relative incidence of viruses infecting soybeans in Iran. *Plant Disease, 88*, 1069–1074.

Harlow, E., & Lane, D. (1988). *Antibodies: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

Hassani-Mehraban, A., Saaijer, J., Peters, D., Goldbach, R., & Kormelink, R. (2005). A new tomato-infecting tospovirus from Iran. *Phytopathology, 95*, 852–858.

Hassani-Mehraban, A., Saaijer, J., Peters, D., Goldbach, R., & Kormelink, R. (2007). Molecular and biological comparison of two Tomato yellow ring virus (TYRV) isolates: challenging the *Tospovirus* species concept. *Archives of Virology, 152*, 85–96.

Hassani-Mehraban, A., Botermans, M., Verhoeven, J. Th. J., Meekers, E., Saaijer, J., Peters, D., et al. (2010). A distinct tospovirus causing necrotic streak on *Alstroemeria* sp. in Colombia. *Archives of Virology, 155*, 423–428.

Jain, R. K., Pandey, A. N., Krishnareddy, M., & Mandal, B. (2005). Immunodiagnosis of groundnut and watermelon bud necrosis viruses using polyclonal antiserum to recombinant nucleocapsid protein of *Groundnut bud necrosis virus*. *Journal of Virological Methods, 130*, 162–164.

Jones, D. R. (2005). Plant viruses transmitted by thrips. *European Journal of Plant Pathology, 113*, 119–157.

Kato, K., Hanada, K., & Hameya-Iwaki, M. (2000). *Melon yellow spot virus*: a distinct species of the genus *Tospovirus* isolated from Melon. *Phytopathology, 90*, 422–426.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23*, 2947–2948.

Lovato, F. A., Nagata, T., Resende, R. de O., De Avilla, A. SC., & Inoue-Nagata, A. K. (2004). Sequence analysis of the glycoproteins of *Tomato chlorotic spot virus* and *Groundnut ringspot virus* and comparison with other tospoviruses. *Virus Genes, 29*, 321–328.

Malapi-Nelson, M., Wen, R.-H., Ownley, B. H., & Hajimorad, M. R. (2009). Co-infection of soybean with *Soybean mosaic virus* and *Alfalfa mosaic virus* results in disease synergism and alteration in accumulation level of both viruses. *Plant Disease, 93*, 1259–1264.

Mandal, B., Csinos, A. S., Martinez-Ochoa, N., & Pappu, H. R. (2008). A rapid and efficient inoculation method for *Tomato spotted wilt tospovirus*. *Journal of Virological Methods, 149*, 195–198.

Nischwitz, C., Mullis, S. W., Gitaitis, R. D., & Csinos, A. S. (2006). First report of *Tomato spotted wilt virus* in soybean (*Glycine max*) in Georgia. *Plant Disease, 90*, 524.

Pappu, H. R., Jones, R. A., & Jain, R. K. (2009). Global status of tospovirus epidemics in diverse cropping systems: Successes achieved and challenges ahead. *Virus Research, 141*, 219–236.

Pietersen, G., & Morris, J. (2002). Natural occurrence of *Groundnut ringspot virus* on soybean in South Africa. *Plant Disease, 86*, 1271.

Plyusnin, A., Beaty, B. J., Elliott, R. M., Kormelink, R., Lundkvist, A., Schmaljohn, C. S., et al. (2011). *Bunyaviridae*. In A. M. Q. King, M. J. Adams, E. B. Carstens, & E. J. Lefkowitz (Eds.), *Ninth report of the international committee on taxonomy of viruses* (pp. 725–742). Oxford: Elsevier.

Seepiban, C., Gajanandana, O., Attathom, T., & Attathom, S. (2011). Tomato necrotic ringspot virus, a new tospovirus isolated in Thailand. *Archives of Virology, 156*, 263–274.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). $MEGA_4$: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*, 1596–1599.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America, 76*, 4350–4354.

Tzanetakis, I. E., Wen, R.-H., Newman, M., & Hajimorad, M. R. (2009). *Soybean vein necrosis virus*: a new threat to soybean production in Southeastern United States. *Phytopathology, 99*, S131.

Wu, J., Yu, C., Yang, C., & Zhou, X. (2009). Monoclonal antibodies against the recombinant nucleocapsid protein of *Tomato spotted wilt virus* and its application in virus detection. *Journal of Phytopathology, 157*, 344–349.

Zhou, J., Kantartzi, S. K., Wen, R.-H., Newman, M., Hajimorad, M. R., Rupe, J. C., et al. (2011). Molecular characterization of a new tospovirus infecting soybean. *Virus Genes, 43*, 289–295.